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Short communication

Effect of steviosides and potassium sorbate on the growth and thermal inactivation of *Zygosaccharomyces bailii* in acidified model aqueous systems

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ABSTRACT

Zygosaccharomyces bailii growth and thermal inactivation curves were obtained in acidified aqueous systems, simulating low sugar products. Growth curves were fitted with Gompertz equation, while thermal inactivation curves were modeled using Baranyi equation. The parameters of the models were estimated and the effect of steviosides (EE) and potassium sorbate (KS) on the growth and survival of *Z. bailii* was established. The addition of KS decreased *Z. bailii* growth rate and increased its inactivation rate. The presence of EE promoted *Z. bailii* growth and protected yeast from thermal treatment. The joint use of KS and EE decreased the growth and nullified the protective effect of the sweetener on thermal inactivation. Results allow the selection of the appropriate concentrations of EE and KS to ensure the microbiological stability of evaluated systems and contribute to the development of low sugar products.

1. Introduction

Obesity has reached epidemic rates worldwide over the past decades. Among others diseases, obese people are at increased risk of diabetes, cardiovascular disease and hypertension (World Health Organization, 2015). As a consequence, the development of healthier food with fewer calories is a must. Apart from providing the sweet taste, sugar contributes to the desired viscosity and texture, controls hygroscopicity and moisture migration and also depresses water activity (Sandrou and Arvanitoyannis, 2000). Therefore, reducing sugar content in food produces changes in chemical, physical and microbiological stability. These problems are usually solved by the addition of sweetener and other additives such as thickeners and antimicrobials (Giemmo, Montagnani, Schelegueda, González, & Campos, 2015).

Due to the growing consumer demand for healthy products, with the least amount of synthetic additives as possible, the use of the sweetener stevia is increasing. The latter is extracted from the leaves of *Stevia rebaudiana* (Bertoni), a herb native from Paraguay. It is sweeter than sucrose and it is also attractive to diabetic people since it has a hypoglycemic response (Jooken et al., 2012). Moreover, its oral antibacterial, hypotensive and vasodilator action has been reported (Ferri et al., 2006). Stevia is generally recognized as safe and its use as food additive is approved in different countries including USA, European Union, China, Brazil, Paraguay and Argentina (Giemmo et al., 2015). According to Hracek, Gliemmo, and Campos (2010), mentioned sweetener is stable in aqueous solutions with pH between 3.0 and 10.0 and under thermal treatment up to 80 °C.

As it was mentioned before, the reduction of sugar content promotes the increase of water activity with the consequent decrease in preservation. Moreover, there is a growing interest of consumers for fresh taste products, therefore it is necessary to avoid the use of single preservation factor. Mentioned problems can be solved up by the use of the hurdle technology. Regarding low sugar foods, the stress factors usually applied are the decrease in pH and water activity, application of a thermal treatment and the addition of preservatives. Sorbic acid or its potassium salt (KS) are preservatives frequently used in acidic foods. Their effectiveness depends on the type of food and the processing and storage

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Table 1
Two-level full factorial design used to evaluate the effects of steviosides (EE) and potassium sorbate (KS) on *Z. bailii* growth and thermal inactivation.

	Independent variable (% w/w)	Coded variable	Level Code		
			−1	0	+1
Growth	KS level	x_1	0.000	0.005	0.010
	EE level	x_2	0.000	0.175	0.350
Thermal inactivation	KS level	x_1	0.000	0.0125	0.0250
	EE level	x_2	0.000	0.175	0.350

conditions (Sofos, 2000). Preservation factors applied in acid low sugar foods prevent the growth of pathogens but are not enough to control the growth of spoilage yeasts, such as *Zygosaccharomyces bailii* (Stiles, Duffy, & Schaffner, 2002). Mentioned yeast is osmophilic, acid tolerant, resistant to low levels of oxygen, high temperatures and preservatives presence. As a consequence it causes numerous economic losses and controlling its growth in acidic product is of interest (Campos, Gliemmo, & Castro, 2014).

Based on the topics discussed and on the lack of information about the effect of the joint use of steviosides (EE) and KS on *Z. bailii* development in acidic aqueous systems, the present study was conducted with the goal of evaluating and modeling the effect of EE and KS on the growth and on the thermal inactivation of *Z. bailii* in aqueous systems with pH 3.00 that model low sugar foods.

2. Materials and methods

2.1. Inoculum preparation

Zygosaccharomyces bailii NRRL 7256 was prepared in Sabouraud broth (Biokar Diagnostics, Beauvais, France) at 25 °C until stationary phase was achieved (30 h).

2.2. Model system formulation

The composition of model systems is shown in Table 1. They were formulated in Sabouraud broth which is a general basal media recommended for yeasts growth (Deák, 2008). The pH was adjusted to 3.00 by citric acid addition. Potassium sorbate (Sigma, St. Louis, Mo., U.S.A.) and citric acid (Merck Química Argentina, Buenos Aires, Argentina) were of reagent grade. Steviosides (90%w/w of a mixture of steviosides and 10%w/w of maltodextrin) (Inmobal Nutrer, Argentina) were of food grade.

2.2.1. Growth

Aliquots of 14.25 ml of each model system were dispensed in triplicate into dark glass flasks and autoclaved. Then, 0.75 ml of inoculated Sabouraud broth was aseptically added, obtaining 1.10^6 CFU/ml. The air headspace was 75% of total volume of flasks. The systems were incubated at 25 °C for 6–7 days.

2.2.2. Inactivation

Aliquots of 99.0 ml of each model system were dispensed in triplicate into 250 ml Erlenmeyer and autoclaved. Then, the systems were tempered at 50 °C and 1 ml of inoculated Sabouraud broth was aseptically added, obtaining 1.10^5 CFU/ml. Systems were incubated at 50 °C and agitated at 60 rpm on an orbital shaker (Shaker Pro, Vicking, Buenos Aires, Argentina) for 60–80 min.

2.3. Sampling and analysis

2.3.1. Growth

The yeast growth was measured by turbidimetry according to Gliemmo, Campos, and Gerschenson (2006a). Briefly, 1 ml was removed from each flask at selected times, diluted if it was necessary, and the absorbance at 540 nm was measured (Spectrophotometer Shimadzu UV-1203, Japan), then, growth curves were constructed.

2.3.2. Inactivation

Aliquots were removed from each Erlenmeyer at selected times and the population of *Z. bailii* was determined by pour plating on Sabouraud agar (Biokar Diagnostics, Beauvais, France). Plates were incubated at 25 °C for 7 days. Colonies were counted and thermal inactivation curves were constructed.

2.4. Data analysis

2.4.1. Growth

Obtained data from *Z. bailii* growth were modeled by means of modified Gompertz equation (Gliemmo et al., 2006a; McMeekin, Olley, Ross, & Ratkowsky, 1993):

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

which express the change of absorbance (y) -produced by the growth of the yeast-vs. time (t). The biological parameters of the yeast growth were the specific growth rate (μ), the lag phase time (λ) and the asymptotic value (A). Since the optical density method is less sensible than the viable method, the evaluated μ does not correspond to the maximum growth (Skara et al., 2012). The parameters of growth of each system were estimated fitting data to mentioned nonlinear regression model (equation (1)).

2.4.2. Inactivation

Obtained data from thermal inactivation were modeled using Baranyi equation (Equation (2)) (Xiong, Xie, Edmondson, Linton, & Sheard, 1999), which expresses the logarithmic relation between the number of microorganisms present at time (N_0) and the number of microorganisms present at time zero (N_0) as a function of time (t)

Table 2
Coefficients of regression models for studied parameters, adjusted determination coefficients (R^2 adj), absolute average deviation (AAD), root mean square error (RMSE) and probability values (p).

Response	Coefficients of regression				R^2adj	AAD	RMSE	p-value		
	β_0	β_1	β_2	β_{12}				x_1	x_2	x_1x_2
λ	14.25	4.25	−0.59	−2.22	90.2	9.08	1.46	<0.0001	0.3081	0.0027
μ	0.13	−0.01	−0.01	−0.03	93.6	4.94	0.01	0.0017	0.0383	<0.0001
A	1.77	−0.22	0.07	−0.15	95.4	2.24	0.05	<0.0001	0.0033	<0.0001
k	0.20	0.05	−0.02	0.01	81.3	10.04	0.02	0.0001	0.0201	0.2044

Coefficients, β_0: independent term, β_1: for KS level, β_2: for EE level, β_{12}: for interaction. Independent variables, x_1: KS level, x_2: EE level and x_1x_2: KS-EE interaction.

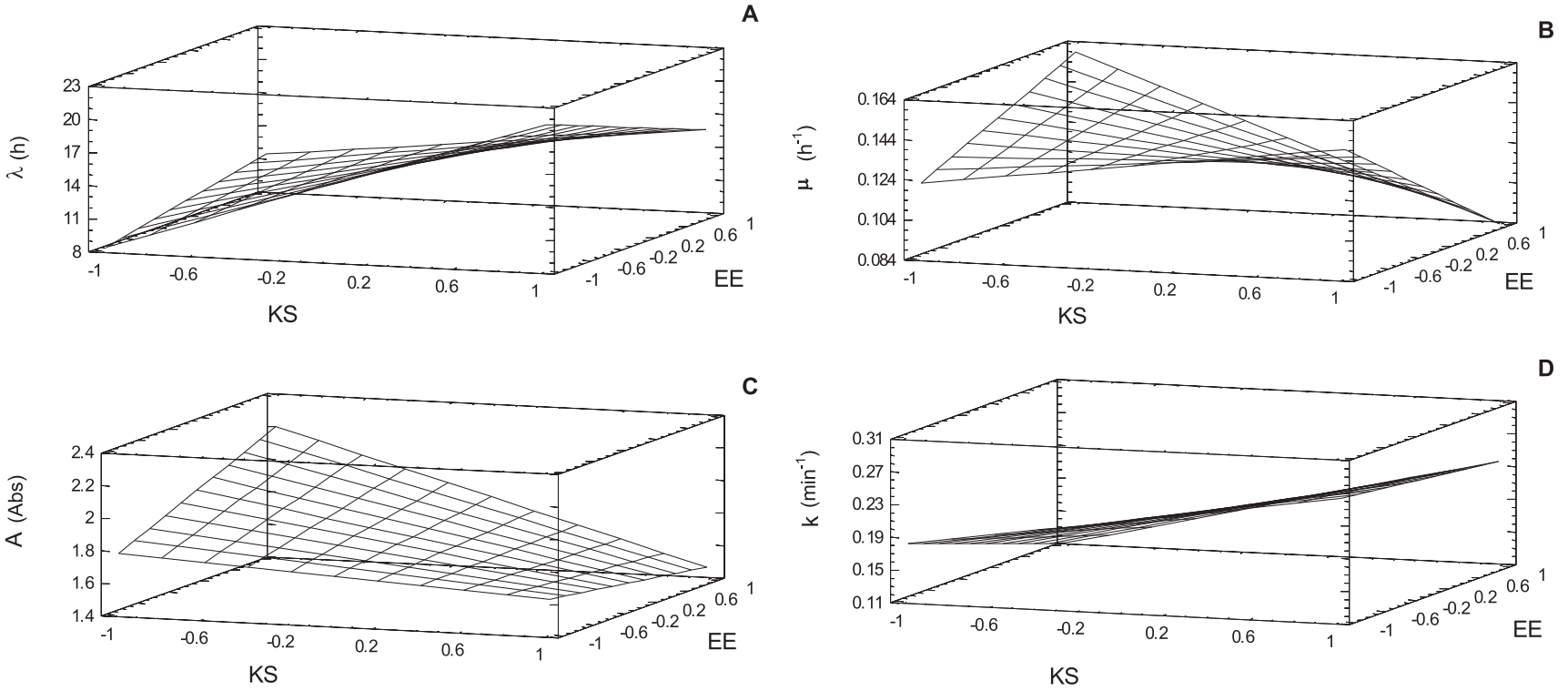

Fig. 1. Estimated response surface graph for the effect of steviosides (EE) and potassium sorbate (KS) on the lag phase time (λ, panel A), the specific growth rate (μ, panel B), the asymptotic value (A, panel C) and the maximum relative death rate (*k*, panel D).

$$\frac{\log N}{N_0} = \log\left[q_B + (1 - q_B)e^{-k(t-B(t))}\right] \quad (2)$$

where $q_B = \left(\frac{(N)_{\min}}{N}\right)$; $N_{min}$is the minimum cell concentration remaining in the tailing phase; k is the maximum relative death rate, and B(t) is the lag time function (Gliemmo, Schelegueda, Gerschenson, & Campos, 2013).

The parameters of survival curves of each system were estimated fitting data to mentioned nonlinear regression model (equation (2)).

2.5. Experimental design and statistical analyses

To assess the effect of EE and KS on the growth and thermal inactivation of *Z. bailii*, two full factorial designs (2^2) in three blocks and a central point were performed, resulting in a total of 15 experimental runs, each one. The studied factors and their levels are shown in Table 1. The maximum levels of KS were sub-inhibitory concentrations selected taking into account the minimum inhibitory concentration determined in previous studies (Hracek et al., 2010).

The effect of each independent variable on the biological parameters and on the maximum relative death rate was evaluated by means of the following regression model:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 \quad (3)$$

Where y are the parameters, x_1 and x_2 are KS and EE levels, respectively, β_0 is the independent term, β_1 and β_2 are the linear coefficients, and β_{12} is the interaction coefficient.

The adequacy of growth and thermal inactivation models and the regression models generated by the factorial design was examined by analysis of variance (ANOVA), adjusted determination coefficients (R^2adj), absolute average deviation (AAD) and root mean square error (RMSE). Also, ANOVA and *p*-value were used to evaluate the significance of the linear and interaction terms of each regression model generated by the factorial design.

Designs' creation and statistical analyses were performed using the statistical program Statgraphics (Statgraphics Plus for Windows, version 5.1, 2001, Manugistics, Inc., Rockville, Maryland, USA). The significance level was 0.05%.

3. Results

Growth and thermal inactivation curves were satisfactorily modeled using Eqs. (1) and (2), respectively, obtaining R^2adj values between 0.92 and 0.99 and low AAD and RMSE values. Regarding thermal inactivation curves, *k* was the most sensitive parameter of Baranyi model, and it was selected as the response for the applied factorial design.

The relations between the independent variables and the studied parameters were adjusted to Eq. (3). The presence of EE and KS significantly affected yeast development and inactivation (Table 2). The goodness of fit between the observed and the predicted values of responses was evidenced by R^2adj, AAD and RMSE values.

3.1. Growth curves

The addition of KS increased the lag phase and decreased the growth rate and the asymptotic value (Fig. 1, panels A, B and C). In the absence of KS, sweetener presence had no effect on the lag phase, but it accelerated the growth rate and increased the maximum population reached at the stationary phase (Fig. 1, panels A, B and C). However, the combined use of EE and KS produced the decrease of the growth rate and the asymptotic value, indicating the existence of interaction between both additives (Table 2).

Applying the regression model, the minimum growth rate and the minimum asymptotic value were achieved by adding 0.350% (w/w) of EE and 0.010% (w/w) of KS. Mentioned formulation presented an asymptotic absorbance value of 1.48, a growth rate of 0.084 h^{-1} and a lag phase of 21.3 h.

3.2. Inactivation curves

Different inactivation curves were obtained depending on the system composition. In most cases, the presence of a tail was detected. In those systems containing EE or KS alone, no shoulder was found. Observed tails suggest the presence of a heat resistant subpopulation. According to Gliemmo, Campos, and Gerschenson (2006b), mentioned subpopulation may be the consequence of the formation of ascospores which are more resistant than vegetative cells.

Inactivation constants increased with KS concentration (Fig. 1, panel D). Moreover, the increase in sweetener concentration protected yeast from thermal treatment. No interaction between variables was detected.

Applying Eq. (3), it was calculated that a concentration of 0.0135% (w/w) of KS would be necessary to nullify the protective effect exerted by 0.350% (w/w) of EE.

4. Discussion

Obtained results indicate that additives affect yeast growth and thermal inactivation. The addition of KS exerted an inhibitory effect on *Z. bailii*, while EE promoted its growth and protected it from thermal treatment. The latter trend suggests that EE could act as nutrient or interact with *Z. bailii* surface, protecting it from environmental stress.

Regarding *Z. bailii* inactivation, no interaction between both factors was found. The sweetener protected *Z. bailii* from thermal treatment but the addition of KS nullified this effect. As a consequence, KS would cooperate with thermal treatment and its combination with EE would be useful to preserve acidic foods with low sugar content. Previous studies reported that aspartame addition increased *Z. bailii* thermal inactivation rate in model aqueous systems, which exhibited a composition similar to those evaluated in the present study (Gliemmo et al., 2013). The latter demonstrates the importance of proper sweeteners selection in order to ensure microbiological stability.

To conclude, the addition of KS to systems sweetened with EE would minimize the effects of the sweetener to enhance the growth of *Z. bailii* and to protect it from thermal inactivation in acidic systems. Exposed trends highlight the importance to know the effect exerted by the interaction between KS and EE on the development of *Z. bailii*. Furthermore, this study allows selecting the appropriate concentrations to ensure the microbiological stability of evaluated systems and to decrease thermal treatment intensity. In this way, results contribute to the development of low sugar products.

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