

Research Paper

Chemicals and lemon essential oil effect on *Alicyclobacillus acidoterrestris* viability

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Abstract

Alicyclobacillus acidoterrestris is considered to be one of the important target microorganisms in the quality control of acidic canned foods. There is an urgent need to develop a suitable method for inhibiting or controlling the germination and outgrowth of *A. acidoterrestris* in acidic drinks. The aim of this work was to evaluate the chemicals used in the lemon industry (sodium benzoate, potassium sorbate), and lemon essential oil as a natural compound, against a strain of *A. acidoterrestris* in MEB medium and in lemon juice concentrate. The results pointed out that sodium benzoate (500-1000-2000 ppm) and lemon essential oil (0.08- 0.12- 0.16%) completely inhibited the germination of *A. acidoterrestris* spores in MEB medium and LJC for 11 days. Potassium sorbate (600-1200 ppm) was more effective to inhibit the growth of the microbial target in lemon juice than in MEB medium. The effect of sodium benzoate, potassium sorbate and essential oil was sporostatic in MEB and LJC as they did not affect spore viability.

Key words: sodium benzoate, potassium sorbate, essential oils, *Alicyclobacillus acidoterrestris*.

Introduction

Alicyclobacillus acidoterrestris has been isolated from spoiled acidic juices and recognized as a spoilage microorganism (Cerny *et al.*, 1984, McIntyre *et al.* 1995, Splittstoesser *et al.*, 1994); it is considered to be one of the important target microorganisms in the quality control of acidic canned foods. The spores of this bacterium can germinate and grow under high acid conditions (pH < 4.5) (Baumgart *et al.*, 1997, Yamazaki *et al.*, 1996), and show a high capacity for survival due to their high heat resistance under the thermal treatments of acidic drinks currently in use (Maldonado *et al.*, 2008, Murakami *et al.*, 1998, Pontius *et al.*, 1998). There is an urgent need for the related industries to develop a suitable method for inhibiting or controlling the germination and outgrowth of *A. acidoterrestris* in such products.

Chemicals, thermal treatments and organic acids have been used since the 1990s to inhibit *A. acidoterrestris* spores (Hsiao and Siebert 1999, Shearer *et al.*, 2000). The use of commercial disinfectants or ethanol was also studied in the past to achieve the same effect (Orr and Beuchat 2000). Nowadays, however, western countries are experi-

encing a trend towards green consumerism in their quest for fewer synthetic additives and more environment friendly compounds (Burt, 2004).

Essential oils are aromatic oily liquids obtained from plant material; some 3000 essential oils are known, out of which about 300 are commercially important for their flavour or fragrance (Van der Braak and Leijten, 1999).

Bevilacqua *et al.* (2008) evaluated the use of cinnamaldehyde, eugenol and limonene to inhibit *A. acidoterrestris* and compared their effectiveness with that of a traditional chemical like sodium benzoate.

This study was aimed at evaluating the use of chemicals in the lemon industry (sodium benzoate, potassium sorbate) and lemon essential oil as growth inhibitors of *A. acidoterrestris* in culture media and LJC. Our work is the first research to study the effect of lemon essential oil against *A. acidoterrestris*.

Materials and Methods

Microorganism

The highly heat resistant *A. acidoterrestris* used in this work was supplied by CIATIAC (Centro de Investi-

gación y Asistencia Técnica a la Industria) and it was kept in orange serum agar (OSA) (Murray *et al.*, 2007) at 4 °C. The strain was confirmed for biochemical tests (mobility, citrate assimilation, catalase and indol production) (Maldonado *et al.*, 2008).

Bacterial activation and spore production

Cells were activated in different culture media to determine the best one for *A. acidoterrestris* growth. They were orange serum medium (OS) (In g/L; tryptein: 10; dextrose: 4; yeast extract: 3; K₂HPO₄: 2.5; orange juice: 200 mL; pH 5.5), malt extract (MEB) (In g/L: malt extract: 17; peptone: 3; pH: 4.5) and bacillus acidocaldarius medium (BAM) (In g/L: yeast extract: 5; peptone: 5; glucose: 5; K₂HPO₄: 4; pH: 4).

The microorganism was spread in the three media and incubated for 48 h at 44 °C. At the end of this period the cells were transferred to a tube with sterile distilled water and Tween 80 until reaching an OD of 0.250 at 540 nm, corresponding to 10⁶ spores/mL. They were kept at 6 °C for 7 days to stimulate sporulation. To destroy the vegetative cells, the spore suspension was shaken for 10 min at 80 °C. The spores thus obtained were used for the tests performed.

Chemicals

Sodium benzoate (500-1000-2000 ppm); potassium sorbate (600-1200 ppm) and lemon essential oil (LEO) (0.08 - 0.12 - 0.16%) were used at concentrations employed in the lemon industry. Stock solutions of sodium benzoate and potassium sorbate in water were freshly prepared before each use and sterilized by filtering through membranes (0.2 µm Millipore).

The lemon oil composition was (%): α- pinene: 1.93; β- pinene 12.26; D-limonene: 67.17; Geranial: 1.25; Sabinene: 2.14; β- Mircene: 1.55; γ- Terpinene:8.33; others: 5.37, determined by HPLC.

The juices, chemicals and LEO were supplied by CITROMAX SACI.

Antimicrobial assay

100 mL of MEB or lemon juice concentrate (LJC) + sodium benzoate (500-1000-2000 ppm) or potassium sorbate (600-1200 ppm) or LEO (0.08-0.12-0.16%), were inoculated separately with 10⁶ spores/mL of *A. acidoterrestris*.

The samples were then incubated at 44 °C, 250 rpm for 11 days and the microbial growth was evaluated every day by measuring absorbance at 540 nm using a UV-visible Beckman DUS30 spectrophotometer.

Inoculated MEB or LJC without chemicals were used as control. To determine the surviving spores after antimicrobial assays, the samples of every day were spread onto the surface of MEA and incubated at 44 °C for 48 h.

Growth data were expressed as Inhibition index evaluated as follows,

$$I.I. = \frac{Ac - As}{Ac} \times 100$$

where Ac represents the absorbance of control sample and As the absorbance of the sample + active compound (Chaibi *et al.*, 1997). All analyses were performed in triplicate.

Results and Discussion

MEB, OSA and BAM were the three media evaluated to determine their suitability to support the microorganism growth. MEB was the best for its higher sugar concentration and it was used for inhibition assays.

Sodium benzoate (500 - 1000 - 2000 ppm) inhibited completely the germination of *A. acidoterrestris* spores (Figure 1). Bevilacqua *et al.* (2008) proposed Na-benzoate (0.1-0.5 g/L) as an antimicrobial to inhibit *A. acidoterrestris* spores, because the acidic pH required by this microorganism could enhance the effectiveness of the compound. The effect of Na-benzoate was dose-dependent and sporostatic, as it did not affect spore viability.

Potassium sorbate was not effective in the spore germination control, because 600 ppm of it inhibited growth for 2 days with a significant absorbance increase after 6 days of storage and population number at the end of the running time was lower, than that recorded in the control sample (Figure 2). A higher concentration of this compound (1200 ppm), however, prolonged the lag phase (time before the beginning of spore outgrowth) to 7 days.

In order to compare the effectiveness of the chemicals the inhibition index in MEB broth + 500 or 600 ppm of chemicals was evaluated. *A. acidoterrestris* was completely inhibited by sodium benzoate 500 ppm after 11 days (inhibition index 99%), without affecting the germination of spores, whereas an inhibition index of approximately 30% was observed in MEB + 600 ppm potassium sorbate after 11 days.

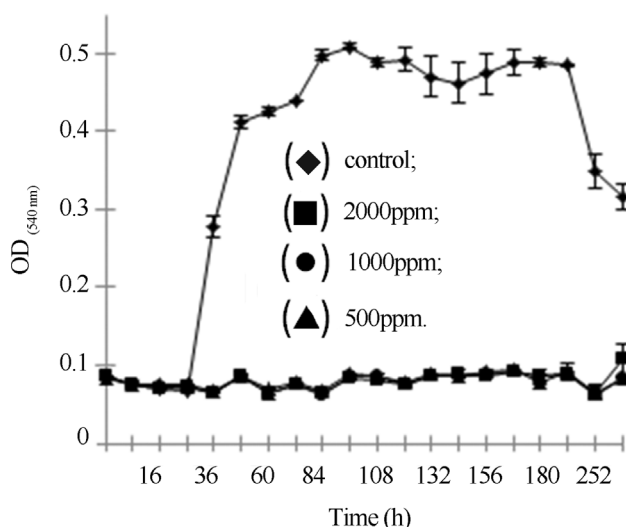


Figure 1 - Germination of *A. acidoterrestris* spores in ME + sodium benzoate. Data are accompanied by the standard deviation.

LEO (0.08 - 0.12 - 0.16%) inhibited completely the germination of *A. acidoterrestris* spores within the running time (11 days) (Figure 3). The oil activity is related to the chemical structure, proportion and interaction of its components (Delaquis *et al.*, 2002, Dorman and Deans 2000, Marino *et al.*, 2001). LEO composition is quite variable, depending on the mode of extraction (enfleurage or distillation), plants and climate. This could explain the difference between our results and those of Bevilacqua *et al.* (2008), because they reported that active compounds of LEO (cinnamaldehyde, eugenol and limonene) were better than oils. However, limonene was not effective against *A.*

acidoterrestris spores while cinnamaldehyde was the most effective compound.

Antimicrobial susceptibility could be strain dependent and probably related to the isolation source or to a different composition and/or distribution of fatty acids in the cytoplasmic membrane. As suggested by Chaibi *et al.* (1997), differences between strains could be explained by a difference in their hydrophobic behavior. The more hydrophobic an organism is, the more sensitive it will be to hydrophobic antimicrobial agents, as the active compounds of LEO. These ideas, however, are only a speculation and need to be confirmed.

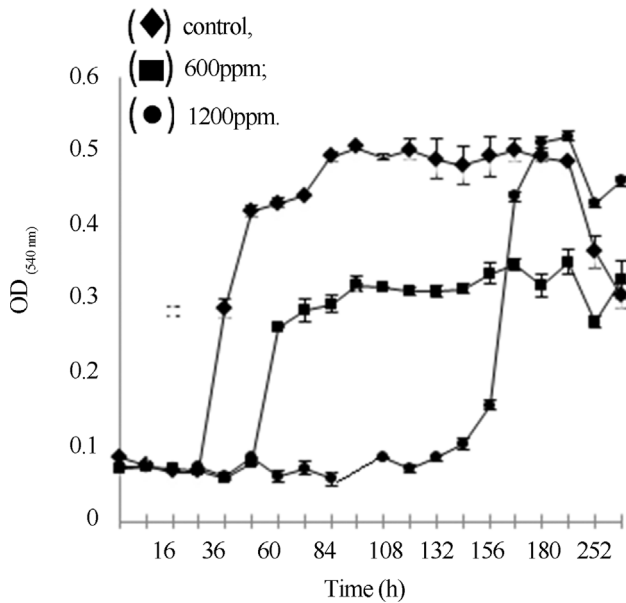


Figure 2 - Germination of *A. acidoterrestris* spores in ME + potassium sorbate. Data are accompanied by the standard deviation.

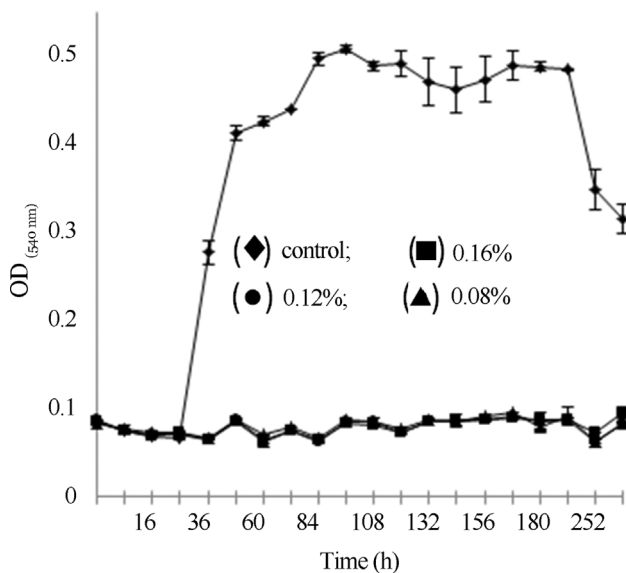


Figure 3 - Germination of *A. acidoterrestris* spores in ME + lemon essential oil. Data are accompanied by the standard deviation.

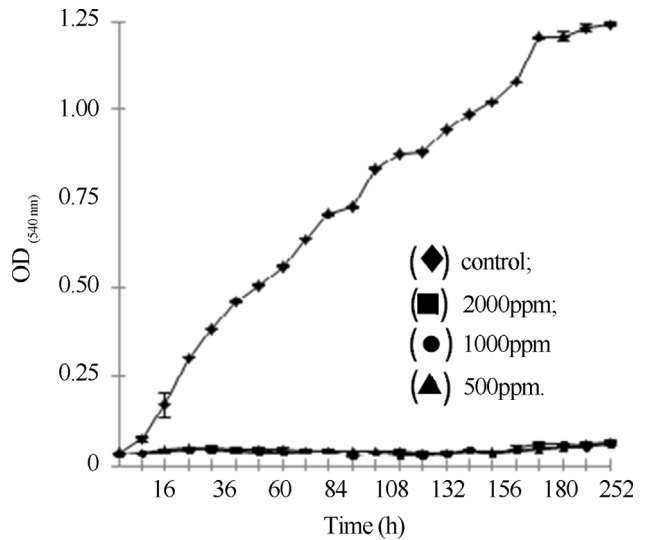


Figure 4 - Germination of *A. acidoterrestris* spores in concentrated lemon juice + sodium benzoate. Data are accompanied by the standard deviation.

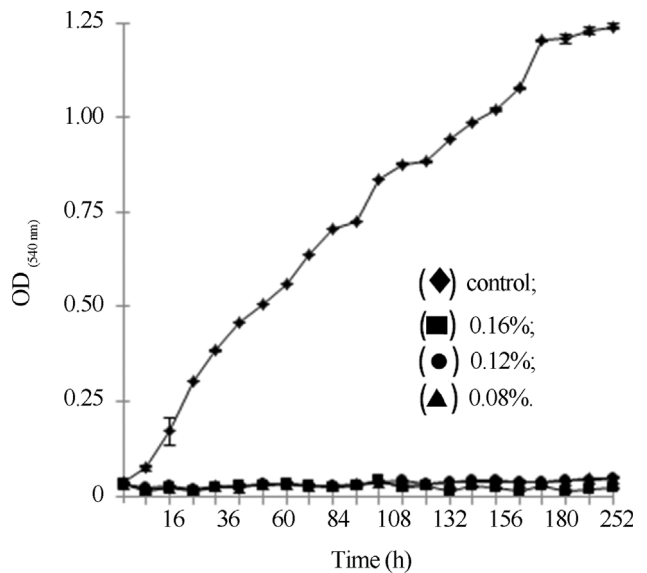


Figure 5 - Germination of *A. acidoterrestris* spores in concentrated lemon juice + lemon essential oil. Data are accompanied by the standard deviation.

Microorganism growth in LJC was twice as high (OD 1.245 nm) as in MEB medium (OD 0.505 nm). The different chemical composition of MEB medium and LJC used for the assays could have a direct influence on *A. acidoterrestris* growth. The germination capacity of the microorganism in high acidity conditions, allows its growth in fruits juices and other foods with a pH lower than 4 (Terano *et al.*, 2005).

Lemon juice + sodium benzoate or LEO in all the concentrations studied, inhibited *A. acidoterrestris* spore germination (Figures 4 and 5). The acidic pH of LJC could enhance the effectiveness of sodium benzoate because its antimicrobial activity rises when pH decreases (Batchelor 1984).

Potassium sorbate was more effective in LJC than in MEB medium because *A. acidoterrestris* growth was controlled, OD: 0.290 and 0.215 nm for 600 and 1200 ppm respectively at the end of the running time (11 days) (Figure 6).

Walker and Phillips (2008) reported that sodium benzoate and potassium sorbate inhibited the germination of 10^3 spores/mL in apple juice; this difference could be attributed to the fact that our inoculums were 10^6 spores/mL.

A study on the acid inhibition of spore germination proposed by Smoot and Pierson (1981), indicated that sorbate could act as a competitive inhibitor of some germinants, like L-alanine and L-cysteine; moreover, its effect seemed to be reversible, as spores germinated after putting them in a fresh medium.

In order to compare the effectiveness of the chemicals, the inhibition index in LJC + 500 or 600 ppm of sodium benzoate or potassium sorbate was evaluated.

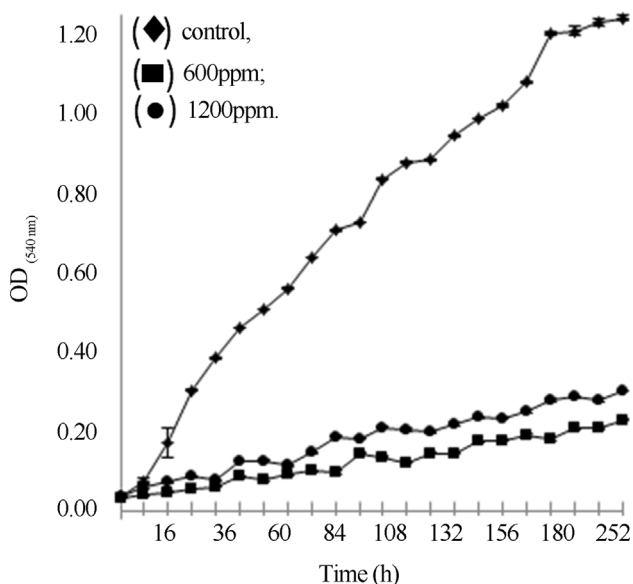


Figure 6 - Germination of *A. acidoterrestris* spores in concentrated lemon juice + potassium sorbate. Data are accompanied by the standard deviation.

A. acidoterrestris was completely inhibited by sodium benzoate 500 ppm after 11 days (inhibition index 99%), without affecting the germination of spores, whereas an inhibition index of approximately 75% was observed in LJC + 600 ppm potassium sorbate after 11 days. The effect of sodium benzoate, potassium sorbate and LEO was spore static in MEB and LJC as they did not affect spore viability.

The use of LEO could be considered an open approach, as juice stabilization could be achieved through some natural molecules, extracted from plants and fruits. Hence, the consumer should not consider these antimicrobials as chemicals, but as natural ingredients of juices; moreover, some of these compounds are usually added to commercial juices (eg. apple juice added with lemon extract) to improve product flavour.

The results of this work show that is possible to control the germination of *A. acidoterrestris* spores in MEB medium and LJC through the use of a natural compound like LEO because it inhibits bacterial spore outgrowth and cell multiplication without affecting germination ability. Accordingly, these results led us to conclude that LEO in lemon juice or its addition is a suitable application for preventing the spoilage caused by *A. acidoterrestris* in most acidic drinks.

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