F-7

the presence of NaCl has been described to enhance killer activity, especially in halotolerant strains, isolated from salted food.

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.

The majority of known K toxins are considered labile proteins, unfitted for biotechnological purposes. 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, especially in the preservation of salted fermented foods. In order to achieve the isolation, purification and further molecular and biochemical characterization of *C. nodaensis* K toxin, a experimental protocol was developed, involving culture supernatant ethanol precipitation followed by exclusion and ionic chromatography. Along with the purification of this zymocin, the study of its mode of action will certainly contribute to evaluate its biotechnological potentialities for several applications, particularly in the preservation of high-salt food products.

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Partial purification and characterization of *Candida nodaensis* killer toxin

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Introduction

The production and secretion of killer toxins is a widespread phenomenon in yeasts. Several killer (K) systems have been investigated; some of them in species considered more or less halotolerant, and its k phenotype display has been, in some cases, associated with the degree of salt-stress in the environment

In order to clarify the possible relation between killer activity and salt-stress tolerance, 58 different yeast strains were assayed as to salt-stress resistance and killer/sensitive phenotypes in the absence and in the presence of NaCl. This survey allowed the identification of several strong salt/dependent killer phenotypes (Table I)

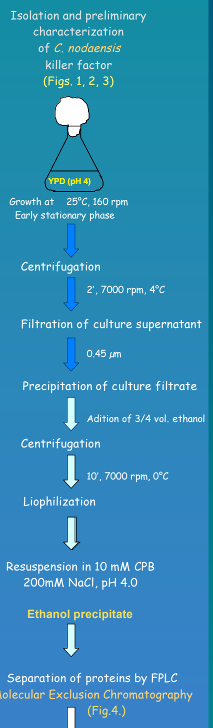
Table I. Killer phenotype variation with salt concentration in the assay. Results are expressed as the percentage of killed strains, from the total assayed as sensitive, at each salt molarity. Only K strains which killed more than one strain were considered.

NaCl - Tolerance Class	K strains	S strains (%)							
	[NaCl] in the assay (M)	0	0.5	1	1.5	2	2.5	3	3.5
	Number of strains tested for S phenotype (100%)	57	56	47	45	20	20	7	5
1 M	*P. jadinii*	38.6%	12.5%	0	n.a.	n.a.	n.a.	n.a.	n.a.
	Z. florentinus	5.3%	7.1%	6.4%	n.a.	n.a.	n.a.	n.a.	n.a.
2 M	*K. lactis* CBS 2359	26.3%	32.1%	4.3%	0	0	n.a.	n.a.	n.a.
	P. anomala IGC 4121	35.1%	48.2%	46.8%	44.4%	50.0%	n.a.	n.a.	n.a.
	S. cerevisiae IGC 4620	8.8%	8.9%	10.6%	4.4%	0	n.a.	n.a.	n.a.
3 M	*C. famata*	0	1.8%	21.3%	28.9%	30.0%	15.0%	0	n.a.
	D. hansenii	14.0%	23.2%	34.0%	35.6%	40.0%	35.0%	0	n.a.
	P. farinosa	10.5%	21.4%	21.3%	13.3%	5.0%	0	0	n.a.
	P. membranaefaciens IGC 3796	8.8%	3.6%	0	0	0	n.a.	n.a.	n.a.
4 M	***C. nodaensis***	**12.3%**	**23.2%**	**34.0%**	**35.6%**	**40.0%**	**25.0%**	**0**	**0**
	C. versatilis	3.5%	3.6%	4.3%	6.7%	15.0%	5.0%	0	0
	P. sorbitophila	5.3%	17.9%	17.0%	11.1%	0	0	0	0

n.a. not assayed

Candida nodaensis was selected for k toxin production and purification

- K activity is tested in methylene blue agar plates (YM-MB)
- Sensitive strain - top-agar layer of *Pichia guilliermondii*
- Activity is determided as routine in the absence or in the presence of 0.5 and 1M NaCl
- Aliquots (50μl) of the sample to be tested are added to sterile paper disks (6 mm)



Toxin stability studies

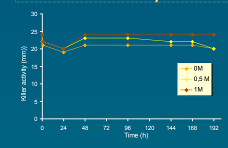


Fig.1. Effect of NaCl on K toxin stability (the ethanol precipitate was resuspended in CPB with NaCl 0, 0.5 and 1M). K activity was assayed in the presence of 1M NaCl.

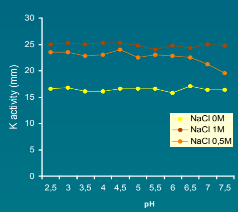


Fig.2. Effect of pH on K toxin activity (K activity was assayed in the absence and in the presence of 0.5 and 1M NaCl).

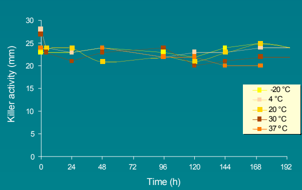


Fig.3. K toxin activity after incubation of the ethanol precipitate at - 20°C, 4°C, 20°C, 30°C and 37°C. K activity was assayed in the presence of 1M NaCl.

Strategy was the **purification** of this yeast k toxin.

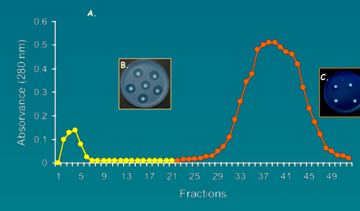


Fig.4. A- Elution profile of K toxin through a Superdex 200 column (Phamacia) equilibrated and eluted with CPB 200 Mm, pH 4.0 (fractions with K activity; fractions without K activity)

B and C - killer activity assay.

I - active fractions 1 to 7
II - active fractions 8 to 21
III – Non-active fractions 22 to 50

Active fractions were pooled in two groups, concentrated and its effect on sensitive cell viability assessed in YEPD plates (Fig.5)

The percentage of S cells remaining alive after treatment with this K toxin fractions (CFU) was quantified comparing with a toxin-free control

The three groups of fractions were analyzed by SDS-PAGE in order to try the identification of the protein involved in k activity phenotype (Fig.6)

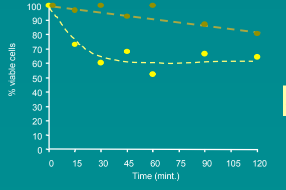


Fig.5. Viability of *P. guilliermondii* under the effect of partially purified K toxin (FPLC active fractions)

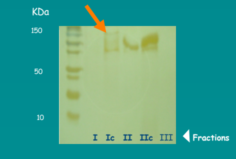


Fig.6. Silver-stained SDS-PAGE (12%) gel.
Lane 2, 4,6 - groups I, II and III
Lane 3, 5 – groups I and II concentrated 2 fold

Preliminary results on k toxin **mode of action**

Using selective fluorochrome assays able to detect different physiological responses of s sensitive strain in the process of dying through the action of *C. nodaensis* K toxin, preliminary results point to a possible Programmed Cell Death mechanism (PCD) involved

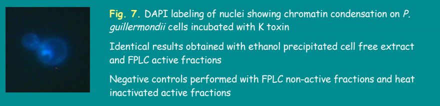


Fig. 7. DAPI labeling of nuclei showing chromatin condensation on *P. guillermondii* cells incubated with K toxin

Identical results obtained with ethanol precipitated cell free extract and FPLC active fractions

Negative controls performed with FPLC non-active fractions and heat inactivated active fractions

Final considerations

Experiments performed to characterize *C. nodaensis* K toxin showed that:

- Toxin stability is maintained after incubation in a relatively broad range of temperature, pH and NaCl concentrations
- Toxin mode of action can involve programmed cell death mechanisms
- The predicted molecular weight of the K toxin from SDS-PAGE of FPLC active fractions lays around 120 KDa

Further work will focus on

→ final steps on protein purification and sequencing
→ detailed study of the mechanisms underlying K toxin induced cell death