As Leveduras "Killer" e a Fermentação Alcoólica

The Killer Yeasts and the Alcoholic Fermentation

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RESUMO

Ao contrário de outras fermentações alcoólicas onde o papel das leveduras "killer" está melhor definido, pouco se sabe sobre o seu papel no processo fermentativo para produção de etanol. Aqui são apresentadas três abordagens ao tema: a distribuição do fenótipo "killer" entre as leveduras do processo; a seleção de linhagens produtoras de etanol com atividade "killer; e a discriminação ("fingerprinting") de linhagens de leveduras, usando a sensibilidade diferencial às toxinas "killer". Meios seletivos foram utilizados para isolar 342 linhagens, das quais 7% apresentaram atividade "killer". Uma linhagem "killer" (CCA 510) apresentou rendimentos em etanol ligeiramente inferiores àqueles apresentados pelo fermento de panificação em caldo de cana, em sistema em batelada com reciclo de células, tanto em frascos quanto em fermentador, mas com alta taxa de viabilidade celular e atividade "killer" (filtrado livre de células) ao longo dos ciclos fermentativos, apesar das condições desfavoráveis do meio de cultura. Esta linhagem também apresentou atividade "killer" contra 101 dentre 110 (92%) linhagens industriais; toxina bruta eficiente a 30-38ºC e baixo pH após 72 horas de incubação, sob condições proliferativas e não-proliferativas; não foi curada (perda do fenótipo "killer") por ciclohexímida e a 37ºC e 40ºC; não apresentou plasmídio "ds-RNA", sugerindo que a toxina "killer" é codificada por genes cromossomais (levedura não-Saccharomyces) e portanto, mais estável. Linhagens de leveduras pertencentes a grupos taxonômicos diferentes, e linhagens similares geneticamente mas isoladas de fontes e locais diversos puderam ser diferenciadas utilizando-se a discriminação pela sensibilidade diferencial às toxinas "killer". Concluiu-se que a seleção de linhagens de leveduras capazes de expressar atividade "killer" nas condições de fermentação é uma abordagem interessante e útil, que pode lhes conferir vantagem competitiva. Além disso, o sistema "killer" pode se tornar também uma ferramenta eficiente e de baixo custo para a discriminação de leveduras ("fingerprinting").

SUMARY

While for other alcoholic fermentations the role of killer yeasts is better understood, a little is known about their significance in the fermentative process for ethanol production. It is presented here three approaches to this subject: the distribution of killer phenotype among the yeasts from the process; the selection of ethanol-producing strains with killer activity; and the discrimination (fingerprinting) of yeast strains using their differential sensitivity to killer toxins. Selective media were used to isolate 342 yeast strains, from which 7% showed killer activity. A killer strain (CCA 510) presented slightly lower ethanol yields comparing to baker's yeast in sugarcane juice in batch system with cell recycle, both in flask and fermentor scale, but a high cell viability and killer activity (cell-free filtrate) along the fermentative cycles in despite of the unfavourable medium conditions. This yeast strain also showed killer activity against 101 among 110 (92%) industrial strains; effective crude toxin at 30-38ºC and low pH after 72 hours of incubation, under proliferative and non-proliferative conditions; was not cured (loss of killer phenotype) by ciclohexímide and at 37º and 40ºC; did not present ds-RNA plasmid, suggesting the killer toxin is encoded by chromosomal genes (non-Saccharomyces yeast) and therefore, more stable. Taxonomically different yeast groups and besides discrimination among similar genetically yeast strains but isolated from different sources and locations could be differentiated using their differential sensitivity to killer toxins. We concluded that the selection of yeast strains capable to express the killer activity in fermentation conditions is an interesting and useful approach, which could give them competitive advantage. Besides, the killer system may be also an effective and inexpensive tool for the yeast fingerprinting.

PALAVRAS-CHAVE
KEY WORDS

fermentação alcoólica, etanol, leveduras "killer" / alcoholic fermentation, ethanol, killer yeasts

1. INTRODUCTION

The killer phenotype was described in 1963 for the first time in laboratory strains of Saccharomyces cerevisiae. There are three basic phenotypes: killer, sensitive and neutral. The killer strains both produce a specific killer factor and resist to this toxin. The resistant or neutral strains are insensitive to one or more killer factors and do not produce any toxin. The sensitive strains are totally susceptible to the killer toxin, which causes inhibition of macromolecule synthesis and cell disruption resulting in cell death (WOODS AND BEVAN, 1968; BUSSEY AND SHERMAN, 1973). YOUNG AND YAGIU (1978) and WICKNER (1979) reported the interactions among killer yeasts from several genera and species, and eleven different toxin groups (K1 to K11) were recognized in relation to the killer activity.

According to STUMM et al. (1977), the wide occurrence of the killer phenomenon suggests that it plays an important role in yeast ecology, altering the distribution of the strains through competition by interference. The killer property is present in other yeast genera as Candida, Debaryomyces, Hansenula, Kluyveromyces, Pichia, Torulopsis and Cryptococcus. In a fermentative process, the ability to produce killer toxin can give a selective advantage over sensitive competitive strains which are contaminant.

The occurrence of killer phenotype is widespread in yeasts from the alcoholic fermentations for beverage production as in brewing (MAULE AND THOMAS, 1973), "saké" (IMAMURA et al., 1974), alcoholic beverages from Andes (CASTILHO et al., 1990), 'cachaça' (MORAIS et al., 1997), wine (NAUMOV et al., 1973; THORNTON, 1986; PETERING et al., 1991; SULO AND MICHALCÁKOVÁ, 1992; CARRAU et al., 1993), and more recently in ethanol-producing units (SATO et al., 1993; CECCATO-ANTONINI et al., 1999, 2004; SOARES AND SATO, 1999, 2000).

In this paper, three approaches allowed us to evaluate the significance/importance of the killer system for the fermentative process for ethanol production. Firstly, the distribution of the killer phenotype among the yeasts from the alcoholic process, followed by the evaluation of the fermentative performance of the killer yeasts isolated. Thirdly, the possibility of using the killer system for fingerprinting of industrial yeast strains.

2. MATERIAL AND METHODS

2.1 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, in two alcohol-producing units (Usina São João and Usina Santa Lúcia, Araras - SP). The isolates were kept in YEPD Agar medium. The procedures used are described in CECCATO-ANTONINI (2004).

2.2 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 (105 cells/mL) with the sensitive strains Saccharomyces cerevisiae NCYC 1006 and Candida glabrata ATCC 15126, at 280 C -300 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 another sugarcane ethanol-producing plant (Destilaria São Marino, Pirassununga-SP) and commercial brand of yeasts (Fleischmann pressed baking yeast) were used to assay their sensitivity to the selected killer strains using the same method, at 260C. An optimization of pH, temperature and incubation time for crude toxin activity was performed under proliferative (citrate-phosphate buffered YEPD broth) and non-proliferative (citrate-phosphate buffered saline solution, 0.85% NaCl), according to BERTOLIN (1995). The curing test was made by cicloheximide and temperature using the method in KITANO et al (1984). The extraction and detection of double strand plasmid RNA (ds-RNA) was performed according GOTO et al (1990) modified by SOARES AND SATO (2000).

2.3 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 a selected killer strain (CCA 510) during the process. The fermentative tests were carried in 500-mL erlenmeyers with 150 mL of clarified sugarcane juice, 12o Brix, 20% inoculum vol/vol, in triplicate, during six 12-hour 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, beginning the second fermentative cycle, and so forth until the sixth cycle. For fermentor scale-up, the inoculum was transferred to the fermentor glass vat (Microferm - New Brunswick) with clarified sugarcane juice, 3.80 Brix, aeration of 1 vvm, at 300 C and agitation, until 10 g of cellular mass/L were obtained. The cells were separated by centrifugation, and recycled to the fermentor for the alcoholic fermentation, for four 12-hour cycles, at 300 C, 100 Brix, initial pH 5.2-6.0, in a final volume of 4 L.

Biotyping of industrial yeast strains by killer system. Thirty-one yeast strains isolated from the alcoholic fermentation were tested against a panel of 29 killer yeast strains to assess their sensitivity to killer toxins using medium and conditions above mentioned. Those strains were already characterized both by physiological/biochemical tests and molecular techniques (TOSTA, 2004) generating clusters of strains which were compared to the clusters originated from the binary data (presence/absence of sensitivity to each killer strain, a matrix of binary data 29X31). The NTSYSpc 2.02i (Applied Biostatistics) was used for Simple Matching (SM) similarity index and the clustering through UPGMA (Unweighted pair group with arithmetic average) for the dendrogram.

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2.4 Analyses

Yeast cell viability was evaluated through Neubauer counting chamber using methylene blue-sodium citrate dye solution. Samples of 5 mL were filtered in Millipore membrane (0,45), the biomass washed and dried at 100oC until constant dry weight. n the cell-free filtrate, total residual reducing sugar analysis was accomplished by the 3,5 dinitrossalicilic acid method; ethanol, by sample distillation and density measure of hydroalcoholic solution; pH; and killer activity against the sensitive strains Saccharomyces cerevisiae NCYC 1006, in buffered YEPD-methylene blue, pH 4.5-4.7 (citrate-phosphate buffer), at 30oC, using the well test assay. Eight mm-diameter wells were produced in culture medium, filled with 80 L of the cell-free filtrate. The biomass (Yx/s) and ethanol (Yp/s) yields were calculated from the data of ethanol produced, cell dry mass and consumed sugar mass, in grams. The fermentative efficiency (%) was calculated based on the alcoholic content of the medium and the initial concentration of total reducing sugar, using fermentation stoichiometry, where 1 g of total reducing sugar produces 0,64755 mL of ethyl alcohol at 200 C.

3. RESULTS AND DISCUSSION

3.1 Isolation of killer strains and characterization of the killer toxins.

Different culture media were utilised aiming the isolation of a greater number of yeast strains, even those in low population numbers, 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), from which a particular 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 that killer yeast, which was also killer against the pressed baking yeast (Fleishmann®). These results may 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. It could be important to remember that the killing action was against yeast strains from the own fermentative process for ethanol production.

The crude killer toxin activity of this yeast strain under proliferative and non-proliferative conditions and different pH, temperature and incubation times was assayed. A remarkable effect of CCA 510 toxin was observed at pH 4.0, 38ºC and 72 hours of incubation, in proliferative and non-proliferative conditions against the sensitive yeast S. cerevisiae NCYC 1006 (Figure 1). It was another good characteristics of this strain, because the conditions above are closer to the ones into alcohol fermentation tanks. 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.

The killer strain CCA 510 was not cured after thermal treatment at 37ºC and 40ºC and in the presence of 0.2 ppm of cicloheximide. It was also assayed a S. cerevisiae killer strain

Figure 1 Cell viability (%) of the sensitive strain S. 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°C and 38°C.


(■) 26ºC with toxin addition
(◆) 26ºC without toxin addition
(✕) 38ºC with toxin addition
(▲) 38ºC without toxin addition
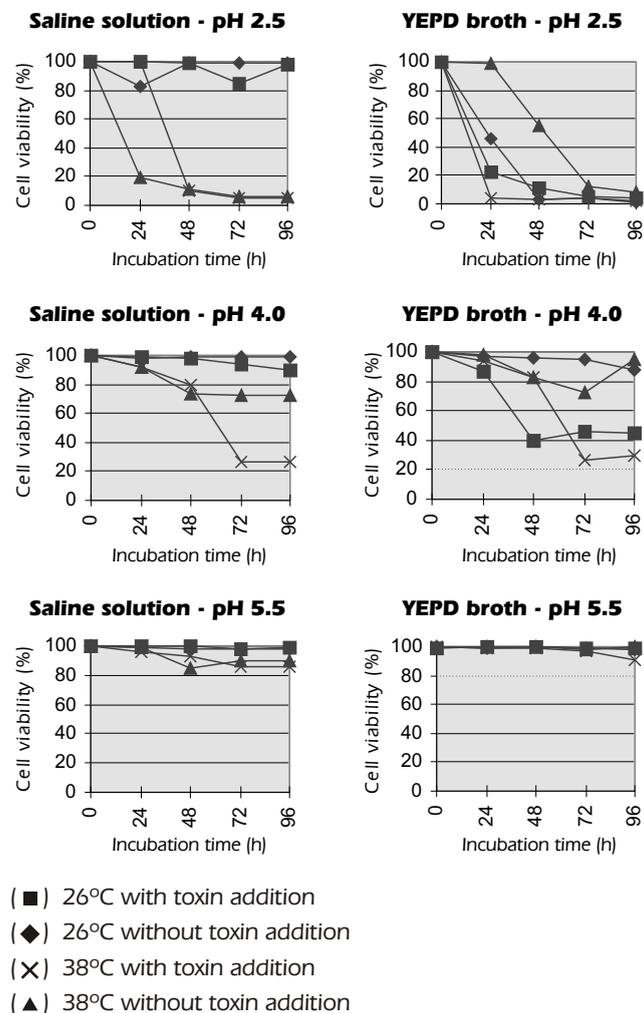
(CCA 118) as a positive control and this strain was cured with cicloheximide and at 37ºC and 40ºC at values of 100%, 71% and 100% respectively, of reduction in the number of killer colonies (comparing to the experiments at 25ºC) as expected, since killer toxin in S. cerevisiae is codified by genes in plasmid, which is susceptible to be cured.

Figure 2 shows that CCA 510 did not present L-dsRNA or M-dsRNA and only total DNA. However, the S. cerevisiae yeast strain showed both dsRNA, around 5.0 Kb and 2.0 KB, for L-dsRNA and M-dsRNA, respectively. These results are in concordance with FIELD (1982), WICKNER (1992), BROWN et al. (1994) and SOARES AND SATO (2000), although only L-dsRNA may be present in some S. cerevisiae strains.

The results obtained with the curing and the detection of dsRNA in CCA 510 (non-Saccharomyces) indicate that the killer character in this strain is codified by chromosomal gene, instead of plasmidial gene. It is also important because of the stability of the killing action.

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Figure 2 *Agarose gel electrophoresis of nucleic acids extracted from the killer yeasts CCA 510 and CCA 118 (S. cerevisiae). Legend: a- Total DNA; b- L-dsRNA; c- M-dsRNA; M- 1 Kb molecular marker.*
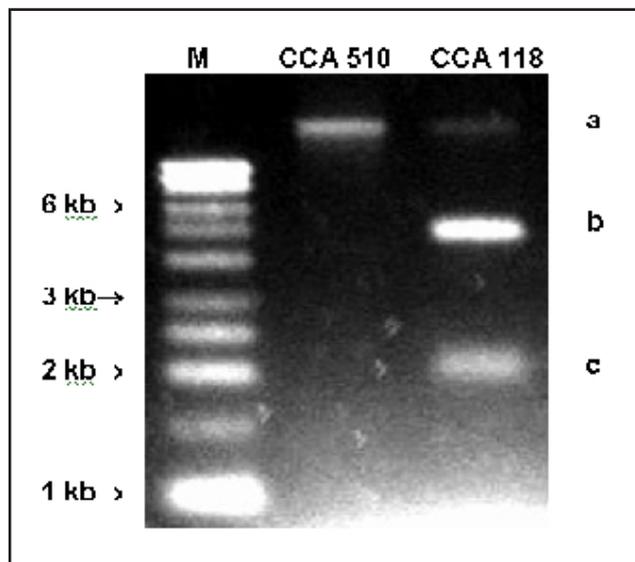
3.2 Fermentative efficiency of killer yeast strain in batch cell recycle system.

The results showed that the strain CCA 510 had lower fermentative efficiency comparing to the baking yeast, which is the most common inoculum used by the producing units, in flasks or fermentor. However, it showed high cell viability 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 (Figures 3 and 4). Although the fermentation conditions are not stated as ideal for the killing activity, concerning large pH variation, higher temperature (30ºC) and low aeration, the strain CCA 510 maintained its killing action both in flasks or fermentor. The commercial brand of yeast did not show any killer activity in any experiment. It is important to stress that not only showing a killer phenotype is an important yeast characteristics, but the expression of this killing action during the fermentation process, in industrial scale, under limiting conditions presented by the culture medium and environmental conditions.

3.3 Biotyping of industrial yeast strains by killer system.

The sensitivity to the killer toxin was demonstrated by the presence of a clear zone around the killer yeast colony, as well as the formation of a bluish line around it, which was originated by the dead cells dyed by the methylene-blue. Figure 5 ilustrates the Petri plates inoculated with the killer yeasts and the test yeasts.

The dendrogram in Figure 6 was constructed with the results obtained for the sensitivity of each test strain to the 29 killer yeast strains. Exception to three S. cerevisiae yeast strains, the other strains from this species were included in the same group, which showed almost 65% of similarity with the second group. There was also a third group with two different species. These results are in great concordance with the fingerprinting obtained using RAPD analysis with OPA-11 primer (TOSTA, 2004).

The variation in similarity inside the first group (S. cerevisiae) may be correlated with the origin of the strains. For example, CCA 007, which was isolated from `saké` showed the lowest similarity percentage in relation to the others, which were isolated from the alcoholic fermentation process.

By the results obtained, the killer sensitivity profile allowed the discrimination of taxonomically different yeast groups and besides the discrimination among strains isolated from different origins, but from the same species (S. cerevisiae). It is really interesting considering that many contaminant strains are S. cerevisiae and could be distinguished from the starter inoculum using this technique. This kind of approach was successfully used by VAUGHAN-MARTINI et al. (1988, 1996), BUZZINI AND MARTINI (2000 a,b).

We concluded that the killer system is an interesting and useful characteristics to be considered for ethanol-making yeasts when in competition with undesirable strains, specially when the killer activity is expressed in fermentation conditions, which is not appropriate for the killer activity. A highly potential killer strain was isolated (CCA 510) with fermentative capability and a stable killing action. Besides, the killer system may be a fast, effective and inexpensive tool for the industrial yeast strain fingerprinting once the discrimination is at intraspecific level. More studies are needed to elaborate an easy-to-use technique which could be used in industrial units.

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Figure 3 Cellular viability (%), ethanol production (g/100 mL), biomass yield (g/g sugar), ethanol yield (g/g sugar), fermentative efficiency (%) and killing inhibition zone (mm)1 during batch alcoholic fermentation, in flasks, at 300 C, using clarified sugarcane juice (120 Brix), 20% inoculum, with cell recycling (six 12-hour cycles), using killer yeast strain (CCA 510) and baking yeast.
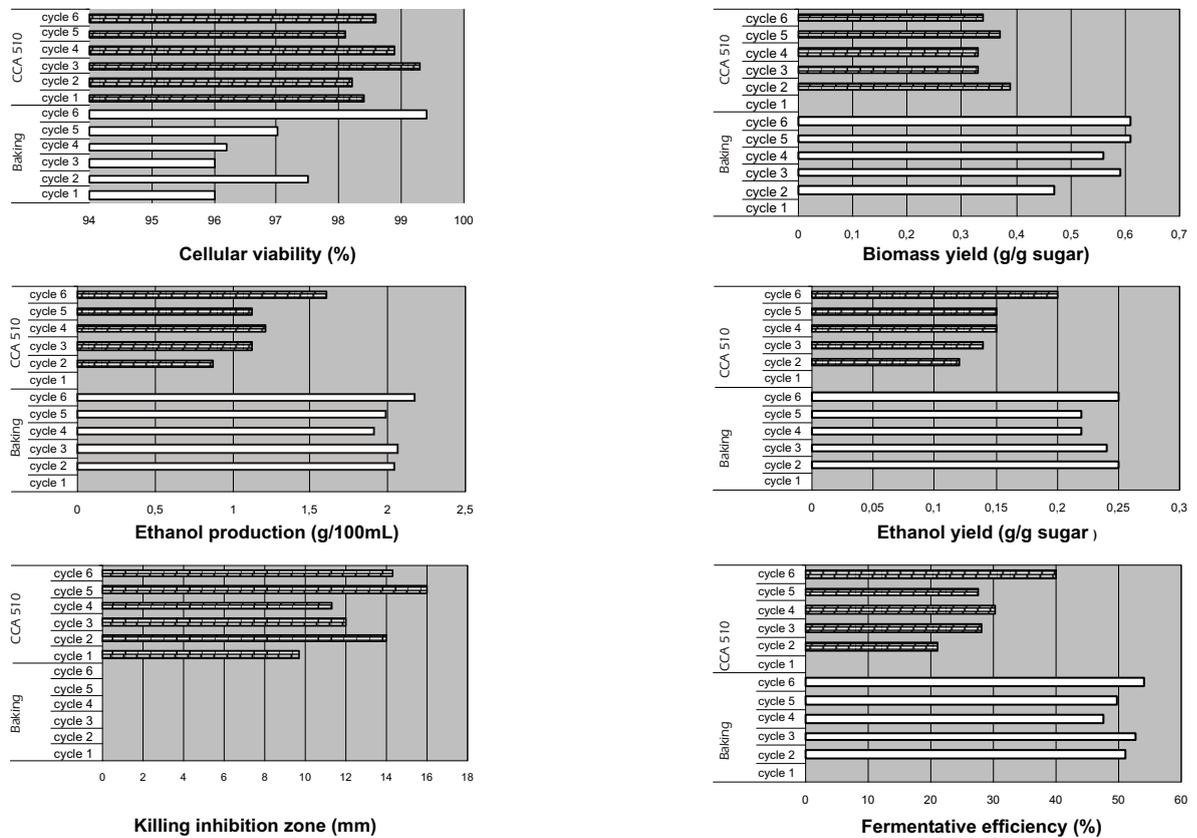   1against S. cerevisiae NCYC 1006

Figure 4 Cellular viability (%), fermentative efficiency (%), ethanol production (g/100 mL) and killing inhibition zone (mm)1 during batch alcoholic fermentation, in 4-liter fermentor, at 300 C, using clarified sugarcane juice (100 Brix), 20% inoculum, with cell recycling (four 12-hour cycles), using killer yeast strain (CCA 510) and baking yeast.
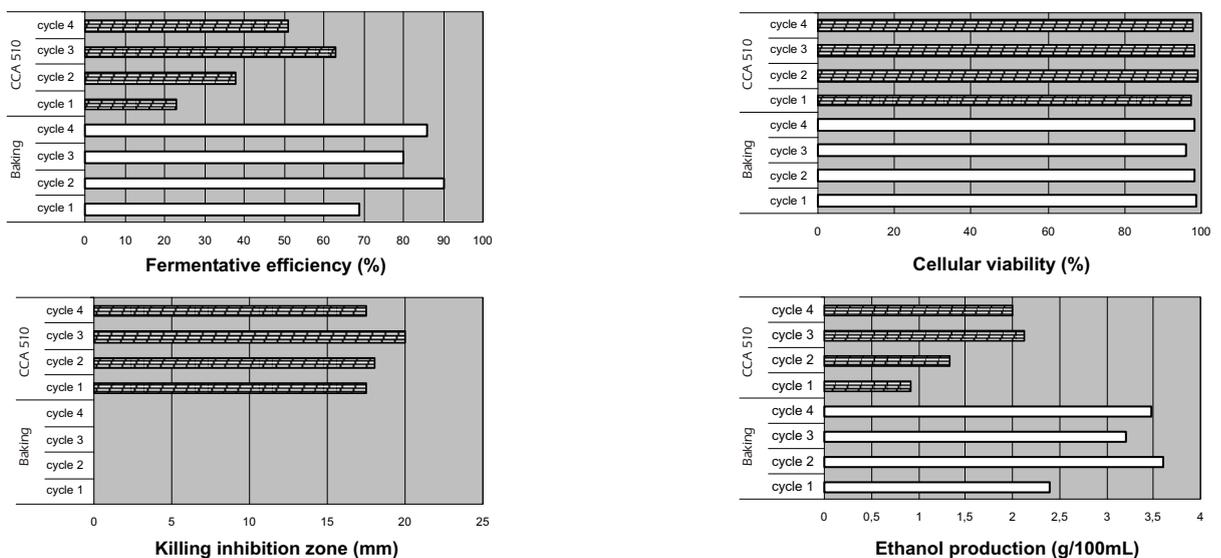   1against S. cerevisiae NCYC 1006

Figure 5 Killer tests in Petri dishes. Differences between the two strains (both S. cerevisiae) relative to the sensitivity to killer yeast strains (inoculated onto the agar surface as small colonies) are observed through the presence of inhibition zones and blue line around the killer strains.
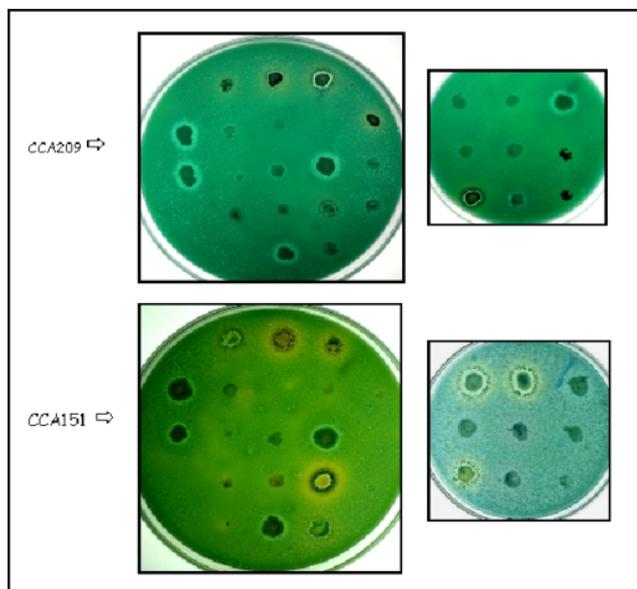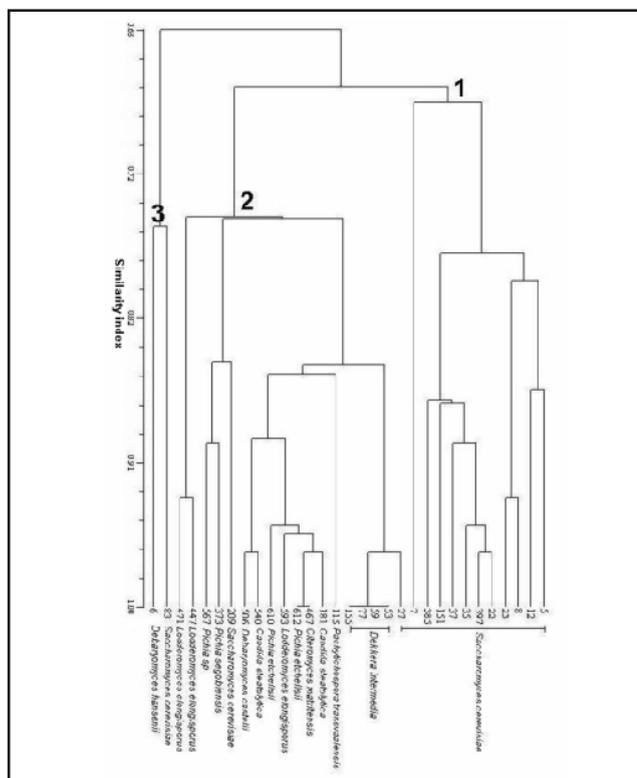

Figure 6 . Dendrogram using the UPGMA method based on the Simple Matching Index with the results of sensitivity of test strains to the 29 killer yeast strains.

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