NEGATIVE H$_2$S CHARACTER AND FLOCCULATION AS YEAST STRAIN MARKERS FOR INOCULUM RECOVERY

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ABSTRACT: Routine identification of yeast behavior is essential to measure the control of the alcohol production process and to maintain product quality standards. This work utilized the non-hydrogen sulfide production and flocculation traits as characteristic strain markers for the evaluation of cell recycling during the alcoholic fermentation process for production of sugarcane alcohol. This study evaluated the behavior of a recombinant yeast bank made by protoplast fusion, for strain screening purposes; strain fermentative kinetics in comparison to commercial baker yeast; viability and recovery of the selected strain on differential media, after five consecutive fermentation batches; and the recovery of the selected strain from fermentation with mixed strain cultures. The strain selected for the H$_2$S negative character kept its viability during successive recyclings, with contamination levels not detected by the method of analysis. It also presented a kinetic behavior similar to that of baker yeast, either in single or mixed culture fermentations, opening new possibilities for further work on quality control of cell recycling in the alcoholic fermentation process.
Key words: cell recovery, hydrogen sulfide, ethanolic fermentation, quality control

O CARATER H$_2$S NEGATIVO E A FLOCULAÇÃO COMO MARCADORES DE LEVEDURAS NA RECUPERAÇÃO DE INÓCULO

RESUMO: A identificação rotineira do comportamento das leveduras é essencial à manutenção do controle e do padrão de qualidade dos processos fermentativos para produção de álcool. Em função da baixa freqüência de ocorrência de leveduras não produtoras de ácido sulfídrico e floculantes, pretendeu-se neste trabalho utilizar estas características como marcadores de linhagem na avaliação da reciclagem de células em processos de fermentação alcoólica do caldo de cana para a produção de aguardente. O trabalho foi elaborado com base no acompanhamento das características de um banco de leveduras recombinantes produzidas por fusão de protoplastos para seleção de linhagem; no acompanhamento da cinética fermentativa da linhagem selecionada em comparação ao fermento de panificação comercial, em função de seu emprego comum em processos fermentativos; na avaliação da viabilidade e da recuperação da linhagem selecionada em meio diferencial após cinco reciclos; na avaliação da recuperação desta linhagem em cultura mista com o fermento de panificação comercial. A levedura selecionada para o caráter H$_2$S negativo manteve sua viabilidade durante os reciclos, com níveis de contaminação não detectados pela metodologia empregada, apresentando comportamento cinético semelhante ao da levedura de panificação tanto em cultura pura quanto em cultivo misto, apresentando uma nova possibilidade para estudos sobre o controle de qualidade de processos de fermentação alcoólica com reciclagem de células.
Palavras-chave: reciclo de células, controle de qualidade, ácido sulfídrico, fermentação alcoólica

INTRODUCTION

Yeast is an important microorganism in the field of applied microbiology, and precise methodologies have been developed to study the growing relationships between yeast for various areas of research and development, technology and health. In the food industry, yeast acts as an agent for production as well as for the breakdown of products. Routine identification of yeast behavior is essential to measure the quality control of the alcohol production process and to maintain product quality standards. Standard industrial procedures routinely use among 80 to 100 morphologic and physiologic tests and the identification methods must be fast and inexpensive (Török & King Jr., 1991).

Through recent progress, easier techniques have been developed for the identification of yeast, such as serologic testing (Campbell, 1971; 1972) and fatty acid chromatographic profiling (Oosthuizen et al., 1987). However, the most acceptable methods are the taxonomy handbooks by Barnett et al. (1983), based upon physiological tests, and by Kreger-van Rij (1984), which describes species morphology without differentiating yeast strains within the same species.

Methods based upon gene expression (phenotypic) can be affected by environmental factors, and their

results depend on the metabolic state of the cells, and require accurate standardization of the procedures to obtain reproducibility of the results. DNA analysis gives results with higher confidence and resolution to distinguish strains within the same yeast species (Ness et al., 1993).

Yeast identification is a complex process and only a few laboratories have the technical competence, resources and qualified personnel to apply molecular techniques utilized to identify yeast. Therefore, the physiologic tests are used more frequently by food analysis laboratories (Velázquez et al., 2001).

Because of the need of microbiological controls in alcoholic fermentation processes, which recycle cells, the best identification method, as an example of other biotechnological processes that utilize yeast, would be one that would allows better resolution, greater accuracy, with the lowest demand for labor, material, space and time. Unfortunately, this method is not yet available (Deák & Beuchat, 1987).

Therefore, the choice of a yeast strain with odd characteristics, that occurs in nature at very low levels or even those that are non-existent in the nature (genetically engineered), would greatly facilitate its identification and the degree of contamination at different stages of the fermentative process. For instance, Hinrichs et al. (1999) produced yeast with the cloned gene that encoded for the barley β-glucanase in the LEU2 gene of *Saccharomyces cerevisiae*, and this allowed for precise identification.

Martins et al. (1999) used protoplast fusion to produce recombinant yeast negative for HI_2S (hydrogen sulfide) production and maintaining the flocculation trait. These characteristics were discussed in the fermentation studies of sugarcane juice (Ribeiro & Horii, 1999), and in the quality of sugarcane spirit (Ribeiro & Horii, 1998). Martins et al. (1999) reported that the first recombinant yeast strains isolated did not show the expected stability for these traits. Other recombinant populations that had not previously been screened for these traits were submitted to a selection study to assess their characteristics on the fermentation of sugarcane must or juice. The objective of this study was to develop a method for the evaluation of the quality control of the inoculum during the alcoholic fermentation process, which recycles cells, using the H_2S trait as a strain marker.

MATERIAL AND METHODS

Microorganisms - This study evaluated 12 recombinant yeast populations obtained from protoplast fusions between auxotrophic mutants derived from a non-hydrogen sulfide producing strain (IZ 987), and the flocculant strain (ABXR 11B), stored as YEPDA slant cultures covered with mineral oil and stored at 5°C (Martins et al., 1999). Thirteen strains isolated by Martins et al. (1999) with the

desired traits and stored on YEPDA slant cultures at 5°C, and a strain isolated from a commercial baker yeast, were also evaluated.

Culture Media - Yeast extract peptone dextrose (YEPD) was used for the selection of the flocculation trait. Yeast extract peptone dextrose agar (YEPDA) was used for storage and isolation of the strains, while Bacto Bismuth Sulfite Agar Dehydrated - BSA (Difco) was used for the selection of the H_2S trait. The non-sulfide producing strains had white colonies, while the H_2S producers presented various colony colors that ranged from light brown to black, depending upon the intensity of the production. Bacto W L Nutrient Medium Dehydrated – WLN (Difco) was used to record cell growth during fermentation assays conducted with a mixture in cultivation. Clarified sugarcane juice (Ribeiro & Horii, 1999), was sterilized (121°C; 15 min.) for the majority of the treatments, with the exception of the assays conducted to recycle the selected yeast strain. In this case, the juice was not supplemented (Barone, 1994).

Selection of non-hydrogen sulfide producing yeast strains - Because of the simplicity of differentiating recombinants, the first trait to be screened during selection was the non-hydrogen sulfide characteristic of colonies. The selected strains were inoculated directly on BSA plates to monitor the stability of this trait. Populations of microorganisms derived from protoplast fusion were inoculated on the surface of the YEPDA medium after eliminating the mineral oil by making a suspension culture and serial dilutions. Plates that had good yeast growth were replicated onto differential BSA media. Growth conditions were 30°C for 24 to 48 h.

Selection of flocculent yeast strains - Flocculation tests were carried out in 250-mL Erlenmeyer flasks with 100 mL of YEPD medium by visual evaluation of floccule formation after 48 h incubation at 30°C on an orbital shaker at 100 rpm. Since the strains were highly flocculent and floccules reached a few mm in size, the visual evaluation was acceptable at this stage. Inoculum from the previously selected strains were obtained from their respective stock tubes and inoculated on YPDA slants. Populations of hybrids and segregants derived from protoplast fusions were plated on BSA medium. Colonies that did not produce hydrogen sulfide were recovered and utilized as inoculum after being stored on YPDA slants.

Growth evaluation of the recombinants in sugarcane juice must - The recombinants that did not produce hydrogen sulfide were stored on YEPDA slant cultures. Inocula were obtained from these stocks and resuspended in distilled, sterile water amended with 1.5 g L^{-1} of dry matter content to inoculate 250 mL Erlenmeyer flasks containing 100 mL of must from sterilized sugarcane juice. The concentration of the inoculum was 15.0 mg L^{-1} dry

matter. Cultures were incubated on a rotatory shaker at 100 rpm, 30°C for 24 h. Aliquots of the wine were then collected, centrifuged three times after washing the pellet with distilled water followed by a 1:10 dilution (v v^{-1}) of the original aliquot to determine the optical density at 600 nm absorbance (Beckman DBG spectrophotometer).

Comparative analysis to assess the kinetic parameters of the selected recombinant

    Inoculum propagation - Pure cultures of the recombinant strains and of the isolated strain from the commercial baker yeast were propagated separately according to the following: a cell suspension culture containing 1.5 g L^{-1} (dry matter) was utilized to inoculate 100 mL of YEPD in 250 mL Erlenmeyer flasks at a concentration of 15 mg L^{-1}. Cultures were incubated for 24 h at 30°C and at 100 rpm in a rotatory shaker. The cellular mass produced was used to inoculate three, 4-L fermentation vats containing sugarcane juice must, clarified and sterilized. The fermentation occurred at 30°C and with reed agitation at 100 rpm. At the end of the fermentation, the cellular mass was separated by centrifugation followed by three consecutive washes in sterilized water.

    **Comparative assay and evaluated parameters - ** Two treatments were conducted in this assay according to the utilized strain: PF132b4 treatment - selected strain from the growth evaluation assay of the recombinants in sugarcane juice must - and the FP treatment - isolated strain from commercial baker yeast. The fermentation process occurred in the fermentation vats under the same incubation conditions. Each treatment had three replications and the concentration of the inoculum was at 4 g L^{-1} dry matter. Each 4-L fermentation vat contained clarified and sterilized must. In all cases, the cessation of the fermentation process was determined by the end of alcohol production. The following determinations and estimations were carried out: Areometric brix (AOAC, 1995) done on the treated juice, must, and must in fermentation; Total reduced sugars (TRS), through the Somogyi & Nelson spectrophotometer method according to Neish (1952); Yeast concentration, via the spectrophotometer method proposed by Stratford & Keenan (1988); Alcohol content, by utilizing a conversion table for the density value found by the digital densimeter PAAR model DmA-45 (Barone, 1994); Cell concentration produced according to the equation: $X = X_f - X_0$, where X = cell concentration produced (g L^{-1} of dry matter); X_f = final cell concentration (g L^{-1} of dry matter); X_0 = initial cell concentration (g L^{-1} of dry matter); Percentage of produced cells $X_{\%} = (X/X_f)*100$; Sugar consumption: $S = - (S_f - S_0)$, where: S = used sugar (g L^{-1} of glucose); S_f = final sugar concentration (g L^{-1} of glucose); S_0 = initial sugar concentration (g L^{-1} of glucose); Conversion factor for the substrate to biomass $Y_{x/s} = X/S$; Produced ethanol: $P = P_f - P_i$, where: P = produced ethanol (g L^{-1}); P_f = final ethanol concen-

tration (g L^{-1}); P_i = initial ethanol concentration (g L^{-1}); Conversion factor for the substrate to ethanol $Y_{\frac{p}{s}} = P/S$; Ethanol yield: PR=P/t, where: PR = ethanol yield (g L^{-1} h^{-1}), t = fermentation period (h); Fermentation efficiency, based upon the theoretical yield obtained from the Gay-Lussac equation (51.1 g of ethanol per 100 g of glucose): $\eta_{P(\%)} = (Y_{\frac{p}{s}} / 51.1) \times 100$, and Maximum specific growth rate according to the angular coefficient of the linear equation of the natural logarithm of the yeast concentration ln[x] in function of the fermentation period t(h) related to the exponential growth phase, which was determined by the linear regression (Pirt, 1985): ln[X] = μt + b, where: μ = slope (maximum specific growth rate h^{-1}); and b = intercept.

**Monitoring the recombinants during cell recycling - ** The cellular mass from the selected strain, necessary for the inoculation of the initial fermentation, was obtained according to the mentioned method. Five consecutive fermentation cycles were carried out with three repetitions each, and cells were recycled until their quantity was enough to reach an inoculum concentration of 4 g L^{-1}. The fermentation conditions were the same mentioned previously. The following parameters were determined at the end of fermentation: cell viability through the methodology described by Borzani & Vairo (1970); recovery percentage of the non-H$_2$S producing [$X_{H2S-}(\%)$] trait, by counting colony forming units (CFU) after plating a serial dilution (10^{-4} to 10^{-7}) of the wine on BSA medium. Each dilution had three replicates. The recovery percentage of non-H$_2$S producing colonies was defined as a result of the following equation: X_{H2S-} = (total number of cfu – number of H$_2$S positive cfus)100 / total number of colonies

Recombinant recovery from a commercial baker yeast mixed culture - The inoculum concentration for this assay was 4 g L^{-1} dry matter, of which 2 g L^{-1} was from the selected strain and 2 g L^{-1} from the strain isolated from commercial baker yeast. Three repetitions were carried out in 4-L fermentation vats of must from sterilized sugarcane juice as mentioned previously. Samples were taken at the initial and end of the fermentation process and were diluted from 10^{-4} to 10^{-7}, plated on WLN medium and after 24 h at 30°C, colonies were replicated onto BSA differential medium. Each plating had three replicates. The mean concentration of the non-H$_2$S (selected strain) and positive H$_2$S producing (commercial baker yeast) strain were given by CFU mL^{-1} of sample.

RESULTS AND DISCUSSION

**Follow-up of the selected strains - ** Six months post-storage with monthly replication, the 13 selected strains were able to maintain the flocculation trait, but not the non-hydrogen sulfide-producing trait.

Selection on the fusion products - The fusion products that showed non-hydrogen sulfide producing colonies were denominated as PF 67b, PF77, PF 132b, and PF170. In all cases, colonies produced this acid and cells derived from these colonies were not able to form visual and detectable flocks in YEPD medium (Table 1).

The instability for the studied yeast traits might have resulted from the taxonomic distance between the original yeast strains (IZ 987 and ABXR11B) (Martins et al., 1999), identified by the differences on their electrophoretic karyotype profiles and by the low similarity coefficient determined by the dendogram derived from RAPD studies. Recent studies suggest that the IZ 987 strain is *Picchia anomala*, which has shown a variable behavior (Anchorena-Matienzo, 2002). This hypothesis is in agreement with Heluane et al. (1993), who reported alterations in their genomes. Therefore, using higher number of original strains when employing this hybridization technique would increase the chances of producing stable recombinants for the desired traits due to a higher probability of obtaining compatibility between parents.

Growth of the strains in sugarcane juice must - Data on the strain growth rates, measured by the optical density (OD) reading at 600 nm, of non-hydrogen sulfide producers in sugarcane juice must 24 h after incubation and at 1:10 dilution (v v^{-1}) are shown in Table 2. The best growth rate was observed for the PF132b4 strain, which was selected to continue the assay, and denominated "treatment PF132b4".

Comparative analysis to evaluate the kinetic parameters of the selected recombinant - Yeast growth (X), ethanol produced (P), and the substrate concentration (S) during the fermentation process are shown in Figures 1 (PF132b4 treatment) and 2 (FP treatment). Data on the

ethanol production utilized the criterium that the fermentation process lasts for 15 h in both treatments. Profiles of yeast behavior were very similar between the strains, and were converted to kinetic parameters (Table 3).

Table 2 - Optical density (OD) reading at $\lambda = 600$ nm, 24 h after fermentation.

Strain	OD$_{600\ nm}$	Strain	OD$_{600\ nm}$
PF67b1	0.013	PF67b19	0.004
PF67b2	0.004	PF132b1	0.004
PF67b3	0.000	PF132b2	0.018
PF67b4	0.004	PF132b3	0.022
PF67b5	0.000	PF132b4	0.538
PF67b6	0.000	PF132b5	0.292
PF67b7	0.013	PF170a1	0.086
PF67b8	0.004	PF170a2	0.004
PF67b9	0.004	PF170a3	0.080
PF67b10	0.004	PF170a4	0.080
PF67b11	0.013	PF170a5	0.031
PF67b12	0.004	PF170a6	0.080
PF67b13	0.008	PF170a7	0.004
PF67b14	0.004	PF170a8	0.004
PF67b15	0.013	PF170a9	0.070
PF67b16	0.004	PF170a10	0.004
PF67b17	0.018	PF170a11	0.004
PF67b18	0.004		

Figure 1 - Fermentation kinetics, Treatment PF132b4.

Table 1 - Selection of the non-hydrogen sulfide producing and flocculant yeast strains.

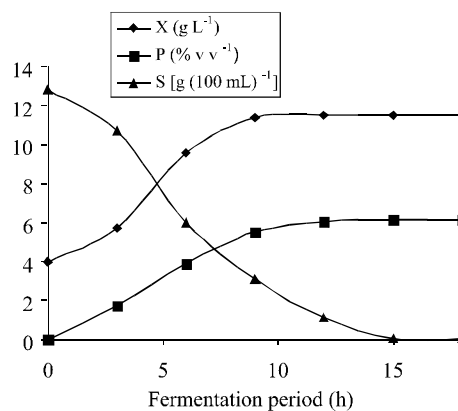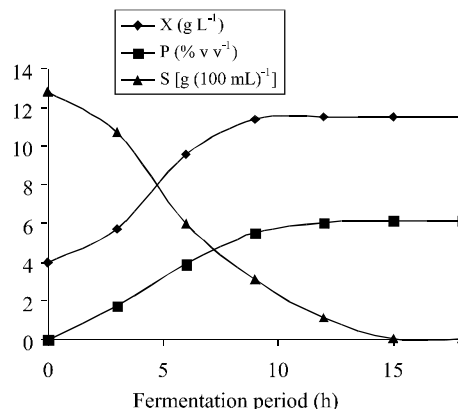|	Colony numbers that expressed the traits	
Fusion Products	Negative H$_2$S	Flocculation
PF 6	0	-
PF 7b	0	-
PF 65	0	-
PF 67b	19	0
PF 70b	0	-
PF 72	0	-
PF 73	0	-
PF 74	0	-
PF 77	9	0
PF 130	0	-
PF 132b	5	0
PF 170	11	0

Figure 2 - Fermentation kinetics, Treatment FP.

Table 3 - Results on the fermentation parameters of the investigated strains (mean of 3 repetitions).

Parameters	PF132b4	FP
t	15	15
X_0	4.00	4.00
X_f	11.7	11.5
X	7.7	7.5
$X_\%$	65.8	65.2
S	127	126
$Y_{\frac{X}{S}}$	0.0606	0.0595
P	50.6	48.8
$Y_{\frac{P}{S}}$	0.398	0.387
EY	3.37	3.25
$\eta_{P(\%)}$	77.9	75.7
μ_{max}	0.158	0.172

*Period between 3 to 6 h after fermentation. t: Fermentation period(h); X_0 = Initial cell concentration (g L^{-1}); X_f = Final cell concentration (g L^{-1}); X = Produced cell concentration (g L^{-1}); $X_\%$ = Percentage of produced cells (%); S = Sugar consumption (g L^{-1}); $Y_{\frac{X}{S}}$ = Conversion factor of substrate to biomass; P= Ethanol produced (g L^{-1}); $Y_{\frac{P}{S}}$ = Conversion factor of the substrate to ethanol (g g^{-1}); EY=Ethanol yield (g L^{-1} h^{-1}); $\eta_{P(\%)}$ = Fermentation efficiency (%); μ_{max} = Maximum specific growth rate (h^{-1}).

The evolution curves for cellular growth of the treatments presented a higher growth rate during the period between the third and sixth hour of fermentation. This period was utilized to estimate the maximum specific growth rate, from linear regressions ln[x] = μ t + b, calculating μ by finite difference: μ = Δln[x]/Δt.

The kinetic parameters did not present differences between strains. Therefore, it was assumed that strain PF132b4 has the same fermentative potential as FP.

Follow-up of the recombinant through cell recycling - At the end of all fermentation processes, the recovery percentage of the non-H_2S producing yeast was 100% and the viability of cells was maintained according to Table 4. The thermal treatment used for the clarification of the must associated with the aseptic conditions of the fermentation and with the metabolism of the strain during the alcoholic fermentation was sufficient to avoid contamination caused by hydrogen sulfide producing microorganisms at detectable levels of the utilized methodology. Moreover, the utilization of the differential BSA medium should not have favoured any positive results. A larger number of cell recycling assays on a larger scale should be used to indicate proper recommendations.

Recombinant recovery from a commercial baker's yeast mixed culture - Recombinant colonies were obtained from the fermentation assays utilizing a mixed culture of a commercial baker yeast and the PF132b4 strain. These colonies were replicated from solid WLN onto BSA medium after a 24 h-incubation period. The number of CFUs at the initial and cessation of fermentation process is illustrated in Table 5.

Table 4 - Cell viability in the recycled inoculum.

Fermentation	Viability
	--------------- % ---------------
1	98
2	96
3	97
4	95
5	97

Table 5 - Colony forming units (CFU) at the initial and end of the fermentation process (mean of three repetitions).

	Negative H_2S	Positive H_2S	Total
	--------------- CFU mL^{-1} ---------------		
At the initial of fermentation	$1.0 . 10^8$	$1.1 . 10^8$	$2.1 . 10^8$
At the end of fermentation	$2.8 . 10^8$	$2.9 . 10^8$	$5.7 . 10^8$

Given that the strains had similar kinetic behavior, they also showed similar growth when plated on solid media, and that did not indicate antagonism between them, since the proportion between the negative and positive H_2S colonies were maintained. This fact confirms the usage of BSA medium as an indicator for the recovery of strains and for the evaluation of the degree of contamination in processes that utilize the non-hydrogen sulfide-producing trait as a marker.

CONCLUSIONS

The strains submitted to the transformation technique through protoplast fusion did not produce recombinants that kept both traits, non-hydrogen sulfide production and flocculation, simultaneously. The selected strain PF132b4 produced similar kinetic parameters in relation to that isolated from a commercial baker yeast, which indicated that the selected strain could be used in biotechnological processes of alcoholic fermentation. The differential BSA medium was suitable for the evaluation of inoculum recycling, and gave coherent results regarding the fermentation utilizing pure cultures of the selected strain as well as with mixed cultures of a strain isolated from a commercial baker yeast.

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