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INFLUENCE OF PHYSICAL AND CHEMICAL FACTORS ON THE SURVIVAL OF *Pichia* ISOLATED FROM GLUCOSE SYRUP

*(Influencia de factores fósicos y químicos sobre la sobrevivencia de **Pichia** aislada de jarabe de glucosa)*

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ABSTRACT

*Yeasts belonging to the genus **Pichia** were isolated from glucose syrup samples. Yeasts were identified as **P. anomala** (it is now **Wickerhamomyces anomala**) and **P. guilliermondii.** These strains did not metabolize the nutritive preservatives, potassium sorbate or sodium benzoate when these were used as the single carbon source. **P. anomala** grew in culture media containing up to 1200 mg/L of both preservatives. Critical temperature and time of exposure for its inactivation were 60 °C and 3 min, respectively. **P. guilliermondii** grew in media containing up to 1400 mg/L of both preservatives.*

*Critical temperature and time for inactivation of this **Pichia** were 80 °C and 2 min. This strain was able to grow in a wide range of temperatures (5 to 30 °C), pH (2.5 to 5.5) and glucose concentrations (200 to 800 g/L). At 5 °C and 800 g/L glucose (osmotic pressure, 0.110 atm), **P.guilliermndii** grew poorly, with no cell death because of its ability to sporulate.*

*We determined that **P. guilliermondii** is a potentially contaminating yeast able to develop in a variety of foods, especially those with low pH or with high sugar concentrations (glucose above 400g/L) such as refreshments, juices, syrups and confected fruits and it is resistant to both food preservatives and low temperatures (5°C)*

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RESUMEN

*De muestras de jarabe de glucosa fueron aisladas levaduras que pertenecen al género **Pichia**. Estas fueron identificadas como **P.anomala** (actualmente **Wickerhamomyces anomala**) y **P. guilliermondii**, ambas cepas no metabolizan los conservantes alimenticios, sorbato de potasio o benzoato de sodio, al ser usados como única fuente de carbono. **P. anomala** desarrolló en medios de cultivo que contenían hasta 1200 mg/L de ambos conservantes. La temperatura y el tiempo crítico de exposición para su inactivación fueron 60 °C y 3 min, respectivamente. **P.guilliermondii** desarrolló en medios que contienen hasta 1400 mg/l de ambos conservantes. La tº y el tiempo crítico de inactivación fueron 80 °C y 2 min. Esta cepa fue capaz de desarrollar en un amplio rango de temperaturas (5 a 30 °C), pH (2,5 a 5,5) y concentraciones de glucosa (200 a 800 g/L). A 5 °C y glucosa 800 g/L (presión osmótica, 0,110 atm), **P. guilliermondii** desarrolló pobremente sin que se produzca la muerte celular debido a su capacidad de esporular. Se determinó que **P. guilliermondii**, es una levadura potencialmente contaminante que puede desarrollarse en una variedad de alimentos, especialmente aquellos con bajo pH o altas concentraciones de azúcar (glucosa, > 400 g/L) como en gaseosas, jugos, jarabes y frutas confitadas, y es resistente a ambos conservantes y bajas tº (5°C).*

INTRODUCTION

Fungi and, exceptionally certain bacteria, are osmotolerant microorganisms. Fungi can be found in a wide variety of natural and artificial environments (1,2,3). The

appearance of filamentous fungi and yeasts in a certain ecological niche is influenced by parameters such as their ability to use different substrates and their tolerance to low pH values, water activity (a_w), oxygen concentration and low temperature (4,5,6,7,8). In products with high sugar concentrations such as syrups, a_w is the most important factor since it determines which microorganisms will develop (9,10,11,12). Only osmophilic microorganisms grow in syrups. Among them, ascogenous and osmophilic yeasts can be found in latent form in syrups used as raw matter in the preparation of foods. These yeasts grow rapidly in acidic or sugary products and their contaminating activity causes flavour changes and excessive gas production in foods. The amount of CO_2 produced can be high enough to cause deterioration of the packaging or make the container explode, which results in financial losses.

Davenport (13), observed that in the deterioration of sweet beverages, contaminants were highly fermentative osmotolerant agents, often fructophiles (preferring fructose); these agents had an excessive gas production, required vitamins and were extremely resistant to food preservatives. He groups microorganisms into four categories according to the food or environment they contaminate. The ones in group 1 cause deterioration, those in group 2 are hygienic indicators and cause deterioration as opportunistic microorganisms, those in group 3 indicate hygiene and the ones in group 4 are aliens. Within Group 1 we can find species of ***Zygosaccharomyces*** and strains of **S*accharomyces*** (14). The genus ***Zygosaccharomyces*** is often synonymous with food contamination due to its resistance to food preservatives such as sorbic acid, benzoic acid and its salts, acetic acid and ethanol. Within this genus, the most common species is ***Zygosaccharomyces bailii***, which can grow at concentrations of food preservatives higher than those legally used in foods. It can also grow at low pH and/or in foods with high sugar concentrations such as tomato sauce, soft drinks (7-12 % w/v), wine, fruit juices (10 % w/v), grape juices (25 % w/v), jellies, honey and sugar syrups (67-80 % w/v). Certain osmophilic yeasts, ***Candida*** and their sexual statements such as ***Pichia*** are potentially pathogenic to humans and animals (8,15,16), have not been investigated as possible contaminants of foods with low water activity.

Usually, preservatives are added to foods to prevent contamination. In some cases, however, they are not adequate and other methods are required to control the development of food spoilage microorganisms. Benzoic acid and its salts, which act on yeasts and fungi, enter the cell in a non dissociated form that exerts an antimicrobial effect. Sorbic acid and its salts inhibit metabolic enzymes such as lactate dehydrogenase, catalase and enolase and bind to the sulphide groups (-SH) of the enzymes.

This paper provides data that can optimize the process of destruction of the isolated yeasts through the combined action of the osmotic pressure generated by high sugar concentrations and the variation in temperature in systems that simulate foods with a pH range of 2.5 to 5.5.

The aim of this paper was to determine the influence of physical and chemical factors such as temperature, pH, osmotic pressure and food preservatives on the survival of the isolated yeast from glucose syrup

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MATERIALS AND METHODS

Isolation and identification.

For the isolation of osmophilic yeasts, 10 g of the glucose syrup sample (resulting from the industrialization of corn starch) were added to 50 ml of medium MY_{40} agar containing 400g/L sucrose; 20, malt extract; 5, yeast extract and 14, agar-agar. The mixture was homogenized and distributed in Petri dishes and incubated at 28 ºC for 2 weeks. This medium was recommended by Kurtzman (17) for the growth of osmophilic yeasts. For identification we used the methods suggested by Verna & Herrero (18), Kurtzman & Fell (17), and the API ID 32 C kit. The studies based on the biochemical properties of yeasts were: assimilation of different carbon sources, nitrogen sources, sugar fermentation, growth at 19, 25, 34, 37 and 40 °C temperatures, saccharate assimilation, growth in NaCl plus 5% (w/v) glucose, gelatin liquefaction, urease production and microscopical characteristics such as vegetative reproduction, ascus production and presence of pseudomycelium. All these studies were performed at a temperature of 28 ºC and at pH 5 a time period of 1 to 4 weeks according to the assay under consideration.

Cell suspension.

In all assays we used a yeast suspension with an optical density (O.D.) of 0.05 (corresponding to 0.09 mg/ml,dry weight) for a wave length of 540 nm measured in a Beckman DU 530 UV/VIS spectrophotometer.

Survival of the yeasts in the glucose syrup at different temperatures.

In industry, temperatures of 120, 80 and finally 60 ° C are used to concentrate the glucose solution in syrup, so we studied the effect of these temperatures to determine the survival of the yeasts in the syrup. For the assay, l ml of concentrated syrup was heated at the above temperatures. When the inside of the syrup reached the desired temperature, 0.1 ml of the yeast inoculum was added and the mixture was incubated for different time periods: 1, 2 and 3 min. Then, agar MY_{40} medium was added to the

treated samples, which were then placed in Petri dishes and incubated at 28 °C for 1 week. The results were expressed as percentage (%) of colonies with respect to the number of colonies obtained with the control (with no thermal treatment), which was considered as 100% survival.

Study of assimilation and resistance to preservatives.

A separate study was made of the assimilation and resistance of each of the food preservatives, sodium benzoate and potassium sorbate, to ***Pichia anomala*** and ***P. guilliermondii***. The preservatives were added to MY_{40} agar and Dextrose agar media to obtain the following concentrations: 600, 800, 1200, 1500 and 2000 mg/L. In order to study the assimilation of the preservatives as the single carbon source, the above media did not contain either sucrose or glucose. In all tests, after the addition of the preservative, the media was adjusted to pH 5.0± 0.1 and 0.1 ml of the yeast suspension was added to the cultures, which were incubated at 28 °C. Readings were taken daily for 4 weeks. The results were expressed as percentage (%) of colonies with respect to the number of colonies obtained with the control (with no preservatives), which was considered as 100 % survival.

Effect of temperature, pH, food preservatives and osmotic pressures in a submerged and shaken system.

We used ***P. guilliermondii*** since is the most resistant strain for the syrup concentration temperature (80°C) and for the food preservatives. A liquid food was simulated and different amounts of glucose syrup were added to it to yield the following glucose concentrations: 200, 400, 600 and 800 ± 0.1 g/L, corresponding to the following osmotic pressures: 0.027; 0.055; 0.083 and 0.110± 0.002 atm, respectively. These assays were incubated at different temperatures: 5, 10, 20 and 30±0.1 °C and different pH: 2.5; 3.5; 4.5 and 5.5 ± 0.2 (adjusted with HCL 1 N or NaOH 1N), with or without the addition of sodium benzoate or potassium sorbate at a concentration of 2000 mg/l. All assays were inoculated with 0.1 mL of a yeast suspension and were incubated for 1 week. These experiments were carried out under submerged culture conditions and shaken at 250 rev/ min. Cell growth was measured by counting cell colonies on malt agar containing, in g/L: 3, malt extrac; 3, yeast extract; 5, proteose peptone; 5, glucose and 15, agar (Britania). Results were expressed as percentage of colonies with respect to the number of colonies obtained at 20ºC which was considered as 100% survival.

These *in vitro* experiments were carried out taking into account the fact that foods are preserved at different temperatures and with different degrees of acidity.

Reproductibility and data treatment.

Effects of temperature, pH, osmotic pressure and their interaction were examined by ANOVA using statistical software 6.0. All assays were carried out in duplicate in separate experiments.

RESULTS

Isolation and identification

Three yeast strains were isolated from the different syrup batches. On the basis of morphological characteristics and biochemical tests, they were identified as belonging to the genus ***Pichia*** and were termed ***Pichia*** MR-1, MR-2 and MR-3. Yeasts MR-1 and MR-3 belong to the species ***P. guilliermondii*** while MR-2 has properties similar to ***Pichia anomala***, it is now ***Wickerhamomyces anomala*** (19), all isolates of ***Pichia*** were confirmed by API ID 32C kit.

Survival of the yeasts in the syrup at different temperatures.

Results obtained in this experiment show that at 60º temperature the survival of ***P. anomala*** was 3 times lower than that of ***P. guilliermondii***, whose values were 20 and 64 %, respectively; these results were calculated in relation to the values found for the control of each yeast and for a residence time of the syrup of 3 min. At 80 °C, cell mortality in ***P. guilliermondii*** was high (98%) with respect to the one obtained at 60 °C (36%). This negative effect of temperature on cell viability was observed after a residence time of 2 min, which suggests that this is the critical exposure time to decrease the growth of ***P. guilliermondii***. ***P. anomala*** does not grow at 80 °C . Critical time and exposure temperature for inactivation of this ***Pichia*** were 3 min and 60 °C, respectively.

Assimilation and resistance to food preservatives of *P. anomala* and *P. guilliermondii.*

Results of the assays of assimilation of sodium benzoate and potassium sorbate as carbon sources indicate that neither of the two ***Pichia*** strains were able to metabolize the preservatives under the assayed conditions. The results of the resistance experiments showed that ***P. anomala*** grew fairly well up to a sodium benzoate concentration of 1200 mg/L while ***P. guilliermondii*** grew up to a concentration of 1400 mg/L of the preservative. This resistance was extremely noticeable in the medium containing yeast extract (MY_{40} agar medium). In contrast, in the dextrose agar medium, which lacked yeast extract**,** yeasts failed to grow. ***Pichia*** exhibited the same behaviour with potassium sorbate.

Effect of temperature, pH, food preservatives and osmotic pressure on the growth of *P. guilliermondii* in a submerged and shaken system.

Statistical analysis of variance (ANOVA) showed

that osmotic pressure and temperature, and their interaction affected significantly survival of ***Pichia*** ($p < 0.05$).

In order to determine osmotolerance, we carried out studies in which we varied the glucose concentration of the syrup and the combined action of factors such as temperature and pH. Table 1 shows the effect of these factors on the growth of ***P. guilliermondii***.

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Table 1. Effect of temperature and glucose concentration on cell survival of *P.guilliermondii* at different pH. Results were expressed as percentage of colonies with respect at 20ºC, which was considered as 100% survival

pH	Glucose (mg/L)	Temperature (°C)		
		5	10	30
2.5	200	16	50	79
	400	21	45	70
	600	21	48	81
	800	16	29	86
3.5	200	16	47	75
	400	15	40	84
	600	12	35	90
	800	10	24	55
4.5	200	29	88	95
	400	16	38	74
	600	14	42	91
	800	11	35	47
5.5	200	18	62	79
	400	19	40	80
	600	19	46	81
	800	14	57	79

DISCUSSION

The results of the assays of assimilation of sodium benzoate and potassium sorbate as carbon sources indicate that the isolated strains were protected against the effect of these antimicrobial agents in media abundant in growth factors. All these experiments were performed with no previous adaptation of the yeasts to the food preservatives. In contrast, Steels *et al.* (20), adapted ***Saccharomyces cerevisiae***, ***Z . bailii*** and ***Z. lentus*** strains to concentrations of 400 and 900 mg/L of benzoic acid for 1 week and later inoculated them in media with concentrations of 700 to 1200 mg/L of acid, noticing that their resistance to benzoic acid had increased. Comparison among the results of these authors to ours suggests that ***Pichia*** strains isolated from the glucose syrup are potentially contaminating such as ***S. cerevisiae, Z. bailii*** or ***Z. lentus***.

Results of the assays of combined action of factors such as osmotic pressure, temperature and pH indicate that ***P. guilliermondii*** grew fairly well at 200 g/L glucose at all pH assayed. In contrast, according to the reports of Steels *et al.* (20), the most tolerant strain to pH was ***Z. lentus***, which grew poorly at pH 2 and fairly well at pH 7. The results of our experiments show that temperature is a parameter that exerts a great influence on the viability of yeasts, especially when that action is enhanced by the increase in osmotic pressure. At 20 and 30 °C, ***P. guilliermondii*** exhibited fairly good growth (high survival) at the glucose concentrations assayed. A marked decrease in its survival was found at 5 °C at all pH assayed and at glucose concentrations ranging from 200 to 800 g/L (Table 1). When the yeast was grown under optimal growth conditions (10 g/L glucose, pH 5 and 28 °C), we found an increase in cell growth (0.11 to 0.70 mg/mL), which shows that at 5°C metabolic activities decreased and the cells remained in a latent state. In contrast, ***Z. bailii*** did not grow at 4 ºC (20) while poor development was observed in ***S. cerevisiae*** at this temperature (6).

When glucose concentration was increased to 400 and 600 g/L, yeast cells were exposed to hyperosmotic stress. These glucose concentrations caused a marked decrease in cell survival even at temperatures of 10, 20 and 30 ºC, with a dramatic drop at 5 ºC, with respect to the values found at 200 g/L.

At a glucose concentration of 800 g/L cell death could be expected due to the effect of cellular dehydration caused by the increase in sugar concentration. However, the results show that cell growth continued at all incubation temperatures and pH assayed (Table 1). This osmotolerance could be due to an increase in the production of glycerol or to the accumulation of intracellular trehalose (these are osmoprotectors produced by the cell in stress situations) according to the reports of Zhang *et al.* (21) and Fillinger *et al.* (22). In contrast with our results, the cell viability of ***S. cerevisiae*** decreased rapidly when cells were added immediately to a hyperosmotic solution of 600 g/L with no previous adaptation to such concentration (5,6), while ***Z. lentus*** developed in media containing 600 g/L glucose at the optimum growth temperature (20).

Our results agree with those reported by Montiel González *et al.* (12), who demonstrated that the effect of high sugar concentrations (higher than 200 g/l) on yeast growth is greater in liquid than in solid media, in the latter due to diffusion problems.

In order to determine the effect of food preservatives on cell viability, we carried out assays simulating

liquid foods with the addition of 2000 mg/l of potassium sorbate or sodium benzoate. The results obtained indicate that these preservatives caused 100% inhibition on the growth of ***P. guillermondii*** at all pH and temperatures assayed and at glucose concentrations of 600 and 800 g/L. The growth inhibition caused by sodium benzoate at 20 °C suggests that this preservative in liquid media totally inhibited the development of this ***Pichia***, keeping it in a latent state. This suggests that the action of preservatives should be enhanced by other treatments, especially in foods with high sugar concentrations. Similar results were obtained with potassium sorbate (2000 mg/L).

In liquid systems, the resistance of ***P. guilliermondii*** to food preservatives decreased but no cell death occurred. This isolated in our work should be considered strongly contaminating, since its growth was not inhibited at pH 2.5 or 3.5± 0.1, so it may be a contaminant of foods having low pH such as soft drinks, fruit juice (10% sugar, pH 2.5) or tomato sauce. It grew at sugar concentrations of 800 g/L so that it is an osmotolerant yeast able to grow in glucose syrups and crystallized fruit (40-67% w/v). As this ***Pichia*** grew at all assayed temperatures, with slow growth at 5 °C, it could contribute to the spoilage of chilled products.

CONCLUSION

Results of the morphological and biochemical tests showed that yeasts isolated in this work belonged to the genus ***Pichia***; they were identified as ***P. anomala* (*Wickerhamomyces anomala*)** and ***P. guilliermondii***.

The critical residence time and critical temperature of exposure of syrup to prevent the growth of ***P. anomala*** and ***P.guilliermondii*** were 2 min and 80 ºC, respectively. These results could be used as references for the selective inactivation of other osmophilic yeasts. In the presence of sodium benzoate or potassium sorbate, ***P. anomala*** and ***P. guilliermondii*** grew even at concentrations of 1200 mg/L and 1400 mg/L, respectively.

In submerged and shaken systems, ***P. guilliermondii*** grew at all pH assayed (2.5 to 5.5) and at temperatures of 10 to 30 ºC, although this strain has the potential to survive in hyperosmotic media (600 to 800 g/L glucose) even at low temperatures (5 ºC) and to remain latent due to its ascospore-forming ability.

Knowledge of the environmental factors influencing yeast growth is important so that storage environments can turn out unfavorable for ***Pichia*** development. Furthermore, considering the pathogenic potential of ***Pichia*** strains (***Candida*** asexual), understanding the effects of environmental factors on yeast growth on products with high sugar concentrations or low pH is necessary to evaluate the consumer health risk.

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