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ORIGINAL PAPER

Temperature, soluble solids and pH effect on *Alicyclobacillus acidoterrestris* viability in lemon juice concentrate

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Abstract *Alicyclobacillus acidoterrestris* is a thermoacidophilic, non-pathogenic, spore-forming bacterium detected in spoiled commercial pasteurized fruit juice. Apple, white grape and tomato are particularly susceptible. *A. acidoterrestris* spores are resistant to lemon juice pasteurization (2 min at 82°C), and they can germinate and grow causing spoilage. This contamination is characterized by a medicinal or disinfectant smell attributed to guaiacol (o-dihydroxybenzene) production and other taint chemicals. The aim of this work was to study the influence of temperature (82, 86, 92 and 95 °C), total soluble solids (SS) (6.20, 9.8, 50 and 68°Brix) and pH (2.28, 2.45, 2.80, 3.25, 3.5) on decimal reduction time (*D*) of the *A. acidoterrestris* in clarified and non-clarified concentrated lemon juice. Once *D*-value was determined, the resistance of *A. acidoterrestris* at the assayed temperatures was confirmed. SS and pH influence spore viability, because spore resistance increases with higher SS (50°Brix 22 min 82 °C–68°Brix 28 min 82 °C) and pH values (pH 2.28, 17 min–pH 4.00, 22 min). Bacterial growth was lower in clarified lemon juice, 26 min at 82 °C, than in non-clarified lemon juice, 51 min at 82 °C. Temperature was the parameter that had the greatest influence on the *D* value.

Keywords Heat resistance · Decimal reduction time · Lemon juice · *Alicyclobacillus*

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Introduction

Alicyclobacillus acidoterrestris, a thermoacidophilic, non-pathogenic, spore-forming bacterium, has been detected in several spoiled commercial pasteurized fruit juices.

Wisotzey et al. [12] characterized this genus by Ω-alicyclic fatty acids as the major natural membrane lipid component. *A. acidoterrestris* spore germination and growth to 10^6 cfu/ml under acidic conditions have been reported in orange juice stored at 44 °C for 24 h [6], and they concluded that 10^5–10^6 cells/ml in apple and orange juices formed enough guaiacol (ppb) to produce sensory taint. Splittstoesser et al. [9] reported growth inhibition of acidic spore-forming bacillus spores with a high content of total SS. Several authors [1, 2] reported the presence of guaiacol in fruits juices.

Visual detection of spoilage is very difficult because *A. acidoterrestris* does not produce gas during growth, and incipient swelling of containers does not occur. Besides, it does not represent a hazard for human health because it is a non-pathogenic microorganism [5].

Due to these facts, spoilage can occur without visible changes [11] during retail storage of the product. Concerning heat resistence, Pontius et al. [7] concluded that the type of organic acid (malic, citric, and tartaric) did not significantly affect *D*-values.

Silva et al. [8] studied the thermal inactivation of *A. acidoterrestris* spores under different temperature, soluble solids and pH conditions in a solution that simulated a fruit system.

There are few published data and no systematic work that simultaneously investigated the influence of pH, SS and temperature (*T*) on the heat resistance of *A. acidoterrestris* spores. The main objective of this work was to investigate the effect of pH, soluble solids and temperature on *D*-values of *A. acidoterrestris* in clarified and non-clarified lemon juice concentrates, because there are no lemon juice data in the references.

Materials and methods

Microorganism

The highly heat resistant *A. acidoterrestris* used in this work was supplied by CIATIac (Centro de investigación y asistencia técnica a la industria), and it was kept in orange serum agar (OSA) [4] at 4 °C.

The strain was confirmed for biochemical tests.

Bacterial activation and spore production

Cells were activated in orange serum medium (OS) in grams/liter: tryptein: 10; dextrose: 4; yeast extract 3; K_2HPO_4: 2.5; orange juice: 200 ml; pH 5.5 at 42 °C for 24 h. The microorganism was spread in an orange serum agar medium (OSA) and incubated for 48 h at 42 °C. At the end of this period the cells were transferred to a tube with sterile distilled water and Tween 80 until reaching a DO = 0.250 at 540 nm, and they were kept at 6 °C for a week. The spores thus obtained were used for the tests performed.

Experimental design

The *D*-value of *A. acidoterrestris* as a function of temperature (82–95 °C), SS (50; 68; 9.8 and 6.2°Brix) and pH (2.28–4) was determined by heating lemon juice. Heating periods were chosen taking into account the time spent in the pasteurization process at 82 °C and the longest time at which spores survived (95 °C). The SS values correspond to clarified lemon juice concentrate (50°Brix), non-clarified lemon juice concentrate (68°Brix), beverages manufactured with clarified (sodas), and seasonings manufactured with non clarified lemon juice concentrate (9.8 and 6.2°Brix respectively).

The *D* values were determined by placing 5 ml of clarified and non-clarified lemon juice concentrate in glass tubes (15 mm × 97 mm), seeding with 50 µl inoculum and heating. SS were adjusted with sterile distilled water and pH values with NaOH 40%.

After setting the temperature, the tubes were placed and stabilized in a thermostated bath for 2 min, necessary time for the temperature to be homogenous in the juice sample. Time was clocked at the end of this period (30, 45 s, 1, 2.5, 5, 10, 15, 20 and 30 min). The tubes were withdrawn and put in an ice bath to stop the thermal treatment. Survival count was carried out by placing 1 ml of the appropriate dilution in 10 ml OSA medium. The inoculated plates were incubated at 42 °C for 48 h, and microorganisms were counted in ufc/ml.

Data evaluation

Survivor curves were plotted as the log of survivors per milliliter versus time in minutes. *D* values (the time necessary to reduce the viable spore population by 90% at a given temperature) were determined as the negative reciprocal of the survivor curve slope. *D* values for a replicate trial were averaged, and decimal reduction time curves (DRTC) were plotted (logarithm of the average *D* values vs. temperature). The reaction rate constant *k* was calculated as $k = 2.303/D$.

Two predictive models were created by using the Mathematics 4 software to describe and visually illustrate the effect of pH and temperature interactions on heat resistance. The statistical model generated a predictive equation which takes into account pH and temperature interaction on *D* values. The equations are as follows:

$$D = 46.7716\,(\text{pH}) - 0.450125\,(\text{pH})(T) \quad (\text{clarified juice}).$$

$$D = -1747.24 - 13.9718\,(\text{pH}^2) - 0.218849\,(T^2) + 98.053\,(\text{pH}) + 37.6104\,(T) \quad (\text{non-clarified juice}).$$

Results and discussion

Low water activity, high sugar concentration and limited O_2 dilution capacity—all physicochemical characteristics of the juice concentrate are important growth inhibitors for most altering and pathogenic microorganisms. Heating at 82–96 °C for 2 min is enough to destroy filamentous fungi and yeast and prevent possible contamination [3]. Our results proved that *Alicyclobacillus* spores can survive at these temperatures in lemon juice. When temperature was raised from 82 to 95 °C, *D* values decreased from 17.36 to 6.20 min in clarified juice with pH 2.28 (Table 1). In non-clarified juice *D* values were 15.50 min at 82 °C and 8.55 min at 95 °C (Table 2). Lemon juice is generally pasteurized at 82 °C for 2 min; our results and those reported for apple, white and dark grape juice [9], orange and apple juice [6] and a fruit juice model [7] evidenced that *Alicyclobacillus* spores can survive the pasteurization process. This study confirms previous findings in other systems, and the novelty lies in using lemon juice matrices, where the conditions the microorganism has to resist are much more adverse.

Table 1 *D* values, reaction rate constants (*K*) and Td values at different pH and temperature for *A. acidoterrestris* in clarified lemon juice concentrates

T (°C)	pH	*K* (min^{-1})	*D* (min)	Td (min)
82°C	2.28	−0.13	17.36	60.36
	2.80	−0.09	25.81	113.79
	3.50	−0.07	33.66	141.51
	4.00	−0.10	21.95	93.95
86°C	2.28	−0.13	18.06	55.46
	2.80	−0.10	22.01	82.90
	3.50	−0.03	68.95	293.47
	4.00	−0.06	35.16	129.76
92°C	2.28	−0.30	7.60	25.98
	2.80	−0.15	15.35	76.98
	3.50	−0.13	16.87	86.22
	4.00	−0.09	23.19	122.57
95°C	2.28	−0.37	6.2	28.43
	2.80	−0.20	11.32	56.68
	3.50	−0.18	12.63	60.34
	4.00	−0.23	9.72	48.18

Table 2 *D* values, reaction rate constants (*K*) and Td values at different pH and temperature for *A. acidoterrestris* in non-clarified lemon juice concentrates

T (°C)	pH	*K* (min^{-1})	*D* (min)	Td (min)
82°C	2.28	−0.15	15.50	66.53
	2.80	−0.04	50.50	229.82
	3.50	−0.06	38.00	181.05
	4.00	−0.08	27.48	113.66
86°C	2.28	−0.16	14.54	63.13
	2.80	−0.07	31.67	126.39
	3.50	−0.02	95.15	360.00
	4.00	−0.04	58.15	260.56
92°C	2.28	−0.26	8.81	37.88
	2.80	−0.06	39.30	179.18
	3.50	−0.04	59.50	310.00
	4.00	−0.03	85.29	451.85
95°C	2.28	−0.27	8.55	33.36
	2.80	−0.10	22.03	108.52
	3.50	−0.13	17.22	81.64
	4.00	−0.09	23.33	119.87

Tables 3 and 4 show the effect of SS on *D* values for both juices. *A. acidoterrestris* was less resistant in clarified juice (50°Brix) than in non-clarified juice (68°Brix). This results coincide with the results reported for Silva et al. [8]. The *D* values were 25.81 and 50.50 min for clarified and non-clarified juice, respectively, at 82 °C and pH 2.8. Similar behavior was observed in juice with pH 3.5 and pH 4 at the temperature studied (Tables 1, 2).

Table 3 *D* values, reaction rate constants (*K*) and Td values at different Brix for *A. acidoterrestris* in clarified lemon juice, pH 3.5

°Brix	*T* (°C)	*K* (min^{-1})	*D* (min)	Td (min)
9.8	82	−0.20	11.23	51.73
	86	−0.22	10.54	49.90
	92	−0.24	9.47	48.90
	95	−0.27	8.55	33.95
50	82	−0.13	17.35	60.36
	86	−0.12	18.06	59.60
	92	−0.30	7.60	25.98
	95	−0.37	6.20	28.43
6.2	82	−0.17	13.21	63.58
	95	−0.24	9.38	48.88

Table 4 *D* values, reaction rate constants (*K*) and Td values at different Brix for *A. acidoterrestris* in non-clarified lemon juice, pH 2.45

°Brix	*T* (°C)	*K* (min^{-1})	*D* (min)	Td (min)
9.8	82	−0.1377	16.72	87.14
	86	−0.2034	11.32	58.99
	92	−0.2177	10.58	55.12
	95	−0.2306	9.98	52.03
50	82	−0.1486	15.50	66.53
	86	−0.1584	14.54	63.13
	92	−0.2614	8.81	37.88
	95	−0.2694	8.56	33.40
6.2	82	−0.1292	17.82	85.15
	95	−0.2483	9.44	45.26

The same thing does not hold true with pH 2.28 lemon juice: differences in *D* values between clarified and non-clarified juice were not significant. In this case, pH effect would play a more important role than SS on *Alicyclobacillus* viability.

To confirm that bacillus behavior in juice with higher pH than 2.28 will depend on SS concentration, clarified and non-clarified juices with pH 2.8 were adjusted at the same SS value (9.8°Brix corresponding to the ones measured in lemon soda) with sterile distilled water and *D* was calculated. No observations were made which confirmed that *D* is influenced by SS. *D* values for the aciduric sporeforming bacilli were almost the same even though the juices differed in pH and SS concentrations.

The effect of these variables on aciduric bacilli resistance is unknown although spores are often more resistant when heated in solutions of higher pH and Brix [9]. Since the industrial pasteurization process currently used does not eliminate *A. acidoterrestris* spores, it was important to understand the behavior of the bacillus in goods manufactured with clarified (sodas) and non-clarified juice

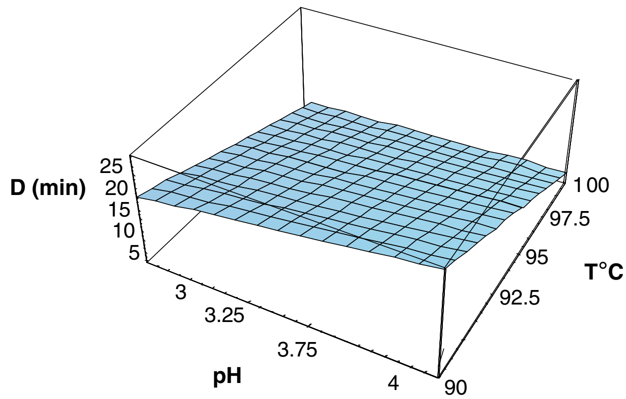

Fig. 1 Statistically generated model of *A. acidoterrestris* in clarified lemon juice using the Mathematics 4 program

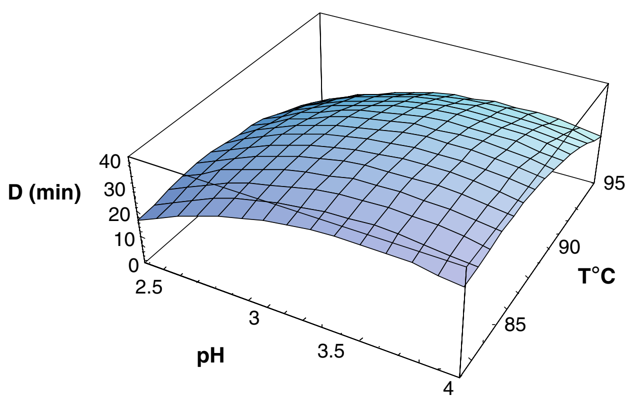

Fig. 2 Statistically generated model of *A. acidoterrestris* in non-clarified lemon juice using the Mathematics 4 program

concentrates (seasonings). Hence, SS and pH characteristics of these products were imitated, and the systems were heated to 82 and 95 °C for different time periods (Tables 3, 4). The results show that *Alicyclobacillus* survives well in the systems studied. Splittstoesser et al. [10] found that when commercial juice beverages were inoculated with *Alicyclobacillus* spores, the results differed depending on the type of juice; apple and tomato juice consistently supported growth, whereas grape juice at both pH 2.9 and 3.3 did not permit it.

Figures 1 and 2 show the model of *A. acidoterrestris* in clarified and non-clarified lemon juice. All the interactions in the equation employed had a statistically significant effect ($p < 0.05$) on the D value. Figure 1 shows that as pH values increase in clarified juice so does D, whereas D values diminish when temperature rises. In non-clarified juice (Fig. 2), maximum resistance to *A. acidoterrestris* occurs at pH 3 to 3.5 at 90 °C approximately. Due to the differences observed between predicted and measured D-values in fruit products, the model obtained cannot be used directly to estimate heat resistance in real fruit systems, but it is useful for predicting the trends and relative changes in D-values brought about by temperature and pH variations.

The results of this work show that *A. acidoterrestris* survives industrial pasteurization (82 °C for about 2 min). Besides, at 95 °C, a much higher temperature than the one used in the hot-fill-hold process, the D value for *A. acidoterrestris* was 6–8.5 min. When the product is held for only 2 min at hot-fill processing temperature, spoilage is likely to occur if the raw product is contaminated even with a modest *A. acidoterrestris* spore population. To check the problem, lemon juice manufacturers should make sure that spore population is sufficiently low by increasing process temperature and/or hold time or adding preservatives to control this organism.

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