

# The killer toxin of the halotolerant yeast *Candida nodansensis*

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The production and secretion of killer (K) toxins is a widespread phenomenon in yeasts. A considerable diversity of yeast killer systems has been investigated, some of them in species considered more or less halotolerant, and which killer phenotype has been, in some cases, correlated with the degree of salt stress in the environment [1, 2, 3, 4]. In a previous survey [5], several strong K phenotypes were identified, some of them even displaying an enhancement of killer action in the presence of salt (Table I).

The great majority of killer toxins are considered labile proteins, unfitted for biotechnological purposes. The possibility that those yeast zymocins could be proteins less labile than the ones described so far, lead to the selection of one of those yeasts for further work. Toxins that are effective *in vivo* at high salinity are probably more stable, thus renewing the interest in these molecules for the development of yeast-based biocontrol strategies in several biotechnological applications, specially in the preservation of fermented foods.

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 strains which killed more than one strain were considered.

| NaCl-Tolerance Class | K-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                  | <i>P. pasteurii</i>                | 38.6%   | 12.5% | 0     | na    | na    | na    | na | na  |
|                      | <i>Z. roulandii</i>                | 5.3%  | 7.1%  | 6.4%  | na    | na    | na    | na | na  |
|                      | <i>C. lusitana</i>                 | 26.3%   | 32.1% | 4.3%  | 0     | 0     | na    | na | na  |
| 2 M                  | <i>P. anomala</i> ECG 4121         | 35.3%   | 48.2% | 46.8% | 44.4% | 50.0% | na    | na | na  |
|                      | <i>S. castellii</i> ECG 4620       | 8.3%  | 8.9%  | 10.6% | 4.4%  | 0     | na    | na | na  |
|                      | <i>C. lusitana</i>                 | 0   | 1.8%  | 21.3% | 25.9% | 30.0% | 15.0% | 0  | na  |
| 3 M                  | <i>D. hansenii</i>                 | 14.0%   | 21.2% | 34.0% | 35.6% | 40.0% | 35.0% | 0  | na  |
|                      | <i>P. farinosus</i>                | 18.5%   | 21.4% | 23.3% | 13.3% | 5.0%  | 0     | 0  | na  |
|                      | <i>P. membranifaciens</i> ECG 3796 | 3.9%  | 3.6%  | 0     | 0     | 0     | na    | na | na  |
| 4 M                  | <i>C. lusitana</i>                 | 12.3%   | 12.3% | 14.0% | 15.6% | 10.0% | 5.0%  | 0  | 0   |
|                      | <i>C. versatilis</i>               | 5.3%  | 3.6%  | 4.3%  | 6.7%  | 15.0% | 5.0%  | 0  | 0   |
|                      | <i>P. caribbicus</i>               | 5.3%  | 17.9% | 17.0% | 11.1% | 0     | 0     | 0  | 0   |

One of the strongest K strains identified above (Table I), the halotolerant yeast *Candida nodansensis* IGC 3198, was chosen for further study and toxin purification.

In order to identify the genetic determinant of this K toxin, some curing experiments (Table II) and nucleic acid extractions (Fig. 1) were performed. The absence of linear DNA plasmids and the impossibility of curing with some known curing agents suggested that this zymocin is encoded by a nuclear gene.

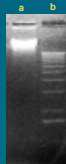


Fig. 1. Nucleic acid extractions from *C. nodansensis* (a). (b) Molecular weight marker X (Boehringer)

Table II. Curing experiments performed in *C. nodansensis*.

| Cycloheximide |          | Temperature |          | Ethidium bromide |
|---------------|----------|-------------|----------|------------------|
| %             | % curing | (°C)        | % curing | % curing         |
| 0.001         | 0        | 35          | 0        | 0                |
| 0.01          | *        | 37          | 0        | 0                |
|               |          | 40          | *        | 0                |

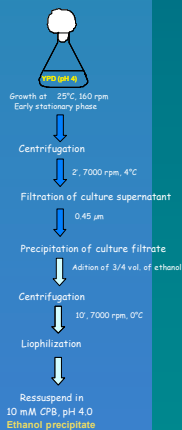


Fig. 2. Experimental protocol developed to obtain *C. nodansensis* K toxin

Thus the following strategy was the isolation and purification of this yeast K factor, now in progress in our laboratories.

Different media and culture conditions were tested to optimise the production of *Candida nodansensis* K toxin. Culture supernatant was concentrated by ethanol precipitation (Fig. 2) and used to preliminary characterize this K factor (Table III and Fig. 3, 4, 5). K activity was tested against the sensitive strain *Pichia guilliermondii*, in methylene blue agar plates (YMA-MB) with or without NaCl.

Table III. K activity of an ethanol precipitate after different enzymatic treatments (20h, 20°C). Control experiments were done with identical samples without the respective enzymes (100% K activity).

| Enzymes                  | Residual K activity |          |
|--------------------------|---------------------|----------|
|                          | Active              | Inactive |
| <b>Endonucleases</b>     |                     |          |
| DNase                    | 100%                | 100%     |
| RNase                    | 100%                | 100%     |
| <b>Proteases (mg/ml)</b> |                     |          |
| Protease K               | 86%                 | 100%     |
| Trypsin                  | 100%                | 100%     |
| Pronase                  | 100%                | 100%     |
| Papain                   | 100%                | 100%     |
| Pepsin                   | 76%                 | 100%     |

Previous results showed that this toxin was stable after 18h incubation at temperatures varying from -80°C to 45°C. Three temperature values were selected to further characterise *C. nodansensis* K factor (Fig. 3, 4, 5).

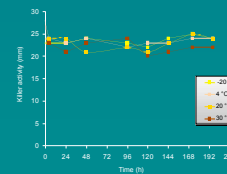


Fig. 3. K activity after 178h incubation at -20°C, 4°C, 20°C and 30°C.

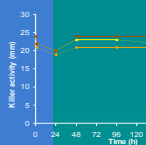


Fig. 4. Effect of the presence of NaCl (0, 0.5 and 1M) on K toxin stability.

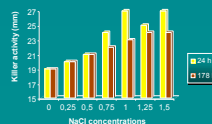


Fig. 5. Effect of the presence of NaCl in the assay medium.

The majority of the K toxins are considered labile proteins, unfitted for biotechnological purposes. Preliminary experiments performed to characterise *C. nodansensis* K factor showed that, besides keeping its biological activity at higher NaCl concentrations, is stable after incubation in a relatively broad range of temperature (up to 45°C) and pH values (2.5 to 7.5). Along with the purification of this protein, the study of its mode of action, will certainly contribute to evaluate its biotechnological potentialities in the preservation of high-salt food products.

[1] Maglian, W., Carré, S., Gerken, M., Borkhoft, D. and Piskunov, L. (1997) Yeast Killer Systems. *Crit Microbiol Rev.*, 15 (3) 265-270.  
 [2] Oliveira, P., Marques, D., Santos, R., Pinheiro, J. M. and Soares-Matias, I. (2007) Effect of salt on the killer phenotype of yeasts from olive brines. *Appl Environ Microbiol.*, 73 1162-1167.  
 [3] Soares, D. (1998) Selection of a Protein Post-Translationally Controlled by NaCl in a Halotolerant Yeast. *Pichia Anomala*. *Yeast*, 15: 123-131.  
 [4] Aguilar, C. and Lucas, C. (2011) Killer-sterility phenotypes and heterodimeric. *Food Technol and Biotechnol.*, 38 (1) 39-45