Vol.47, n. 1 : pp. 13-23, March 2004
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
AN INTERNATIONAL JOURNAL

Determination of Yeast Killer Activity in Fermenting Sugarcane Juice Using Selected Ethanol-making Strains

Sandra Regina Ceccato-Antonini*, **Christiann Davis Tosta and Ana Cláudia da Silva**
Departamento de Tecnologia Agroindustrial e Sócio-Econômica Rural; Centro de Ciências Agrárias; Universidade Federal de São Carlos; antonini@cca.ufscar.br; C. P. 153; 13600-970; Araras - SP - Brazil

ABSTRACT

Twenty-four yeasts out of 342 isolated from the fermentative process showed killer activity and three of them were selected for the fermentative efficiency evaluation in batch system with cell recycle, flask and fermentor experiments. The selected three killer strains did not present similar results to those of pressed (baking) yeast concerning ethanol (0.07-0.18; 0.12-0.20; 0.10-0.13; 0.22-0.25 g/g, respectively) and biomass (0.19-0.26; 0.33-0.39; 0.13-0.27; 0.47-0.61 g/g, respectively) yields and fermentative efficiency (12.3-36.3; 21.0-40.0; 19.3-26.3; 47.6-54.0 %, respectively) in sugarcane juice, in flasks. In fermentor, similar behaviour was observed. However, the selected strains showed high cellular viability and killer activity (using cell-free filtrate) along the fermentative cycles, in spite of the unfavourable conditions of the medium, like high pH variation of the medium (from 5.5-6.0 to 3.0-4.0), low aeration and higher temperature (30^0 C), which were not the ideal ones for the production/activity of killer toxins. A Pichia *strain (CCA 510) showed the best results among the killer yeasts tested, exhibiting a killer activity against 92% of isolated fermentative yeasts of the process and against the pressed(baking) ferment. It also demonstrated killer activity (using crude toxin preparation) at higher temperatures (38^0C) and low pH (4.0) after 72 hours of incubation, under proliferative and non-proliferative conditions. The results indicated that the killer activity should be a characteristic to be looked for in the strain selection for ethanolic fermentation, beside other important productivity-based characteristics, since it assure the permanence of the selected strain during the process.*

Key words: Killer yeasts, alcoholic fermentation, ethanol, killer toxin

INTRODUCTION

The phenotype killer was first described in 1963 in *Saccharomyces cerevisiae* (Bevan and Makower, 1963, Somers and Bevan, 1969), that certain strains of such species could be classified in one of three phenotypes: killer, sensitive and neutral. When sensitive and killer cells grew in the same culture medium, a high proportion of the sensitive cells died. The neutral cells did not kill sensitive cells, nor were killed by the killer ones. Killer yeast was immune to the action of its own killer factor. The killer reaction can even happens when the cells are not in contact: The culture medium in which the killer cells was developed previously and removed by centrifugation retained its ability to kill sensitive cells (Somers and Bevan, 1969).
According to Woods and Bevan (1968), the killer activity of a K1 toxin was only expressed in a narrow pH range (4.6-4.8), unstable in temperatures above 25°C and inactivated by agitation. Subsequent studies (Philliskirk and Young, 1975; Young and Yagiu, 1978) of several killer toxins showed that the properties of K1

* Author for correspondence

toxin and another were similar, indicating that all the toxins had proteic nature. A temperature increase (37-40^{0}C) can lead to the “cure” phenomenon, when killer strains lose their killer activity. This is due to the non-chromosomal killer genom loss or mutation (Wickner, 1974). The occurrence of killer phenotype in yeasts is widespread in alcoholic fermentations for beverage production such as in breweries (Maule and Thomas, 1973), saké (Imamura et al., 1974; Ouchi et al., 1979), wine (Naumov et al., 1973; Thornton, 1986; Petering et al., 1991; Sulo and Michalcáková, 1992; Carrau et al., 1993) and recently in sugarcane producing plants (Sato et al., 1993; Morais et al., 1997; Ceccato-Antonini et al., 1999; Soares and Sato, 1999, 2000). Many of these fermentative processes use non-pasteurised medium, which can allow the predominance of wild yeast strains coming from the raw material outnumbering the starter yeast. These contaminations can bring about the fermentation slowness or blockage, acidity increase, fusel oil production, and ethanol productivity decrease. So, the killer system may be a way to avoid the effects caused by undesirable yeasts in the fermentative processes. The utilisation of killer strains with good fermentative yields can bring undoubtedly advantages to the process, once it can guarantee competitive advantages to the starter ethanol-making yeast. We aimed to isolate killer yeast strains from the fermentative process for ethanol production, to evaluate both their fermentative efficiency and killer activity expression under industrial conditions during the fermentation process, in flasks and fermentor, and to assess their potential competitive advantage assaying the sensitivity of a panel of fermentative industrial yeasts to the selected killer strains.

MATERIALS AND METHODS

Isolation of yeasts using differential media

The following culture media were used: WLN and WLD (Green and Gray, 1950); Lysine medium (Morris and Eddy, 1957) and Lin Wild Yeast Medium (Lin, 1975) for the isolation of yeast strains from sugarcane juice, recycled ferment, fermented broth, molasses and water, of two alcohol-producing units. The samples were homogenised by agitation, transferred 10 mL to sterile centrifuge tubes and centrifuged at 1000 g for 5 minutes. Supernatants were discarded, adding sterile saline solution to ressuspend the cells. This procedure was repeated once. Serial dilutions and surface plating of 0.1 mL sample aliquots were done. Incubation was at 28^0 C for 72 h, when morphological evaluations of the colonies were accomplished. Visually different colonies were described and isolated in YEPD slants. The maintenance of the cultures was made through periodic transfer on YEPD under sterile mineral oil at room temperature.

Killer activity of the isolated yeast strains

The conventional method of observing a clear/blue killing zone around the test yeast streaked on buffered (pH 4.5-4.7, citrate-phosphate buffer) YEPD-methylene blue seeded (10^5 cells/mL) with the sensitive strains *Saccharomyces cerevisiae* NCYC 1006 and *Candida glabrata* ATCC 15126 at 28-30^0 C was used (Woods and Bevan, 1968) in the first evaluation. Secondly, a panel of 110 yeast strains isolated from the sugarcane fermented broth from a sugarcane ethanol-producing plant and commercial brands of yeasts (Fleischmann® pressed baking yeast) were used to assay their differential sensitivity to the selected killer strains using the same method at 26^{0}C.

Killer activity optimization of a selected strain

An optimization of pH, temperature and incubation time for crude toxin activity of a selected killer strain was performed under proliferative and non-proliferative conditions. Crude toxin preparation was obtained transferring loops of yeast cells (CCA 510) into tubes with 20 mL of YEPD broth (1% yeast extract; 2% peptone; 2% glucose), pH 4.0, following incubation for 48 h, at 26^0 C. Then, filtration through Millipore membrane (0.22 μ) was carried out and kept the filtrate (crude toxin) at 5^0 C. For the proliferative assay, 10% (v/v) of the crude toxin filtrates were added to tubes with citrate-phosphate buffered YEPD broth, at pH of 2.5; 4.0; 5.5, inoculated previoulsy with a cell suspension prepared from the sensitive strain *Saccharomyces cerevisiae* NCYC 1006 at a concentration of 10% (v/v) and 10^7 cells/mL. The incubation period was extended to 96 h, at 26 and 38^0 C, and sampled three times at 24-h periods. The cells were stained with methylene blue-citrate dye solution and counted in Neubauer chamber to estimate cell viability. For non-proliferative assay, citrate-phosphate buffered saline solution (0.85% NaCl) was used instead of

YEPD broth. Control tubes were made utilizing sterile distilled water (10% v/v) replacing crude toxin filtrates in all the treatments.

Preliminary evaluation of the killer strain fermentative capability

Using 24 selected killer strains and the Fleischmann® pressed baking yeast, the initial fermentative tests were accomplished in 250-mL erlenmeyers with 50 mL of clarified sugarcane juice, 14^0 Brix, 20% of inoculum v/v, at 30°C for 18 hours in triplicate. After the incubation period, the ethanol production was evaluated by sample distillation and density measure of hydroalcoholic solution (Amorim et al., 1979). Inoculum was prepared with the cell transfer to tubes with 10 mL of sugarcane juice 5° Brix, at 30° C for 24 h. The content was transferred to 1L-erlenmeyers with 300 mL of sugarcane juice, 10°Brix at 30°C with forced aeration (sterile air injection). The flasks were maintained under refrigeration for 12-24 h for cell sedimentation. Supernatant was discarded and cells were used as inoculum for the fermentative tests.

Fermentative efficiency of selected killer strains

Tests were accomplished both in flasks and fermentor for the evaluation of fermentative efficiency and killer activity expression of 3 selected strains during the process. For flask experiment, inoculum was prepared by yeast cell loop transfers to tubes with 20 mL of sterile saline solution, homogenised and transfer of 1 mL aliquots to 250-mL flasks with 150 mL of clarified sugarcane juice (3.8° Brix) under agitation of 250 rpm for 24 h 30° C (initial pH 5.5-6.0). The cycle duration was defined in this work according to Parazzi (1995) in results obtained previously that showed the most appropriated periods with minimal sugar residues in the fermented juice. The cellular mass was separated by centrifugation and again incubated under the same conditions. The procedure was repeated once. The fermentative tests were carried in 500-mL flasks with 150 mL of clarified sugarcane juice, 12° Brix, 20% inoculum v/v, in triplicate, during six 12-h cycles. At the end of each cycle, a 5% volume sample was taken for analysis. The remaining fermented juice was centrifuged and the biomass ressuspended in a new fermentation medium, with the same characteristics. As on the beginning the second fermentative cycle, and so forth until the sixth cycle. For fermentor experiment, only 2 killer strains were used (CCA 510 and CCA 449), because one of them (CCA 574) did not produce enough cellular mass to begin the fermentative process. The inoculum was produced previously in the same conditions described, and the inoculated tubes were transferred to the 10 L-fermentor glass vat (Microferm - New Brunswick) with clarified sugarcane juice, 3.8^0 Brix, aeration of 1 vvm at 30^0C and agitation of 120 rpm until 10 g of dry cellular mass/L were obtained. The cells were separated by centrifugation and recycled to the fermentor for the alcoholic fermentation for four 12-h cycles, at 30^0 C, 10^0 Brix, initial pH 5.2-6.0, in a final volume of 4 L.

Analyses

Initially, yeast cell viability was evaluated through Neubauer counting chamber (Lee et al., 1981), using methylene blue-sodium citrate dye solution. Samples of 5 mL were filtered in Millipore® membrane (0,45 µm), washing the biomass 3 times with distilled water and drying at 100°C until constant dry weight. In the cell-free filtrate, total residual reducing sugar analysis was accomplished by the 3,5 dinitrossalicilic acid method according to Miller 1959; ethanol by sample distillation and density measure of hydroalcoholic solution; pH and killer activity against the sensitive strains *Saccharomyces cerevisiae* NCYC 1006 and *Candida glabrata* ATCC 15126 in buffered YEPD-methylene blue, pH 4.5-4.7 (citrate-phosphate buffer) at 30°C using the well test assay. Eight mm-diameter wells were produced in culture medium, filled with 80 µL of the cell-free filtrate. The biomass (Yx/s) and ethanol (Yp/s) yields were calculated from the data of ethanol and dry cell mass produced, and consumed sugar mass, (Duarte, 1994). The fermentative efficiency (%) was calculated based on the alcoholic content of the medium and the initial concentration of total reducing sugar using practice fermentation stoichiometry, where 1 g of total reducing sugar produces 0.461 g of ethyl alcohol at 20^0 C according to Vailant (1980).

RESULTS AND DISCUSSION

Isolation, killer activity and preliminary evaluation of the fermentative capability of killer yeast strains from the ethanol fermentation process.

Different culture media were utilised aiming the isolation of yeast strains, according to studies previously accomplished (Ceccato Antonini and Silva, 2000). Three hundred and forty two yeast strains were isolated in different culture media and samples from two sugarcane producing units. Twenty four strains presented killer activity against the sensitive yeasts (7% of the total). Only eight killer strains presented fermentative capability in sugarcane juice, which were selected for further tests. Two killer strains were discarded for their flocculation characteristics. The results obtained in the preliminary evaluation of the killer strain fermentative capability are shown in Table 1, comparing to pressed baking yeast.

Table 1 - Ethanol production (g/100 mL) of the fermentative killer strains, in sugarcane medium, 14^0Brix, 20% inoculum, 30^0C, 18 hours, in flasks

Yeast strain	Ethanol (g/100 mL)[1]
CCA 574	5.62 ± 0.16
CCA 510	3.97 ± 0.28
CCA 449	3.74 ± 0.14
CCA 377	3.61 ± 0.24
CCA 369	3.42 ± 0.16
CCA 476	2.30 ± 0.09
Baking yeast	5.52 ± 0.24

[1] The values refer to the average and standard deviation (n=3).

The results pointed out a killer strain (CCA 574) with alcohol production similar to baking yeast. However, other two killer strains (CCA 510 and CCA 449) were selected for fermentative efficiency evaluation because CCA 574 did not multiply efficiently. The cause of the absence of multiplication was not elucidated. The same medium was tested with another strain and it worked out. A probable explanation could be that this strain lost its fermentative capability by the constant reactivation and it is known that these operations can induce alterations in yeasts. The killer strain CCA 510 showed a powerful killer activity against 101 out of 110 yeast strains isolated from the ethanol fermentation process, i.e., 92% of these yeast strains were sensitive to this killer yeast, which was also killer against the pressed baking yeast (Fleischmann®). These results could indicate a higher competitive advantage of CCA 510 as a starter ethanol-making yeast against other yeasts when inoculated in a non-pasteurised sugarcane medium. None of the killer fermentative strains isolated showed so wide killing activity. It could be important to remember that the killing action was against yeast strains from the own fermentative process for ethanol production, what better assured CCA 510 killer efficiency. Taxonomical tests indicated it as *Pichia* genus. The assays of crude killer toxin activity of this yeast strain under proliferative and non-proliferative conditions and different pH, temperature and incubation times are showed in Fig 1. In the proliferative assay at pH 2.5 there was a decrease in the cell viability, which was independent of the crude toxin addition at both temperatures. However, at 38^0C, a remarkable effect of the toxin could be observed. Indeed, at this temperature, we could observe a great killer activity of this yeast at pH 4.0, showing a remarkable thermotolerance of this toxin. It was another good characteristics of this strain, that is, good killer activity at higher temperatures at low pH values, which were conditions much similar to those into alcohol fermentation tanks. In non-proliferative assay, we observed the benefical effect of higher temperatures on the killer activity of CCA 510, mainly at pH 4.0. At pH 5.5, we did not observe significant killer activity in any assay. Best results were obtained after 72 h of incubation in both assays.

Fermentative efficiency of killer yeast strains in batch cell recycle system

The results showed that none of the killer yeasts tested had fermentative efficiency similar or superior to that presented by the baking yeast, which was the most common inoculum used by the producing units, in flasks or fermentor (Fig. 2 to 5). The cell-free filtrate after the first cycle could not be analysed, so the results were not showed in Fig 2 and 3. The strain CCA 510 presented the best performance. Whatever the yeast strain used, best results were performed in fermentor. However, the strain CCA 510 showed high cell viability presented along the cycles and persistent killer activity in the cell-free filtrate, in spite of high pH variation of the culture medium, from 5.5-6.0 to 3.0-4.0 at the end of each cycle. It showed the killer activity against *S. cerevisiae* NCYC 1006 for the largest inhibition zones (clear region/bluish coloured cells).It is known that the Killer activity is pH dependent, better expressing in buffered medium, and under low temperatures, around 20-25^0C (Woods and Bevan, 1968; Young, 1987).

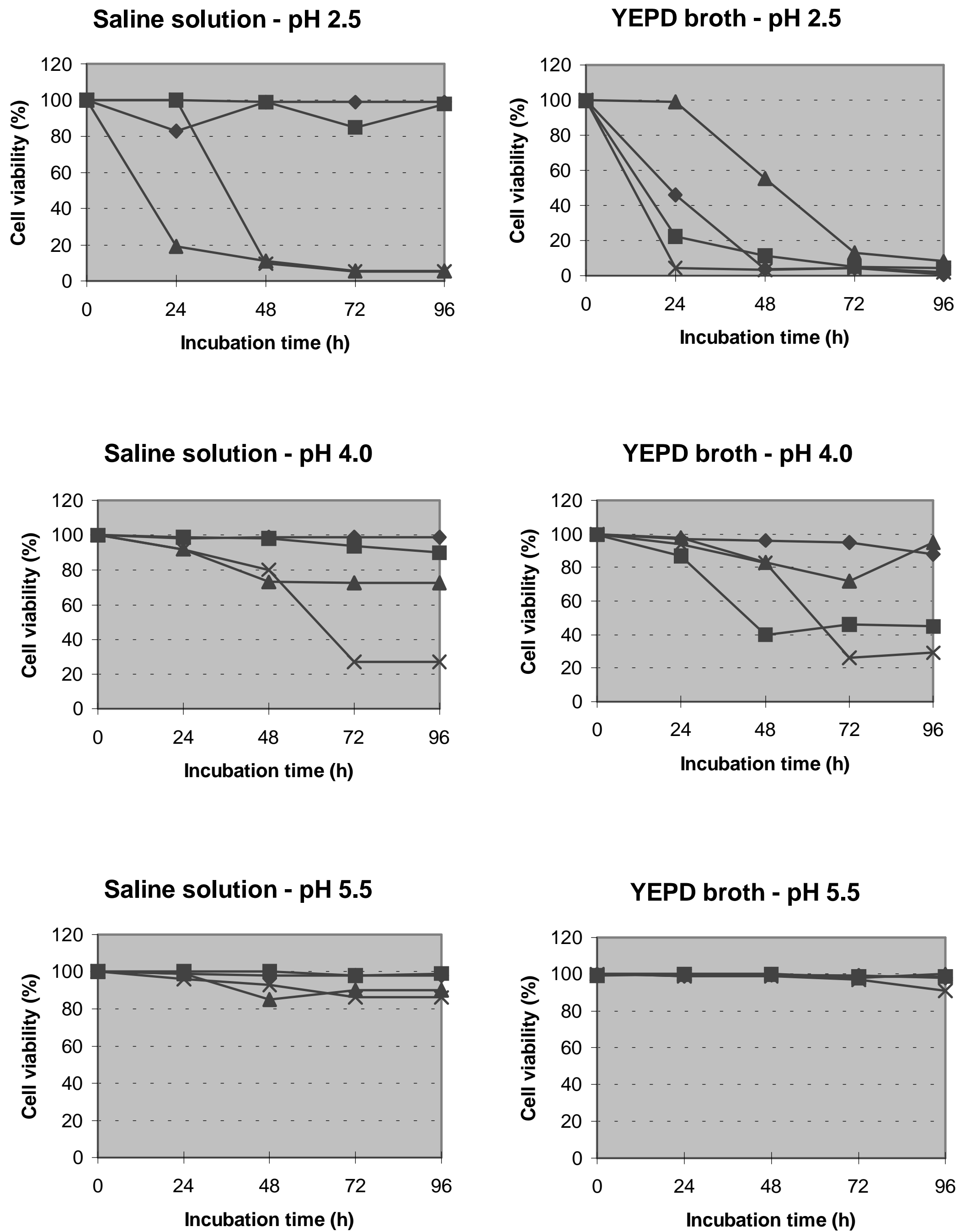

Figure 1 - Cell viability (%) of the sensitive strain *Sacch. cerevisiae* NCYC 1006 with and without toxin addition of CCA 510 yeast strain, under proliferative (YEPD broth) and non-proliferative conditions (saline solution), at pH 2.5, 4.0 and 5.5, at 26^{0}C and 38^{0}C

(■) 26^{0}C with toxin addition (✕) 38^{0}C with toxin addition
(◆) 26^{0}C without toxin addition (▲) 38^{0}C without toxin addition

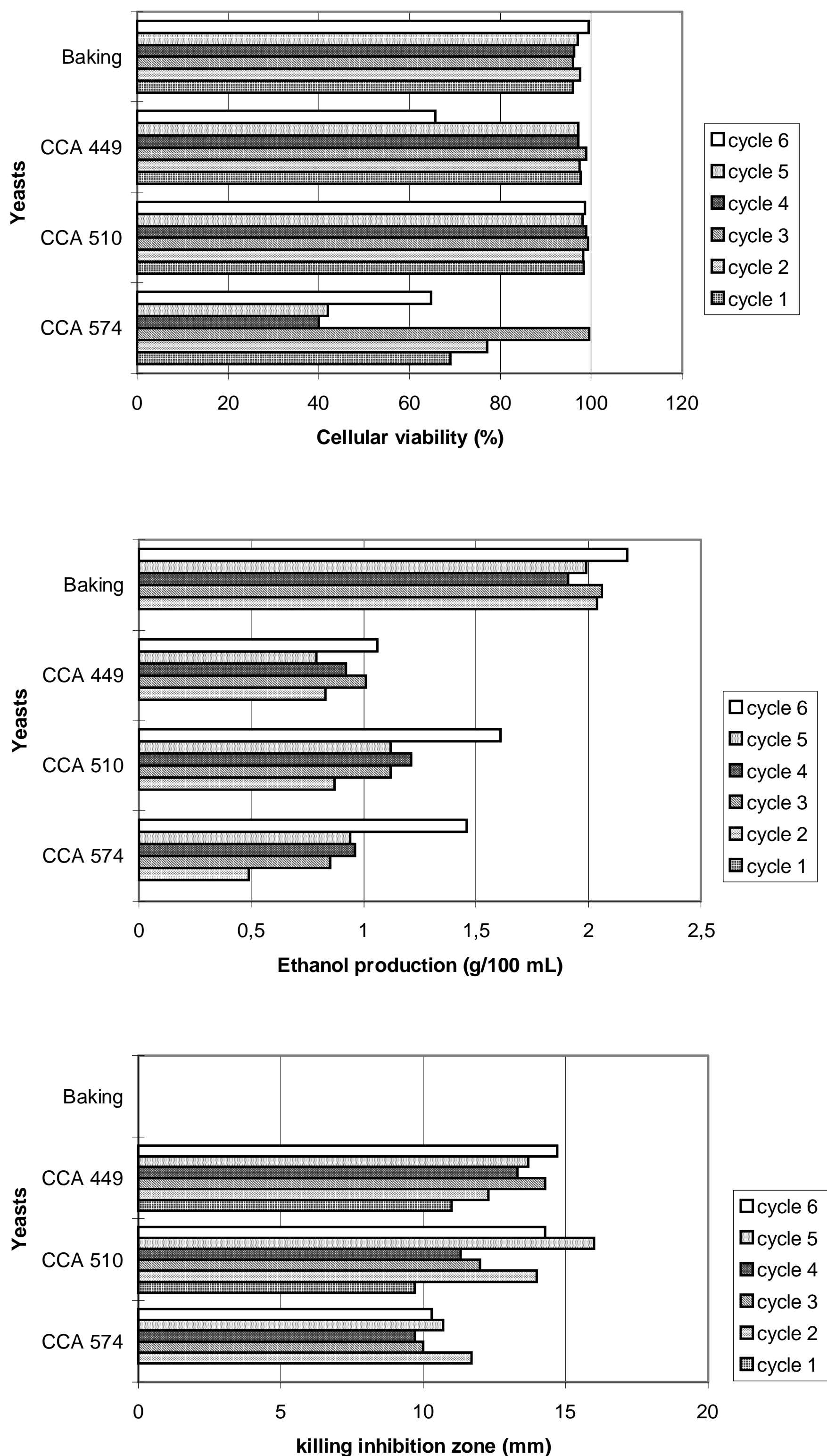

Figure 2 - Cellular viability (%), ethanol production (g/100 mL) and killing inhibition zone (mm)[1] during batch alcoholic fermentation, in flasks, at 30^0 C, using clarified sugarcane juice (12^0 Brix), 20% inoculum, with cell recycling (six 12-hour cycles), using killer yeast strains and baking yeast.
[1] against *Sacch. cerevisiae* NCYC 1006

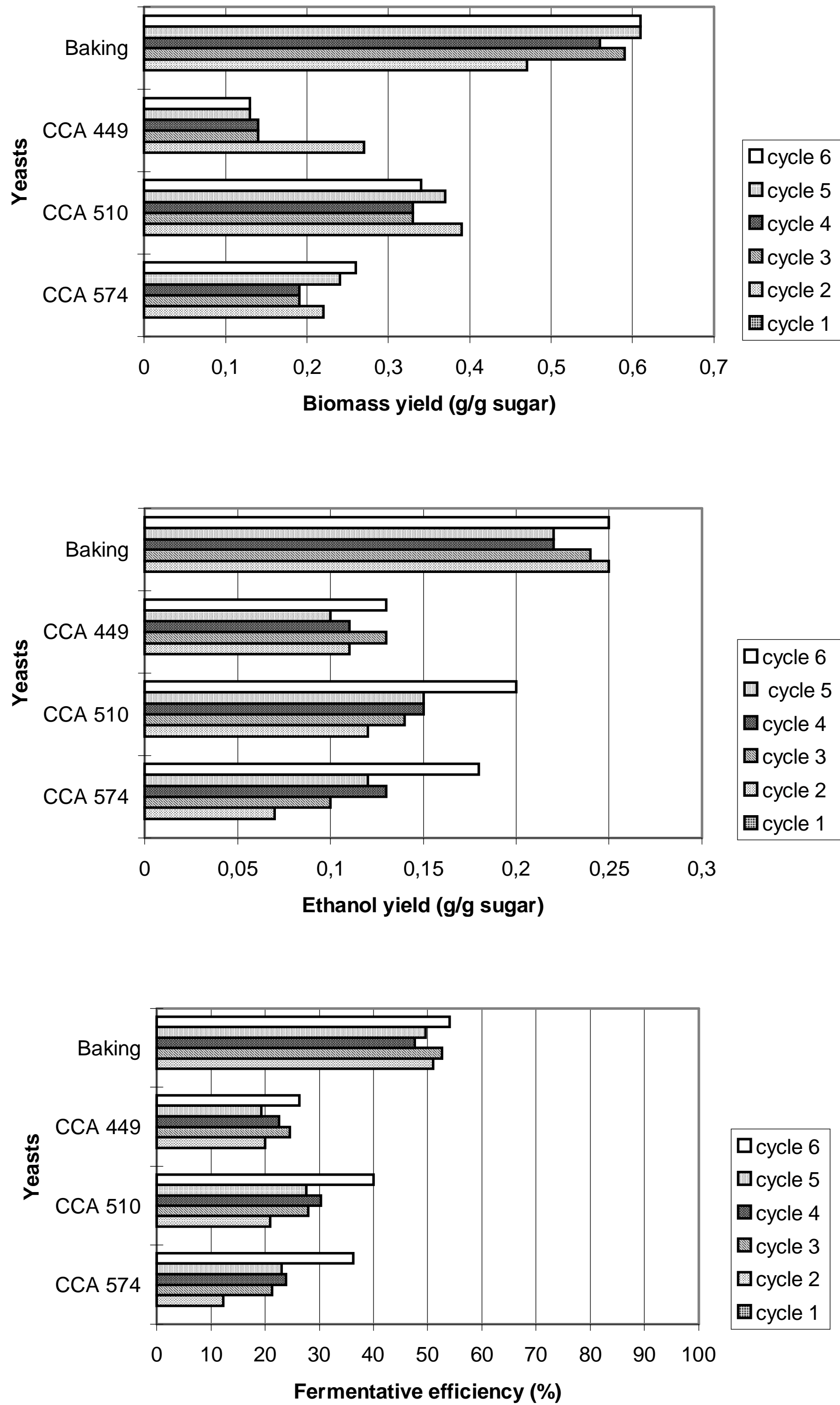

Figure 3 - Biomass and ethanol yields (g/g sugar) and fermentative efficiency (%) during batch alcoholic fermentation, in flasks, at 30^0 C, using clarified sugarcane juice (12^0 Brix), 20% inoculum, with cell recycling (six 12-hour cycles), using killer yeast strains and baking yeast.

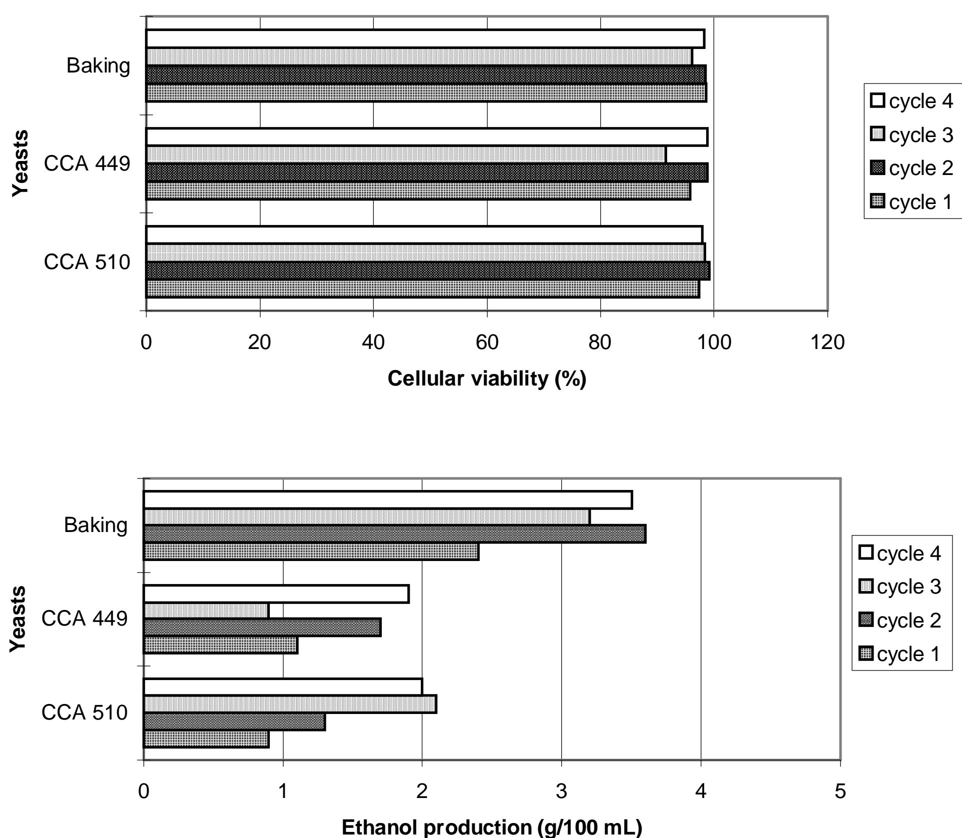

Figure 4 - Cellular viability (%) and ethanol production (g/100 mL) during batch alcoholic fermentation, in 4-liter fermentor, at 30^0 C, using clarified sugarcane juice (10^0 Brix), 20% inoculum, with cell recycling (four 12-hour cycles), using killer yeast strains and baking yeast.

Although the fermentation conditions are not stated as ideal for the killing activity, concerning large pH variation, higher temperature (30^0 C) and low aeration, the strain CCA 510 maintained its killing action both in flasks or fermentor. Otherwise, the strain CCA 449 did not show any killing activity in fermentor experiment as well as the pressed baking yeast. The commercial brand of yeast did not show any kind of killer activity in any experiment. The killer system was the most important and decisive factor for the yeasts to establish during the fermentative process, since there were many yeast strains presenting neutral phenotype, as already showed for some wild yeasts presenting pseudohyphal morphology (Ceccato-Antonini et al., 1999).

The point is not only to show a killer phenotype, but to express this killing action during the fermentation process, in industrial scale, under limiting conditions presented by the culture medium and environmental conditions. From the results obtained, we concluded that this characteristics was more an exception than a rule for the yeast strains from the ethanol fermentation process. But selected ethanol-making yeasts possessing killer activity like that could be a way to assure their predominance in the process. A little has been published about the meaning of yeast killer system for alcoholic fermentation to produce ethanol, differently for wine and other alcoholic beverages, in which yeast killer system has performed important and expressive results. Other tests seeking the culture media optimisation could take that strain (CCA 510) to improve its fermentative yields, which in association with the killer activity performance along the fermentative process, could indicate it as potential for the industry.

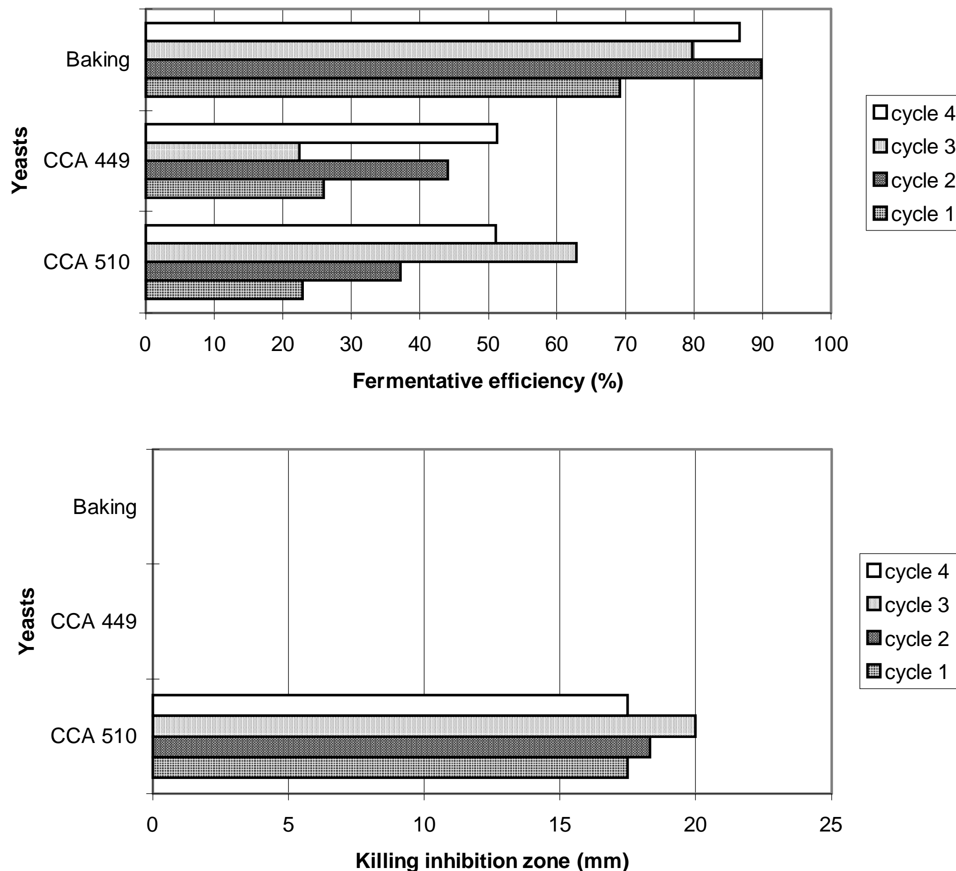

Figure 5 - Fermentative efficiency (%) and killing inhibition zone (mm)[1] during batch alcoholic fermentation, in 4-liter fermentor, at 30^0 C, using clarified sugarcane juice (10^0 Brix), 20% inoculum, with cell recycling (four 12-hour cycles), using killer yeast strains and baking yeast.
[1]against *Sacch. cerevisiae* NCYC 1006

ACKNOWLEDGMENTS

We wish to thank FAPESP (98/06034-2 and 98/06036-5) for the grants to the second and third authors.

RESUMO

A atividade 'killer' poderia garantir às leveduras fermentativas uma vantagem competitiva sobre outras linhagens durante a fermentação etanólica, no entanto, pouco se sabe sobre o papel do sistema 'killer' nesse tipo de fermentação alcoólica. A seleção de linhagens 'killer' altamente produtivas pode ser uma abordagem interessante. Vinte e quatro dentre 342 leveduras isoladas do processo fermentativo mostraram atividade 'killer' e 3 delas foram selecionadas para a avaliação da eficiência fermentativa em sistema de batelada com reciclo de células, em escala de frascos e fermentador. As 3 linhagens 'killer' selecionadas não apresentaram resultados similares àqueles apresentados pelo fermento prensado (panificação) em relação aos rendimentos em etanol (0,07-0,18; 0,12-0,20; 0,10-0,13; 0,22-0,25 g/g, respectivamente) e biomassa (0,19-0,26; 0,33-0,39; 0,13-0,27; 0,47-0,61 g/g, respectivamente) e eficiência fermentativa (12,3-36,3; 21,0-40,0; 19,3-26,3; 47,6-54,0 %, respectivamente) em caldo de cana-de-açúcar, em frascos. Em fermentador,

observou-se o mesmo comportamento. No entanto, elas mostraram alta viabilidade celular e atividade 'killer' (usando filtrado livre de células) ao longo dos ciclos fermentativos, a despeito das condições desfavoráveis do meio, como alta variação de pH do meio (de 5,5-6,0 a 3,0-4,0), baixa aeração e temperatura mais alta (30^0C), as quais não são as ideais para a produção/atividade das toxinas 'killer'. Uma linhagem de *Pichia* (CCA 510) mostrou os melhores resultados entre as linhagens 'killer' testadas, além de exibir uma poderosa atividade 'killer' contra 92% de leveduras fermentativas isoladas do processo e contra o fermento prensado. Demonstrou também atividade 'killer' (usando preparado de toxina bruta) em temperaturas elevadas (38^0C) e baixo pH (4,0) após 72 horas de incubação, sob condições proliferativas e não-proliferativas. Os resultados permitem indicar que a atividade 'killer' deve ser uma característica a ser avaliada na seleção de linhagens para a fermentação etanólica, além de outras características baseadas na produtividade, uma vez que pode assegurar a permanência da linhagem selecionada durante o processo.

REFERENCES

Amorim, H. V.; Zago, E. A. and Gutierrez, L. E. (1979), Método prático para controle da fermentação e destilação. *Saccharum STAB*, **2** : (4), 31-34.

Carrau, F. M.; Neirotti, E. and Gioia, O. (1993), Stuck wine fermentations: effect of killer/sensitive yeast interactions. *J. Ferment. Bioeng.*, **76**, 67-69.

Ceccato-Antonini, S. R.; Cremonini, L. C. M. and Regenfuss, C. (1999), 'Killer' character of yeasts isolated from ethanolic fermentations. *Sci. Agric.*, **56**, 631-635.

Ceccato-Antonini, S. R. and Silva, D. F. (2000), Eficiência de meios diferenciais no isolamento de cepas de leveduras de processos industriais de fermentação alcoólica. *Stab*, **18**, 40-46.

Duarte, M. C. T. (1994), Efeito da lecitina e do óleo de soja sobre o desempenho fermentativo e a reserva de carboidratos em Saccharomyces cerevisiae IZ 1904. Tese de Doutorado, Universidade Estadual Paulista, Rio Claro, São Paulo.

Green, J. R. and Gray, P. P. (1950), A differential procedure applicable to bacteriological investigation in brewing. *Wallerstein Lab. Comm.*, **13**, 357-368.

Imamura, T.; Kawamoto, M. and Takaoka, Y. (1974), Characteristics of main mash infected by killer factor. *J. Ferment. Technol.*, **52**, 293-299.

Lee, S. S.; Robison, F. M. and Wang, H. Y. (1981), Rapid determination of yeast viability. *Biotech. Bioeng.*, **11**, 641-649.

Lin, Y. (1975), Detection of wild yeasts in the brewery. Efficiency of differential medial. *J. Inst. Brew.*, **81**, 410-417.

Miller, G. L. (1959), Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry.*, **31**, 426-428

Maule, A. P. and Thomas, P. D. (1973), Strains of yeast lethal to brewery yeasts.*J. Inst. Brew.*, **79**, 137-141.

Morais, P. B.; Rosa, C. A.; Linardi, V. R.; Pataro, C. and Maia, A. B. R. A. (1997), Characterization and succession of yeast populations associated with spontaneous fermentations during the production of Brazilian sugarcane aguardente. *World J. Microbiol. Biotechnol.*, **13**, 241-243.

Morris, E. O. and Eddy, A. A. (1957), Method for the measurement of wild yeast infection in pitching yeast. *J. Inst. Brew.*, **63**, 34-35.

Naumov, G. I.; Tyurina, L. V.; Buryan, N. I. and Naumova, T. I. (1973), Winemaking, an ecological niche of type k2 killer. *Bio. Nauki*, **16**, 103-107.

Ouchi, K.; Wickner, R. B. and Toh-E, A. (1979), Breeding of killer yeast for sake brewing by citoduction. *J. Ferment. Technol.*, **6**, 483-487.

Parazzi, C. (1995), *Fermentação alcoólica com leveduras floculantes*. Tese de Doutorado. UNESP, Instituto de Biociências Universidade Estadual Paulista, Rio Claro, Brazil. 164 pp.

Petering, J. E.; Symons, M. R.; Langridge, P. and Henschke, P. A. (1991), Determination of killer yeast activity in fermenting grape juice by using a marked *Saccharomyces cerevisiae* wine yeast strain. *Appl. Environ. Microbiol.*, **57**, 3232-3236.

Philliskirk, G. and Young, T. W. (1975), The occurrence of killer character in yeasts of various genera. *Ant Leeuwenhoek*, **41**, 147-151.

Sato, H. H.; Pastore, G. M. and Park, Y. K. (1993), Study of some characteristics of newly isolated killer yeast. *Rev. Microbiol.*, **24**, 71-72.

Soares, G. A. M. and Sato, H. H. (1999), Killer toxin of *Saccharomyces cerevisiae* Y500-4L active against Fleischmann and Itaiquara brands of yeast. *Ver. Microbiol.*, **30**, 253-257.

Soares, G. A. M. and Sato, H. H. (2000), Characterization of the *Saccharomyces cerevisiae* Y500-4L toxin. *Braz. J. Microbiol.*, **31**, 1-11.

Sommers, J. M. and Bevan, E. A. (1969), The inheritance of killer character in yeast. *Genet. Res.*, **13**, 71-83

Sulo, P. and Michalcáková, S. (1992), The K3 type killer strain of genus *Saccharomyces* for wine production. *Folia Microbiol.*, **37**, 289-294.

Thornton, R. J. (1986), Genetic characterization of New Zealand and Australian wine yeasts. *Ant. Leeuwenhoek*, **52**, 97-103.

Vailant, L. P. M.; Gonçalves, L. A. C.; Lucas, S. M. A. and Gomes, C. H. F. (19--), *Tecnologia da fabricação do álcool*, IAA-Planalsucar, Coord. Regional Leste. 129 pp.

Wickner, R. B. (1974), 'Killer character' of *Saccharomyces cerevisiae:* curing by growth at elevated temperatures. *J. Bacteriol.*, **117**, 1356-1357.

Woods, D. R. and Bevan, E. A. (1968), Studies on the nature of the killer factor produced by *Saccharomyces cerevisiae. J. Gen. Microbiol.*, **51**, 115-126.

Young, T. W. (1987), Killer yeasts. In: Rose, A. H. and Harrison, J. J. (eds.) *The yeasts*. Academic Press : London. pp.131-164.

Young, T. W. and Yagiu, M. (1978), A comparison of the killer character in different yeasts and its classification. *Ant. Leeuwenhoek*, **44**, 59-77.

Received: November 20, 2001;
Revised: August 14, 2002;
Accepted: August 27, 2003.