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# SUSCEPTIBILITY OF *SACCHAROMYCES CEREVISIAE* AND LACTIC ACID BACTERIA FROM THE ALCOHOL INDUSTRY TO SEVERAL ANTIMICROBIAL COMPOUNDS

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## ABSTRACT

The antimicrobial effect of several products including commercial formulations currently used in sugar and alcohol factories was determined by adapted MIC (Minimal Inhibitory Concentration) test on *Saccharomyces cerevisiae* and on natural contaminants *Lactobacillus fermentum* and *Leuconostoc mesenteroides.* The MIC test by macrodilution broth method was adapted by formulating of the culture medium with cane juice closely simulating industrial alcoholic fermentation must. Acid penicillin V (MIC 0.10-0.20 µg/ml) and clindamycin (MIC 0.05-0.40 µg/ml) were most effective against bacterial growth in 24 h. Among the chemicals, sulphite (MIC 10-40 µg/ml), nitrite (MIC <117 µg/ml) and copper sulphate (75-300 µg/ml) were the most effective. Zinc and manganese ethylene-bis-dithiocarbamate and dimethyldithiocarbamate did not show good inhibitory effect on bacteria (MIC > 50 µg/ml). Methyldithiocarbamate was efficient only on *L. fermentum* (MIC 2.5 µg/ml) and *S. cerevisiae* (MIC 5.0 µg/ml). Thiocianate (MIC 1.2-5.0 µg/ml), bromophenate (MIC 9-18 µg/ml) and n- alkyldimethylbenzylammonium cloride (MIC 1-8 µg/ml) affected *S. cerevisiae* at similar inhibitory concentration for *L. mesenteroides* or *L. fermentum.* Formaldehyde was more effective on bacteria (MIC 11.5 - 23 µg/ml) in both pH (4.5 and 6.5) than yeast (MIC 46-92 µg/ml). Several tested formulated biocides seriously affect *S. cerevisiae* growth in the similar dosages of the bacterial inhibition, so these products should be avoided or used only in special conditions for the bacterium control of fermentation process. For this step, the control of these contaminants by antibiotics are more suitable and effective.

**Key words:** Antimicrobial compound, MIC, *S. cerevisiae*, lactic acid bacteria

## INTRODUCTION

Lactic acid bacteria, *Lactobacillus* and *Leuconostoc*, are common contaminants of yeast alcoholic fermentation and are frequently associated with process problems (10,16). Commercial fuel ethanol in Brazil is currently produced by fed-batch or continuous fermentation process of sugar cane by *Saccharomyces cerevisiae* with cell recycle. Microbial contaminants are also recycled with yeast and this may cause many problems due to the competition between bacteria and yeasts for the same substrate. *Lactobacillus* is adapted to the alcoholic and nutritional conditions of the process (17), but *Leuconostoc* is more sensitive to alcohol and usually does not persist for long period in alcoholic fermentation (14). In addition to acid production, *Lactobacillus* causes serious problems of yeast flocculation in the alcoholic fermentation (20,24). The antagonism between *Lactobacillus* and *Saccharomyces cerevisiae* is due to organic acids produced by the bacterial cells. Lactic acid can strongly inhibit yeast metabolism and decrease alcoholic yield. Essia-Ngang *et al.* (8) observed 30% decrease in ethanol yield by yeast fermentation of beet sugar with 5 g lactic acid/l produced by lactic acid bacteria contamination. Maiorella *et al.* (15) noted an 80% reduction in

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the yeast population in the presence of 40 g lactic acid/l. Alcoholic fermentation and yeast viability are strongly reduced by *L. fermentum* after a few cycles in a fed-batch process with cell recycles, if a method of bacterial control is ommitted (17).

The bacterial control in industrial fuel alcoholic fermentation in Brazil is currently done by sulphuric acid washing of yeast cell suspension (5,22). Sometimes, this process helped by addition of biocides in wort such as carbamates, quaternary ammonium compounds, halogenated phenols and antibiotics (penicillin, virginiamicin, Kamoran HJ).

Some antibiotics such as tetracycline (2) and chloramphenicol (1) have been tested in alcoholic fermentations but have proven to be unsuitable for industrial applications. Bacterial contaminants are frequently adaptable to the products used for their control particularly antibiotics which makes industrial control difficult (7). Recently 3,4,4' trichlorocarbanilide imobilized in calcium alginate was proposed to control *Lactobacillus fermentum* in alcoholic fermentation (19). This product showed bacterial inhibition in dosage that did not affect *Saccharomyces cerevisiae*. Biocides currently used in industrial fuel alcoholic fermentation in Brazil were usually effective against growth of bacterial contaminants, but could affect yeast at similar concentration. This paper shows the results of the antimicrobial ability of several products including commercial formulations used in industrial alcoholic fermentation. The effect on bacterial contaminants from alcohol industry was compared to the effect on *Saccharomyces cerevisiae*.

## MATERIALS AND METHODS

### Microorganisms

The cultures used in this work were obtained from Tropical Culture Collection (CCT), Campinas - Brazil: *Saccharomyces cerevisiae* CCT 0472, *Lactobacillus fermentum* CCT 1400 and *Leuconostoc mesenteroides* CCT 0367 (both were isolated from Brazilian distilleries with serious problems of bacterial contamination), *L. fermentum* CCT 0559 and *L. mesenteroides* CCT 0582 (from American Type Culture Collection, ATCC 9338 and ATCC 10830, respectivelly). The maintenance medium was Man Rogosa & Sharpe (Difco) for bacteria and Yeast Malt Extract (Difco) for yeast.

### Minimal Inhibitory Concentration (MIC)

MIC of the products was determined by adapted macrodilution broth method (13), in tubes with 6 ml medium containing 40 g/l dry cane juice (concentrated and dryed natural cane juice) and 5.0 g/l yeast extract (Difco) dissolved in distilled water and pH 4.5 corrected with 1 N HCl (18). As the results were intended for application in sugar cane and alcohol factories, the medium formulation was designed as closely as possible to duplicate commercially extracted cane juice. The tubes were sterilized at 121ºC during 15 min. The inoculum was standardized according to Macfarland 0.5 standard (13) in aseptic conditions. The cultures were incubated at 30ºC. The bacterial and yeast cells growth were asepticaly measured by absorbance at 600 nm with a spectrophotometer. MIC for each product was performed in two or three replications and shown as averages. Statistical analysis was carried out to compare averages in treatments (Unpaired t test, ANOVA and Tukey-Krammer Multiple Comparison test) with the Graphpad Instat statiscal program (Rutgers University). MIC of all products including commercial formulations were calculated by amount of active product in pure base.

The list of formulated chemicals tested for minimal inhibitory concentration (MIC) on yeast and bacterial cells is shown in Table 1. Most of them were commonly recommended by different companies for use in microbial control in sugar and alcohol factories. Other tested chemicals were lysozyme (Sigma), copper sulphate (Vetec, Brazil), trisodium polyphosphate (Cinética

**Table 1.** Formulated chemicals tested for minimal inhibitory concentration (MIC) on yeast and lactic acid bacteria.

| Active chemicals | Trade name | Company |
|---|---|---|
| Zinc Manganese ethylene bis dithiocarbamate | - | Dithane-Mancozeb |
| Methyl dithiocarbamate | Buzan 40 | Buckmann Lab. |
| Dimethyldithiocarbamate | Buzan 85 | Buckmann Lab. |
| 3 Methyl 4 chlorine phenol | Preventol CMK | Bayer |
| 2 Benzyl 4 chlorine phenol | Preventol BP | Bayer |
| o-phenyl phenol | Preventol o extra | Bayer |
| Bromophenate | Biopen 400 | Aquatec |
| 2 chlorine acetamide | - | Sigma |
| Benzyl alcohol mono (poly) formaldehyde | Preventol D2 | Bayer |
| Thyocyanate | Buzan 110 | Buckmann Lab. |
| n-alkyl-di-methyl-benzyl ammonium chloride | - | Indústria Química Arujá |
| Formaldehyde | - | Cinética Química Ltda. |
| Glutaraldehyde | - | Labsynth Prod. Lab. |

Química Ltda. Brazil), sodium sulphite (B.Herzog, Brazil), sodium sorbate (Fluka, AG), sodium phosphate (Ecibra, Brazil), sodium nitrite (MB Lab. Quim., Brazil) and tannin (Sigma). The following antibiotics were also included in the experiments: acid penicillin V (Squibb, Brazil), frequently used by different alcohol industries, clindamycin (Upjohn, Brasil) and cephamandole (Sigma).

## RESULTS AND DISCUSSION

MICs of several products on *Leuconostoc mesenteroides* and *Lactobacillus fermentum*, microorganisms involved in contamination of sugar cane extraction plant and alcoholic fermentation measured in comparison with MIC on *Saccharomyces cerevisiae* (Tables 2 and 3). For the antibiotic acid penicillin V, MIC average at pH 4.5 and 24 h. for *L. fermentum* (0.15 µg/ml or 158 IU/l) was not different ($p > 0.05$) than for *L. mesenteroides* (0.14 µg/ml). In contrast, Cruz *et al.* (7) reported that 500-1000 IU/L of this antibiotic was necessary in the must to prevent bacterial infections, while Bayer *et al.* (3), working with 40 strains of *Lactobacillus*, reported a MIC value of 0.48 µg/ml and MBC (maximum bactericide concentration) of 10 and 100 µg/ml of penicillin respectively to kill 22 and 100%. Currently, the industrial process of alcoholic fermentation uses 1 to 4 µg/ml of penicillin in the must every 2 weeks, to control the bacterial infection within $10^5$ to $10^7$ cell/ml.

Clindamycin (Table 2) was an efficient bacterial growth inhibitor with MIC of 0.05 and 0.40 µg/ml, for both bacteria genera, but the cost is more expensive than penicillin. Cephamandole was less active ($p < 0.05$) in *L. mesenteroides* (MIC average 1.16 µg/ml) than in *L. fermentum* (MIC average 0.31 µg/ml). In contrast, Bayer *et al.* (4) found that 20 µg/ml of cephamandole inhibited the growth of 97% strains of *Lactobacillus*.

Sodium sulphite (MIC 10-40 µg/ml), sodium nitrite (MIC <58 to 117 µg/ml) and copper sulphate (75-300 µg/ml) were most effective salts for lactic acid bacteria tested in pH 4.5 (Table 2). The last two chemicals inhibited the yeast in dosage similar to the bacteria, indicating that they were not suitable for control of lactics in alcoholic fermentation. However, only 5,000 µg/ml of sodium sulphite was active on *Saccharomyces cerevisiae*, which was very different ($p < 0.001$) of bacteria MIC. Sodium sulphite is present in concentrated cane molasses about 500-700 mg/l and probably affect more bacteria than yeast cells in mixed wort (cane juice and molasses) depending on their ratio. The average of bacteria MIC of sulphite was significatively ($p < 0.03$) greater in pH 6.5 (410 µg/ml) than pH 4.5 (22.5 µg/ml), which agreed with Foegeding and Busta (9) who indicated that the best pH was below 4.0. The antibacterial action of sulphite in water solution at various pH was investigated by Carr *et al.* (6). Within pH 5 and 9, a mixture of $HSO_3^-$ and $SO_2^{-2}$ was observed and by decreasing pH, sulphite form increased, which was responsible

**Table 2.** Minimal inhibitory concentration (MIC), in µg/ml, of several products on lactic acid bacteria and *Saccharomyces cerevisiae* at pH 4.5 and 6.5 and 30ºC.

| Product | Time (h) | pH | *S. cerevisiae* | *L. fermentum* | | *L. mesenteroides* | |
|---|---|---|---|---|---|---|---|
| | | | CCT 0472 | CCT 1400 | CCT 0559 | CCT0582 | CCT 0367 |
| Copper sulphate | 24 | 4.5 | 75 | 75 | 300 | 75 | 150 |
| | 24 | 6.5 | 140 | 70 | 70 | 65 | 140 |
| Three sodium polyphosphate | 24 | 4.5 | 5000 | 2500 | 2500 | 5000 | 5000 |
| | 24 | 6.5 | 1250 | 625 | 625 | 2500 | 1250 |
| Sodium sulphite | 24 | 4.5 | 5000 | 40 | 20 | 20 | 10 |
| | 24 | 6.5 | 5000 | 625 | 625 | 312 | 78 |
| Sodium sorbate and sodium phosphate (1:1) | 24 | 6.5 | 1250 | 1250 | >2500 | >1250 | 1250 |
| Sodium phosphate | 24 | 6.5 | >12500 | 6250 | 12500 | 3125 | 3125 |
| Sodium nitrite | 24 | 4.5 | 234 | 117 | 117 | <58 | 117 |
| | 24 | 6.5 | 3750 | 1875 | 469 | 234 | 234 |
| Tannin | 24 | 6.5 | >302 | >302 | >302 | >302 | 302 |
| Lysozyme | 24 | 4.5 | >124 | >124 | >124 | >124 | >124 |
| Acid penicillin V | 15 | 4.5 | OF* | 0.10 | 0.05 | 0.05 | 0.10 |
| | 24 | 4.5 | OF | 0.20 | 0.10 | 0.10 | 0.20 |
| Clindamycin | 15 | 4.5 | OF | 0.10 | 0.05 | 0.20 | 0.05 |
| | 24 | 4.5 | OF | 0.10 | 0.05 | 0.40 | 0.05 |
| Cephamandole | 15 | 4.5 | OF | 0.40 | 0.20 | 0.40 | 0.20 |
| | 24 | 4.5 | OF | 0.36 | 0.26 | 1.45 | 0.36 |

OF* - no effect.

**Table 3.** Minimal Inhibitory Concentration (MIC), in µg/ml, of commercial formulations, for lactic acid bacteria and *Saccharomyces cerevisiae*, 30ºC for 24 hours.

| Product | pH | *S. cerevisiae* | *L. fermentum* | | *L. mesenteroides* | |
|---|---|---|---|---|---|---|
| | | CCT 0472 | CCT 1400 | CCT 0559 | CCT0582 | CCT 0367 |
| Zinc manganese ethylene bis dithiocasbamate | 6.5 | 250 | 250 | >250 | >250 | >250 |
| Methyldithiocarbamate | 4.5 | 5.0 | 2.5 | 2.5 | >40 | >40 |
| Dimethyldithiocarbamate | 4.5 | 50 | >50 | >50 | >50 | >50 |
| 3 Methyl 4 chlorine phenol | 4.5 | 37 | 150 | 300 | 150 | 37 |
| 2 Benzyl 4 chlorine phenol | 6.5 | 60 | 30 | 60 | 60 | 60 |
| o-Phenyl phenol | 4.5 | 250 | 62.5 | 62.5 | 125 | 62.5 |
| Bromophenate | 4.5 | 9.0 | 18.0 | 18.0 | 9.0 | 9.0 |
| 2 chlorine acetamide | 6.5 | >300 | >300 | >300 | >300 | >300 |
| Benzyl alcohol mono (poly) hemy formaldehyde | 4.5 | 250 | 62.5 | 62.5 | 125 | 62.5 |
| | 6.5 | 62 | 125 | 125 | 62 | 62 |
| Thiocyanate | 4.5 | 2.5 | 5.0 | 1.2 | 5.0 | 5.0 |
| Formaldehyde | 4.5 | 46.2 | 23.1 | 11.5 | 23 | 23 |
| | 6.5 | 92.5 | 11.5 | 23 | 23 | 5.7 |
| Glutaraldehyde | 6.5 | >300 | >300 | >300 | >300 | >300 |
| n-alkyldimethylbenzyl ammonium chloride | 4.5 | 8.0 | 8.0 | 8.0 | 1.0 | 1.0 |

for major antibacterial effect. *Lactobacillus malli* and *Leuconostoc mesenteroides* showed a fast decrease of ATP at pH 4.0 when they were submitted to 1 mM sulphite. The antibacterial action decreased at pH 6.0, and 2 mM sulphite at pH 5.0 prevented entirely their growth (12). Lysozyme did not produced any growth inhibitory effect up to 124 µg/ml in bacteria and yeast (Table 2), although according to Shan and King (21) it destroyed 60-70% cells of the *Micrococcus lysodeikticus* within 5 min.

The biocides MIC tested in this work are presented in Table 3. Zinc and manganese ethylene bis dithiocarbamate and dimethyldithiocarbamate did not show a satisfactory performance (MIC > 50 µg/ml). The first compound was described (23) as effective on *S. aureus* (MIC 2 µg/ml), but not on *S. cerevisiae* (MIC 200 µg/ml). The optimum pH range for zinc and manganese ethylene bis dithiocarbamate is 5-9 and the probable reason for the ineffectiveness is the unstability in acid medium. Dimethyldithiocarbamate acts better in basic pH. Methyldithiocarbamate was efficacious only on *L. fermentum* (MIC 2.5 µg/ml) and *S. cerevisieae* (MIC 5.0 µg/ml). Thiocyanate (Busan 110) showed MIC on bacteria of 1.2-5.0 µg/ml, and on yeast of 2.5 µg/ml, which indicated it was unsuitable for alcohol industry. Bromophenate (Biopen 400) was effective on bacteria (MIC 9-18 µg/ml) and on yeast (MIC 9 µg/ml). The MIC average of formaldehyde for *S. cerevisiae* (69.3 µg/ml) was significantly ($F=9.998$, $p < 0.05$) higher than for *L. fermentum* (20.2 µg/ml) and *L. mesenteroides* (18.7 µg/ml) at pH 4.5 and 6.5. This product inhibited Gram positive and negative bacteria (*S. aureus* and *E. coli*) with 20 µg/ml and caused disruption of cells of several Gram positive and negative bacteria when treated with twice the concentration of MIC (11). Glutaraldehyde (bacterial MIC > 300 µg/ml) probably reacted with aminated compounds of the must (e.g. protein) and lost the antibacterial activity. Benzyl alcohol mono (poly) hemi formaldehyde was worse than the formaldehyde (MIC 62.5-125 µg/ml) on bacteria and the effect was close to that on yeast (62-250 µg/ml). n-Alkyl-di-methyl-benzyl ammonium chloride also showed similar MIC on tested microorganisms (bacteria MIC 1-8 µg/ml and *Saccharomyces cerevisiae* MIC 8 µg/ml). Commercially formulated biocides for alcohol industry are normally recommended for application in dosages 10-40 µg/ml. They should not inhibit yeast cells of the process, which is one of the most important limitations of their use in alcoholic fermentation. This work showed that current biocides used in industrial fuel alcoholic fermentation in Brazil could affect yeast. In the fermentation step, the antibiotics should be combined with usual methods for controlling bacterial contaminants such as acid washing of yeast in the cell recycle process.

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## RESUMO

### Susceptibilidade de *Saccharomyces cerevisiae* e bactérias láticas provenientes de indústrias alcooleiras a vários compostos antimicrobianos

O efeito antimicrobiano de vários produtos incluindo formulações comerciais usualmente utilizadas em usinas de açúcar e álcool foi determinado pelo teste da Concentração Mínima Inibitória (CMI) adaptada para S*accharomyces cerevisiae* e os contaminantes naturais *Lactobacillus fermentum* and *Leuconostoc mesenteroides.* O teste da CMI foi feito pela adaptação do método da Macrodiluição em caldo pela formulação de um meio de cultivo com caldo de cana em condições similares ao mosto da fermentação alcoólica. Penicilina V Ácida (CMI= 0,10-0,20 µg/ml) e clindamicina (CMI = 0,05-0,40 µg/ml) foram os mais efetivos contra o crescimento bacteriano em 24 horas. Entre os produtos químicos, sulfito (CMI = 10-40 µg/ml), nitrito (CMI <117 µg/ml) e sulfato de cobre (CMI = 75-300 µg/ml) foram os mais efetivos. Etileno-bis-ditiocarbamato de zinco e manganês e dimetilditilcarbamato não apresentaram efeito inibitório satisfatório (CMI > 50 µg/ml). Metilditiocarbamato foi eficiente apenas para *L. fermentum* (CMI= 2,5 µg/ml) e *S. cerevisiae* (CMI= 5,0 µg/ml). Tiocianato (CMI= 1,2-5,0 µg/ml), bromofenato (CMI= 9-18 µg/ml) e n-alquildimetilbenzil cloreto de amônio (CMI= 1-8 µg/ml) afetaram o crescimento de *S. cerevisiae* em concentrações inibitórias similares à *L. mesenteroides* ou *L. fermentum*. Formaldeido foi mais efetivo contra as bactérias (CMI= 11,5-23 µg/ml) em ambos pHs (4,5 e 6,5) em relação à levedura (CMI= 46-92 µg/ml). Vários biocidas testados afetam seriamente o crescimento de *S. cerevisiae*, nas dosagens similares àquelas que inibem as bactérias, portanto estes produtos deveriam ser evitados, ou usados somente em condições especiais, para o controle bacteriano do processo de fermentação. Para esta etapa, o controle destes contaminantes por antibióticos é mais apropriado e efetivo.

**Palavras-chave:** Compostos antimicrobianos, CMI, *S. cerevisiae*, bactérias láticas

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