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Effect of several humectants and potassium sorbate on the growth of *Zygosaccharomyces bailii* in model aqueous systems resembling low sugar products

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Abstract

The effect of solute type (glucose and polyols) and potassium sorbate (KS) level on the growth of *Zygosaccharomyces bailii* was evaluated in model aqueous systems resembling low sugar products. Growth curves were modeled using Gompertz equation. Parameters of the models were estimated and applied to analyze the effect of water activity (a_w), solute used and KS on growth of *Z. Bailii*.

It was observed that as KS concentration increased, the yeast growth diminished gradually.

Use of polyols in the absence of KS generally decreased growth. In particular, xylitol was the solute that promoted the highest decrease in yeast growth.

Depression of a_w in the presence of KS produced different effects on growth rate depending on a_w level, solute added and concentration of the preservative.

In several cases, a synergism between humectants (glucose and polyols) and KS was observed in relation to growth inhibition. This behavior would allow to maintain the biological activity of the preservative diminishing the amount used.

Keywords: Humectants; Potassium sorbate; *Zygosaccharomyces bailii* growth; Aqueous systems; Low sugar products

1. Introduction

Diet foods, like juices, jams and jellies are generally preserved by the application of a combination of hurdles such as low pH, depression of water activity by solute addition, a thermal treatment and the use of preservatives (Stiles Battey, Duffy, & Schaffner, 2002). These products are intrinsically resistant to growth of pathogens but they are susceptible to spoilage by yeasts, specially by osmophilic, acid tolerant and preservative resistant yeasts such as the case of *Zygosaccharomyces bailii* (Jenkins, Poulus, Cole, Vandeven, & Legan, 2000). The latter can grow in the presence of 600 mg/L of sorbic acid and at pH levels less than the p*K*a of the preservatives (Warth, 1977). The yeast growth and its sorbate resistance could be affected by the type of solute used to reduce the water activity (a_w) as well as by other solutes present in the food (Lenovich, Buchanan, Worley, & Restaino, 1988). For these reasons, *Z. bailii* is a major concern to manufacturers of acidic products.

Formulation of low calorie products involved the replacement of sucrose by an alternative sweetener and by a bulking agent. Addition of polyols can fulfill both requirements. Sorbitol, mannitol and xylitol are the polyols most used for production of candy and chewing gum. According to FDA, its use allows to reduce the nutritional input of 4 kcal/g of sucrose to 2.6, 2.4 or 1.6 in the case of adding sorbitol, xylitol or mannitol respectively (O'Brien Nabors, 2002). Clinical studies have shown that its use

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reduces caries and plaque white (O'Brien Nabors, 2002). Moreover, xylitol, may help to reduce the chance of acute otitis media in children (Pszczola, 1999). From another point of view, they act as humectants, depressing the water activity and improving texture and mouthfeel (O'Brien Nabors, 2002).

Preservatives such as sorbic and benzoic acids and its salts are usually added to low sugar products: in particular, potassium sorbate is reported to have lower toxicity to mammals, to impart a lower aftertaste sensation and to be more inhibitory for controlling growth of spoilage yeasts than sodium benzoate (Deuel, Slater, Weil, & Smyth, 1954; Dryden & Hills, 1959; Praphailong & Fleet, 1997). According to previously mentioned reasons, KS is most frequently used. Anyhow, it must be highlighted that nowadays consumers demand products free of chemical preservatives or with reduced levels of them. For these reasons, it is important to study the effect of other addtives on antimicrobial action; as an example, elevated salt or sugar concentration and pH depression were shown to act synergistically enhancing sorbates action (Bills, Restaino, & Lenovich, 1982; Praphailong & Fleet, 1997; Castro, Garro, Campos, & Gerschenson, 2002) and allowing to use reduced levels with equal effect.

The type of solute used to reduce a_w of growth media has been shown to influence both the growth and death rate of yeast. In particular, polyols and sugars increase the heat resistance of bacteria, yeast and fungal spores (Corry, 1976; Lenovich et al., 1988). But, this behavior depends on solute used as humectant and on the nature of the microorganism (Almagro et al., 2000).

There is no information about the effect of polyols on growth of *Z. bailii* and on the antimicrobial action of sorbates. Therefore, this study examines the effect of some solutes (polyols and glucose) and potassium sorbate on the growth of *Z. bailii* in aqueous systems modeling low sugar products.

2. Materials and methods

2.1. Inoculum preparation

Z. bailii NRRL 7256 inoculum was prepared in Sabouraud broth (Biokar Diagnostics, Beauvais, France) at 25 °C until stationary phase was achieved (24 h).

2.2. Model system formulation

Model systems were formulated in Sabouraud broth and the different composition is given in Table 1. Polyol concentrations were selected to give a final a_w of 0.985 (system A, B and C). A glucose level of 10% (w/w), which depressed a_w to 0.988 (system D), was chosen according to the maximum level admitted by Argentine Food Code for modified jams, jellies and fruit stews. In some cases, enough glucose was added alone or in combination with a polyol to get a final a_w of 0.971 (system E to H).

Water activity was measured with an Aqualab dewoint electronic humidity meter (Decagon Devices Inc, Pullman, Washington, USA). The experimental error in a_w determination is $\pm$ 0.005 a_w units when using electronic humidity meters according to Roa and Tapia (1991) and, as a consequence there are no significant differences between a_w values of 0.985 and 0.988.

Different concentrations of KS (0.005–0.010%, w/w) were added to each system. Control system without preservative and or humectants were prepared for comparison purposes. The pH was adjusted to 3.0 by addition of citric acid before autoclaving.

A volume of 14.25 ml of each model system was dispensed in duplicate into dark glass flasks and autoclaved. Previous studies have shown that pH and KS content did not change significantly by autoclaving. Then, 0.75 ml of Sabouraud broth containing the inocula was aseptically added to obtain a population of 1.10^7 CFU/ml. The air headspace was kept the same in all the flasks (75% of total volume of flask). The systems were incubated at 25 ($\pm$ 1) °C.

3. Sampling and analysis

The yeast growth was measured by turbidimetry (Dalgaard, Ross, Kamperman, Neumeyer, & McMeekin, 1994). For that purpose, 1 ml aliquots were removed from each flask at selected times and the absorbance at 540 nm was measured (Spectrophotometer Shimadzu UV-1203, Japan). According to McMeekin, Olley, Ross, and Ratkowsky (1993), the relationship between concentration and absorbance/turbidity is only linear over a limited range corresponding to approximately to a tenfold increase in cell numbers. To achieve this condition, the systems that had

Table 1
Model system composition

Composition[a] (% w/w)	System								
	A	B	C	D	E	F	G	H	I
Sorbitol	12.90	–	–	–	12.90	–	–	–	–
Xylitol	–	11.00	–	–	–	11.00	–	–	–
Mannitol	–	–	13.00	–	–	–	13.00	–	–
Glucose	–	–	–	10.00	10.00	10.00	10.00	22.00	–
a_w	0.985	0.985	0.985	0.988	0.971	0.971	0.971	0.971	1.000

[a] Sabouraud broth: enough quantity for 100 g.

an absorbance greater than 0.3 were diluted with the supernatant obtained after centrifugation of each sample (3000 RPM for 10 min at room temperature). Besides, this supernatant was used as blank for measurements. All determinations were conducted in duplicate.

3.1. Data analysis

The yeast growth curves were modeled using the modified Gompertz equation (McMeekin et al., 1993):

$$y = A \cdot \exp\left\{-\exp\left[1 + \frac{\mu}{A}(\lambda - t)\right]\right\}$$

which express the change of the turbidity (y) produced by the yeast population vs. the time (t). The turbidity is evaluated through the measurement of the absorbance of the sample at 540 nm. The biological parameters of the yeast growth are the specific growth rate (μ), the lag phase time (λ) and the asymptotic value (A). The specific growth rate evaluated does not correspond to the maximum growth rate, as the optical density method is less sensible than the viable method (McMeekin et al., 1993).

The parameters of growth were estimated for each system by nonlinear regression analysis of data and using the modified Gompertz equation. In the cases of the joint presence of xylitol and KS (0.10%, W/W), yeast growth curves could not be satisfactory modeled by the modified Gompertz equation and specific growth rate (μ) was determined by linear regression of data. But no information about lag phase time and the asymptotic value could be provided.

Analysis of varianza (ANOVA) and the least significant difference (LSD) test were applied to establish significant differences between parameters calculated for all systems. Besides, an ANOVA of two factors (humectant and KS level or a_w and KS level) was performed to establish possible interactions (Sokal & Rohlf, 1969). In all cases, statistical significance was evaluated at a 5% level ($\alpha = 0.05$).

The statistical analysis were performed using the Statgraphics computer program (Statgraphics Plus for Windows, version 3.0, 1997, Manugistics, Inc., Rockville, Maryland, USA).

4. Results and discussion

Yeast growth curves were satisfactory modeled by Gompertz equation as it was proved throughout an ANOVA and by the correlation coefficient (R^2) which showed values of 0.98–0.99 for all systems. The exception to this behavior were observed for systems containing xylitol and 0.01% of KS (system B and F) in these systems, absorbance increased slowly with time and population did not reach the stationary phase even after 180 h of storage.

Experimental data and modeled curves for the systems containing 13% (w/w) of mannitol and different levels of KS (0–0.010% w/w) are shown in Fig. 1 as an example. Also, data for the system free of humectants and preservative is reported. For the rest of the systems analyzed, modeled curves showed a similar pattern.

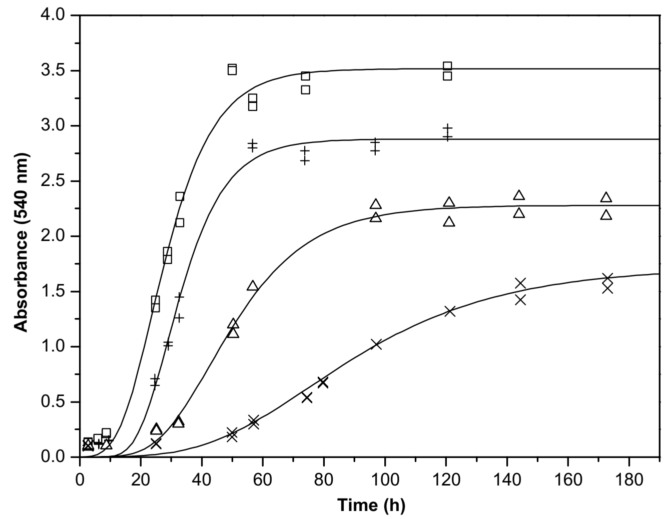

Fig. 1. *Z. bailii* growth. Experimental data and modeled curves for the system free of humectants and potassium sorbate (KS) (system I) and for the mannitol containing system (C) with different levels of KS. Symbols: (□) system I; (+) system C without KS; (△) system C with 0.005% (w/w) of KS; (x) system C with 0.010 % (w/w) of KS.

In general, it could be observed that yeast grew in the systems without the preservative and that growth was not inhibited neither by stress factors (pH and a_w depression) nor by the humectants used (glucose, polyols or their combination), with the exception of systems containing xylitol (systems B and F) as it will be discussed later in which a_w depression with xylitol diminished yeast growth (Figs. 2 and 3).

In all systems, as potassium sorbate concentration increased, yeast growth diminished gradually. The minimum inhibitory concentration (MIC) for studied systems had been previously determined and it was within the range of 0.020–0.025% (w/w) (Gliemmo, Campos, & Gerschenson, 2004).

4.1. Effect of potassium sorbate on yeast growth parameters

As it was mentioned above, in all systems, an increase in sorbate concentration produced a decrease of yeast growth rate (Fig. 2, panel a). In the case of xylitol, it can be observed the deepest decrease in growth rate (systems B and F), even in the absence of the preservative growth rate got to be insignificant for sorbate concentrations equal to 0.010% (w/w). This trend suggests that this humectant exerted an inhibitory effect on *Z. bailii* growth. Effect of KS on yeast growth is well documented by other authors: *Z rouxii* growth rate was substantially reduced by the addition of 0.03% (w/w) of KS to the medium (Golden & Beuchat, 1992). In the case of *Z. bailii*, 0.025% (w/w) of sorbic acid was the maximum level that allowed yeast growth at pH 3.0 in culture media (Praphailong & Fleet, 1997). Moreover, the minimum inhibitory concentration

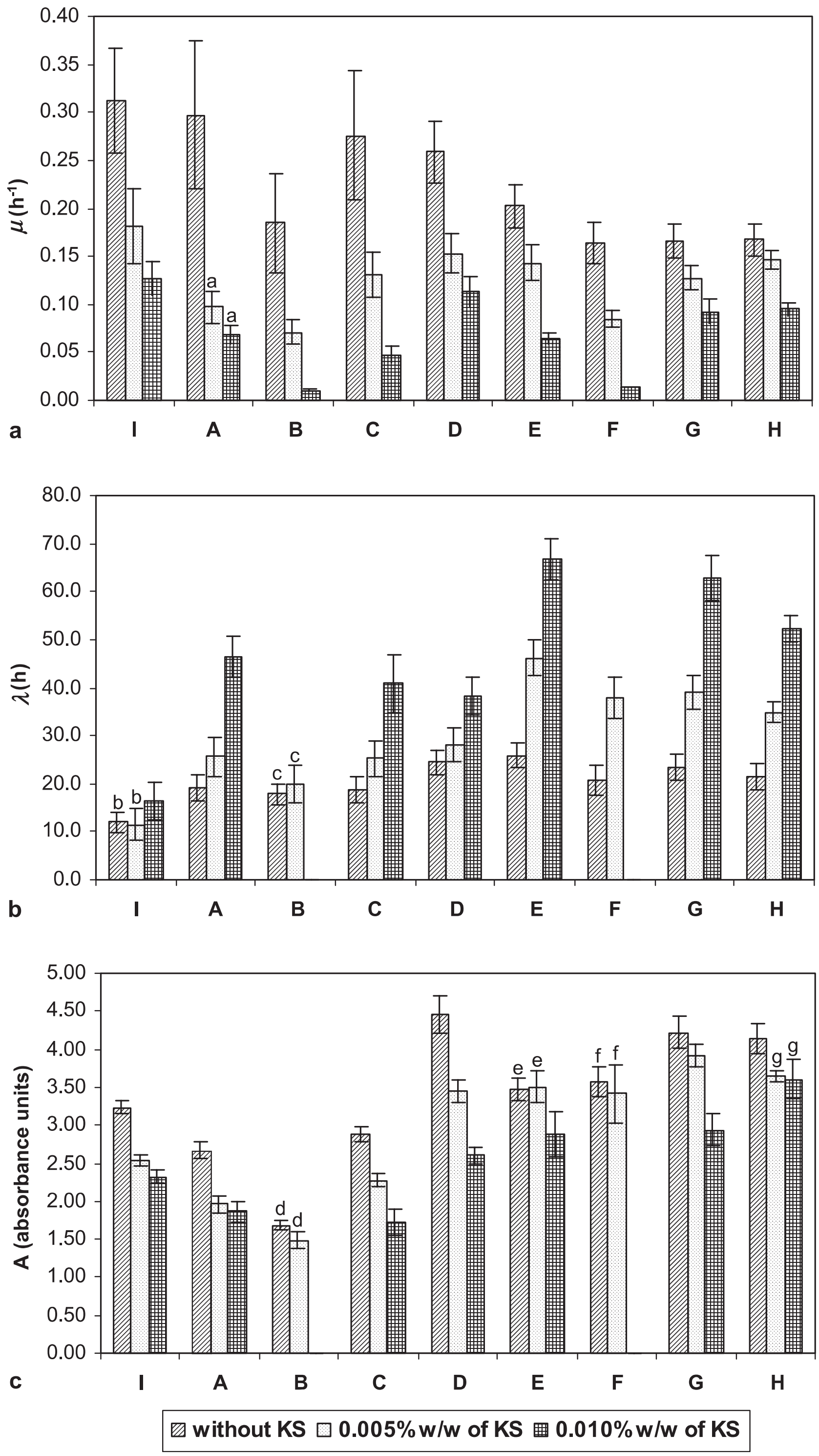

Fig. 2. Growth parameters of *Z. bailii* in model systems as a function of potassium sorbate (KS) content. Panel a growth rate (μ), panel b lag phase (λ), panel c asymptotic value (A). Columns followed by the same letter are not significant different ($P \leqslant 0.05$). Vertical bars represent standard deviation of the mean.

for inhibiting *Z. bailii* growth was 0.030% (w/w) at pH 3.5 in model aqueous systems resembling salad dressings (Castro et al., 2002).

In general, the lag phase increased with sorbate level, with the exception of the system free of humectants (system I) and the one containing xylitol (system B). In these cases, no significant change in the lag phase was observed when sorbate concentration increased from 0% to 0.005% (w/w) (Fig. 2, panel b). Lambert and Stratford (1999) observed that increasing concentrations of a weak acid preservative led to increased lag time phase and they suggested that the microorganism has to pump out excess

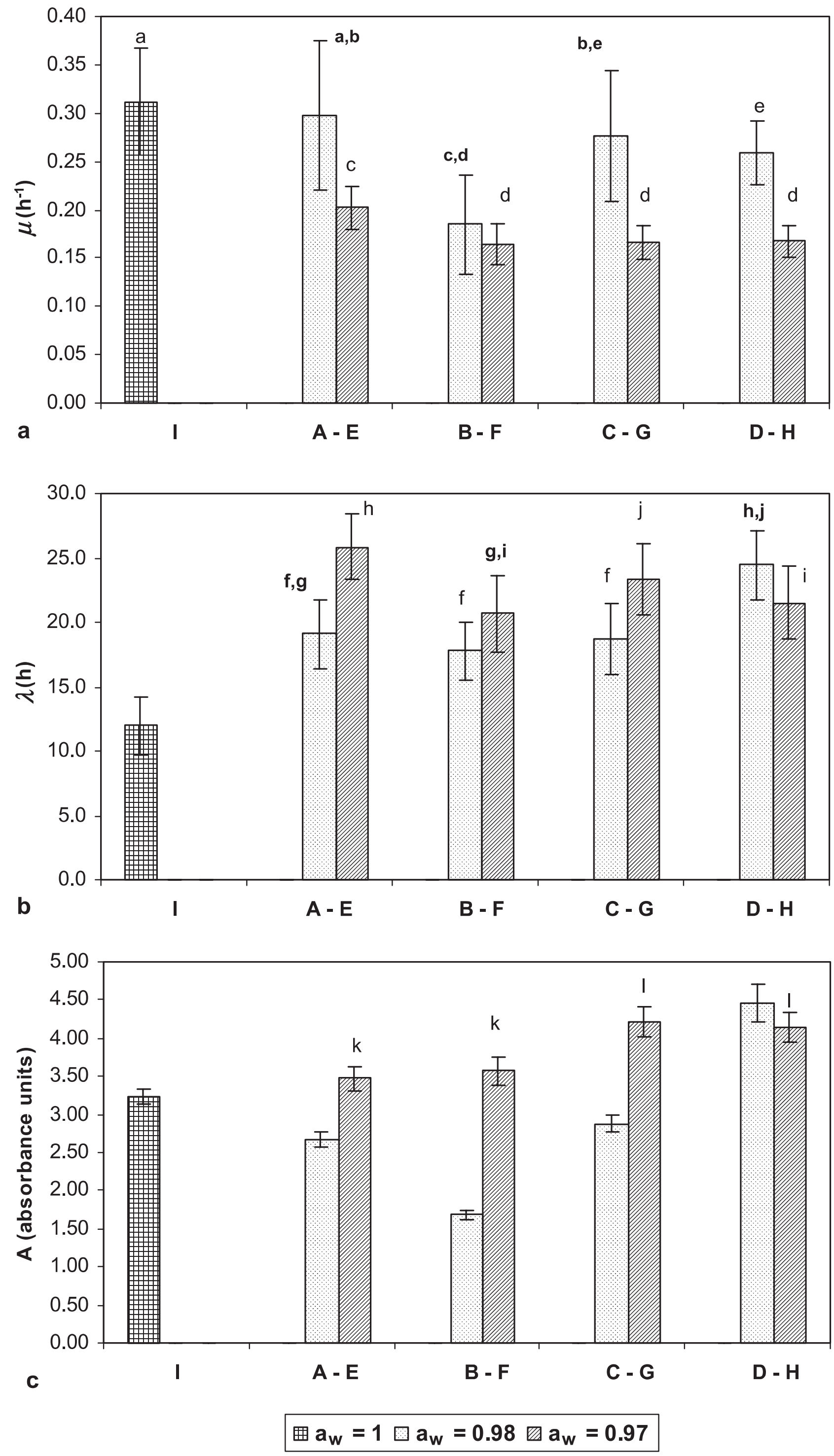

Fig. 3. Growth parameters of *Z. bailii* in model systems as a function of water activity (a_w) depression and humectant added in preservative free systems. Panel a: growth rate (μ), panel b: lag phase (λ), panel c: asymptotic value (A). Columns followed by the same letter are not significant different ($P \leqslant 0.05$). Vertical bars represent standard deviation of the mean.

protons to achieve the optimum growth pH to enter in the exponential phase and that since there is a direct relation between the time necessary to remove protons and the duration of the lag phase.

It must be stressed that estimation of lag phases through turbidimetry is useful for comparison purposes. But they are larger than the ones estimated from viable data (Augustin, Rosso, & Carlier, 1999) due to the fact that turbidimetry does not allow the detection of populations of less than 10^6–10^7 CFU/ml (Dalgaard et al., 1994).

As sorbate concentration increased, the parameter A, which represents the turbidity when the population reached the stationary phase, diminished (Fig. 2, panel c). This trend shows the inhibitory effect of sorbates on yeast growth modifying the population in the stationary phase. In some cases, such as in the sorbitol, xylitol, sorbitol/glucose or the xylitol/glucose containing systems (A, B, E and F, respectively) no change in the asymptotic value was detected with the increase in the preservative level from 0% to 0.005% w/w. In addition, an increase in KS level from 0.005% to 0.010% in a 22% glucose containing system (H) did not modify asymptotic value. These trends can be the expression of yeast cells adapted to weak acid stress through different mechanisms as it was suggested by Kubo and Lee (1998).

4.2. Effect of a_w depression on yeast growth parameters in free preservative systems

As can be observed in Fig. 3, a_w depression from 1.000 to 0.988, 0.985 or 0.971 diminished μ significantly in all cases (system I vs. B, C, E, D, F, G, and H) with the exception of the system of a_w 0.985 and containing sorbitol (system A); in this case, no effect of a_w depression was observed. This trend is coincident with the findings of (Almagro et al., 2000), who reported that addition of sorbitol to depress a_w to 0.982 produced no effect on the growth of *D. hansenii* and *S. cerevisiae*.

The change in a_w from 0.985 or 0.988 to 0.971 due to glucose addition diminished μ for all humectants used (systems A, C, D vs. E, G, H, respectively) with the exception of xylitol; in this case, no effect of a_w depression was observed (system B vs. F). However, no significant differences in growth rate were detected between the systems containing glucose, xylitol or mannitol at an a_w of 0.971 (systems H, F and G). This trend suggests that at this level of a_w the effect of a particular solute on growth rate is masked by the effect of a_w depression.

In all studied systems, lag phase increased when water activity was depressed from 1 to 0.988 or to 0.971 (Fig. 3, panel b). Lag phase length was independent of the polyol used to depress water activity to 0.988, but this trend was modified when a_w reached a level of 0.971 by the addition of glucose. At this a_w level, the combined use of

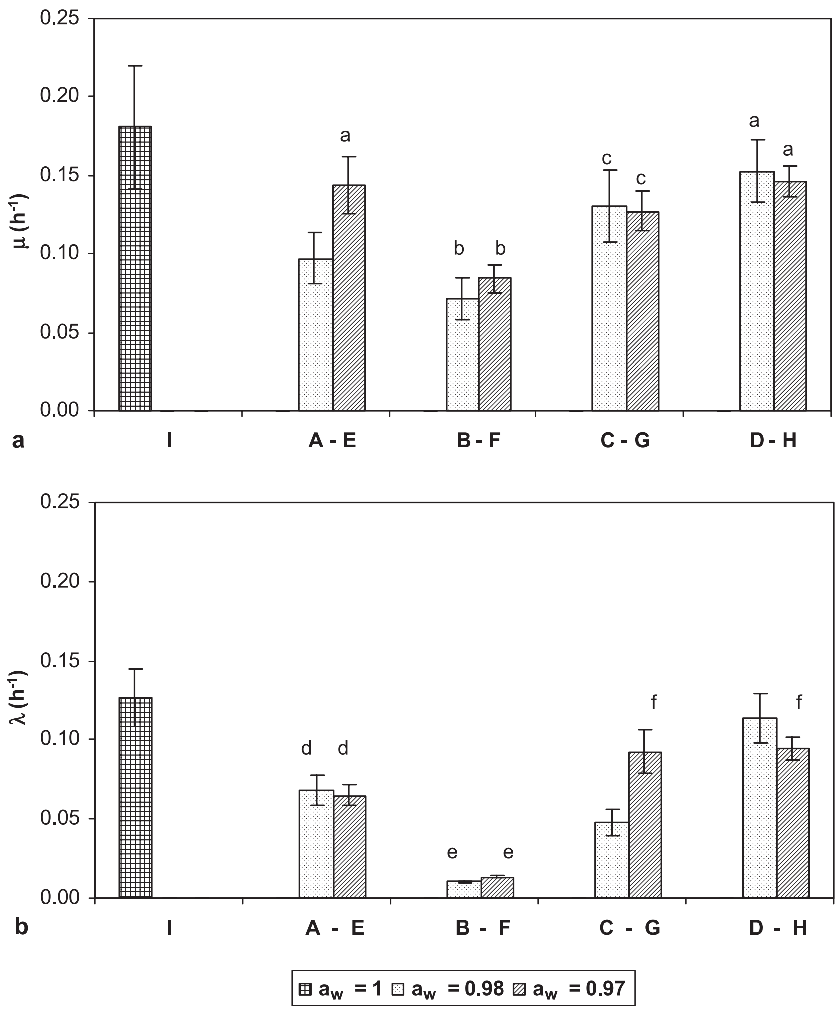

Fig. 4. Growth rate (μ) of *Z. bailii* in model systems as a function of potassium sorbate (KS) level. Panel a: systems containing 0.050% (w/w) of KS, panel b: systems containing 0.010% (w/w) of KS. Columns followed by the same letter are not significant different ($P \leqslant 0.05$). Vertical bars represent standard deviation of the mean.

glucose and sorbitol or mannitol promoted the greatest increase in the lag phase.

Maximum level of growth was modified by a_w depression, as it can be seen in Fig. 3, panel c. A_w depression from 1 to 0.985 through polyol addition diminished the asymptotic value and the use of xylitol promoted the highest decrease. On the contrary, addition of glucose to get a a_w of 0.988 or 0.971 increased the asymptotic value for the systems which contained polyol and glucose jointly. This behavior of glucose could be related to the use of glucose as a carbon source and had been previously observed in the growth of *Z. rouxii* in culture media of 0.93 a_w and pH 4.5 (Golden & Beuchat, 1992).

In general, the presence of polyols decreased growth rate, extended lag phase and diminished the level of population in the stationary phase. In particularly, xylitol was the solute that promoted the highest decrease in growth rate and in the level of population in the stationary phase.

It must be remarked that according to Lenovich et al. (1988) the response of the cells to a_w depression by different solutes include variations in the cellular permeability and the accumulation of intracellular polyols changing synthesis or activity of enzymes. However, polyols are compatible solutes for yeasts and their inhibition of the enzymatic activity decreases with the chain length of the polyol (Gould, 1985). On the other hand, Vaamonde, Scarmato, Chirife, and Parada (1986) reported that a_w depression is not the only determinative regulatory factor in relation to biological response of *Staphylococcus aureus;* the solute nature plays an important role and use of erythritol or xylitol inhibited the growth at a water activity as high as 0.92–0.94, raising considerably the minimal a_w for aerobic growth of *Staphylococcus aureus*. Katsuyama (2001) reported that xylitol is effective in external preparation for the treatment of atopic dermatitis caused by *S. aureus*.

4.3. Effect of a_w depression on yeast growth parameters in the presence of KS

In systems containing 0.005% or 0.010% w/w of KS, a_w depression from 1 to 0.985 through polyol addition

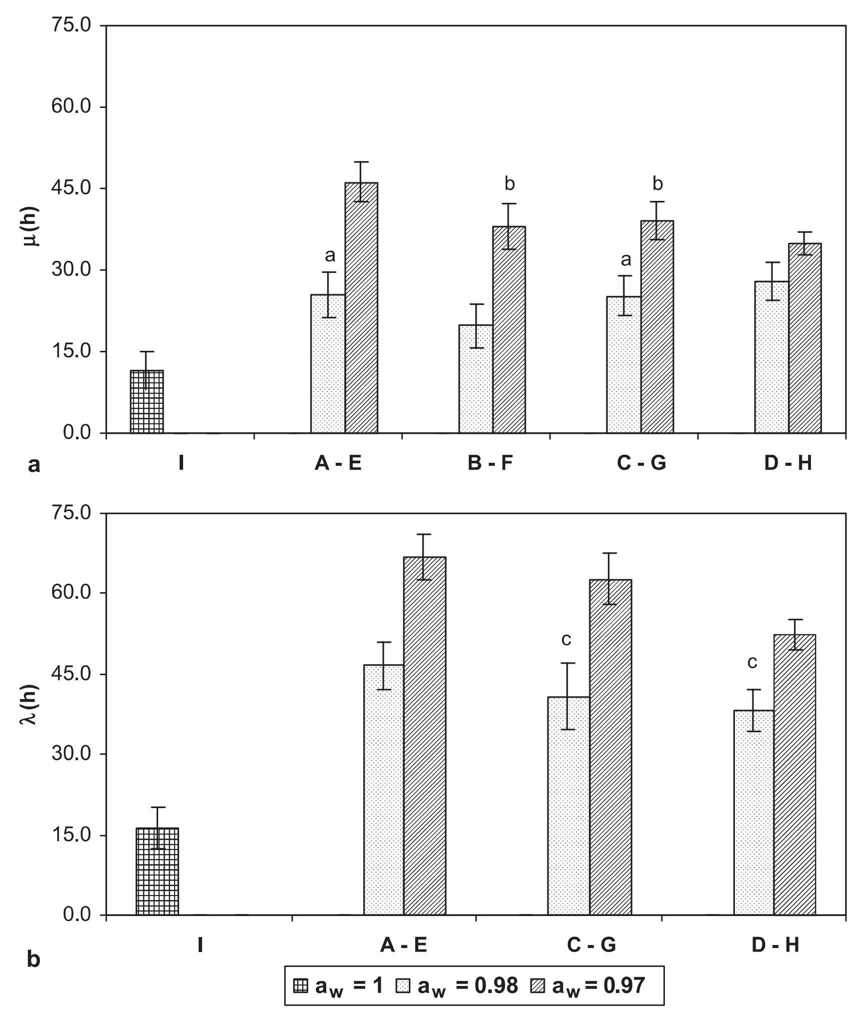

Fig. 5. Lag phase (λ) of *Z. bailii* growth curve in model systems as a function of potassium sorbate (KS) level. Panel a: systems containing 0.050% (w/w) of KS, panel b: systems containing 0.010% (w/w) of KS. Columns followed by the same letter are not significant different ($P \leqslant 0.05$). Vertical bars represent standard deviation of the mean.

(systems I vs. A, B and C) or to 0.988, in the case of glucose (system I vs. D), promoted a decrease in growth rate (Fig. 4, panel a and b). In relation to polyols, xylitol was the one that produced the highest decrease in growth rate for systems containing KS.

Water activity (a_w) depression from 0.985 or 0.988 to 0.971 due to glucose addition to systems containing 0.005% w/w of KS, xylitol, mannitol or glucose produced no effect on μ (Fig. 4, panel a: systems B, C, D vs. F, G, H). However, an increase in growth rate was observed for the sorbitol system (Fig. 4, panel a: systems A vs. E).

When KS content raised to 0.010% w/w, different trends were observed in relation to a_w depression: in the system containing only glucose as humectant growth rate decreased (systems D and H); in the one with mannitol, it increased (system C vs. G), for the one with sorbitol it suffered no change (systems A vs. E) (Fig. 4, panel b). Finally, the presence of xylitol (systems B and F) exhibited the lowest growth rate. Mentioned trends remarked that effect depends strongly on a_w level and solute added.

Lag phase increased as water activity decreased independently of KS content (Fig. 5 panel a and b) being the system containing sorbitol and glucose (system E) the one that possessed the biggest lag phase for both levels of KS.

Addition of a polyol to depress water activity from 1 to 0.985 in systems containing 0.005% or 0.010% w/w of KS produced a decrease in the asymptotic value of growth curve (Fig. 6, panel a and b, systems I vs. A, B, C). For the contrary, an increase in the asymptotic value was observed when glucose was used alone (system D and H) or in combination with a polyol (system E, F and G). This trend was independent of KS content (Fig. 6, panel a and b). The same behavior was previously reported in the absence of the preservative.

In order to evaluate the combined effect of a_w depression and KS addition or the effect of solute used as humectant and KS addition on growth rate, a two factors ANOVA analysis was performed. It was observed an interaction between depression of a_w by the use of glucose and KS addition (Fig. 7). As a result of this behavior, the combined use

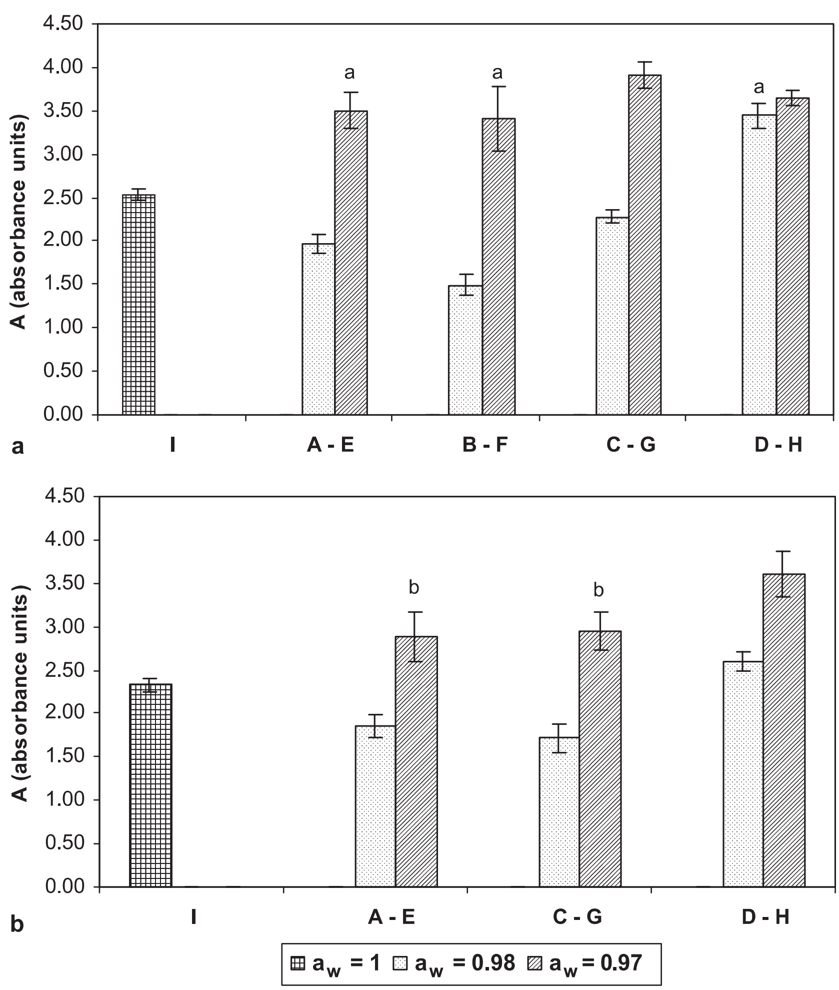

Fig. 6. Asymptotic value (A) of *Z. bailii* growth curve parameter in model systems as a function of potassium sorbate (KS) level. Panel a: systems containing 0.050% (w/w) of KS, panel b: systems containing 0.010% (w/w) of KS. Columns followed by the same letter are not significant different ($P \leqslant 0.05$). Vertical bars represent standard deviation of the mean.

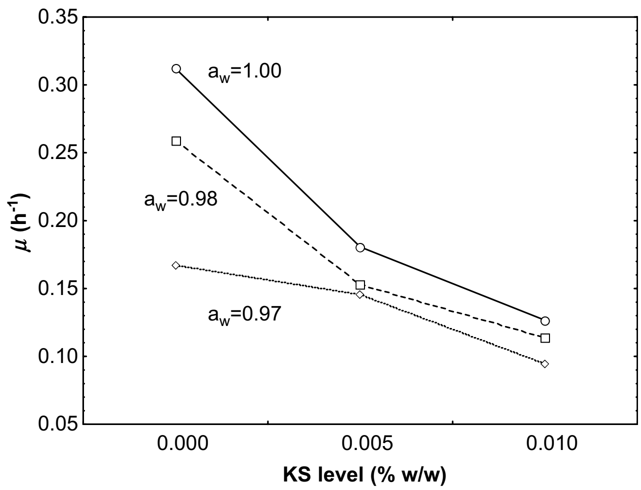

Fig. 7. Interaction between water activity (a_w) depression and potassium sorbate (KS) level on the growth rate (μ) constants of *Z. bailii* in systems containing glucose.

of glucose and KS promoted a greater decrease in the growth rate than each factor (a_w depression by glucose addition and KS) by itself.

When data obtained for a_w 0.971 were analyzed, it was observed an interaction between the solute used to depress a_w and KS (Fig. 8). This effect might be the expression of the synergic action of the combined use of glucose 22% or glucose–polyol and KS on growth rate. Mentioned synergism can be of technological relevance and it could lead to a diminishing of the level of preservative that is necessary for food stability. The strategy based on the combined use of KS with other additives to increase the biological activity of the preservative has been reported; for example, Kubo and Lee (1998) combined sorbate with polygodial, a potent fungicidal of *Saccharomyces cerevisiae*, and

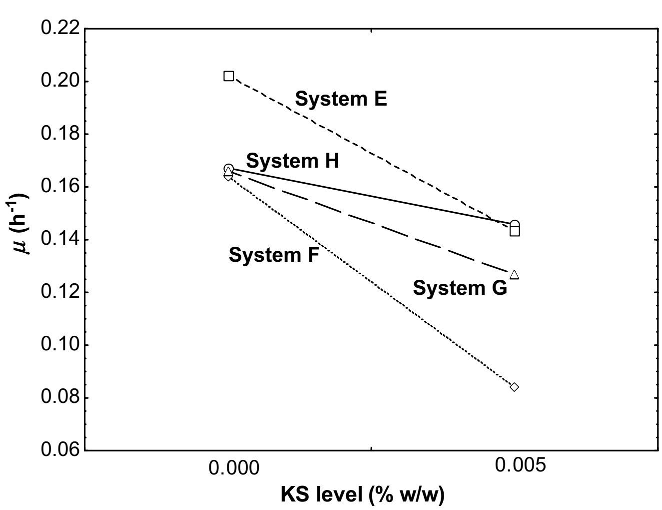

Fig. 8. Interaction between the solute used to depress water activity (a_w) to 0.971 and potassium sorbate (KS) level on the growth rate constants (μ) of *Z. bailii*.

observed that sorbate increased 64-fold its activity on the growth of this yeast.

In summary, parameters of *Z. bailii* growth seem to depend on a_w depression, solute used as humectant, presence of KS and also on the interaction of these factors. It must be highlighted as useful for preservation of low sugar products, the combined use of a_w depression through xylitol addition and KS. These recommendations are based in the assumption of the similarity in behavior among aqueous model systems and a food product; however it must be keep in mind that food components such as pectins and starches probably present in raw vegetal material can modify the performance of the preservative while acting as microorganism substrate or complexing with sorbates (Kurup, Wan, & Chan, 1995).

5. Conclusions

Water activity (a_w) depression, humectant used, presence of KS and interaction among mentioned factors influence growth of *Z. bailii*. Concerning this influence, the following facts need to be remarked:

- As potassium sorbate concentration increased, the yeast lag phase increased, growth rate and maximum population at the stationary phase diminished.
- Use of polyols in the absence of KS generally decreased growth rate, extended lag phase and diminished the level of population in the stationary phase.
- Use of polyols in the presence of KS produced a decrease in the asymptotic value of growth curve. On the contrary, the use of glucose alone or in combination with a polyol promoted an increase in the asymptotic value independently of KS content.
- Depression of a_w in the presence of KS increased lag phase and produced different effects on growth rate depending on a_w level, solute added and concentration of the preservative.
- A synergism between humectants (glucose and polyols) and KS was observed in relation to growth inhibition. This behavior would allow to diminish the amount of preservative used preserving the product microbial stability.
- Xylitol produced per se an inhibitory effect on *Z. bailii* growth. This fact would allow formulation of a system without or with a smaller amount of preservative using xylitol as additive.

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