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# Activity of Na-Benzoate and Ethyl-Paraben Against Osmotolerant Yeasts at Different Water Activity Values

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ABSTRACT

To preserve high sugar products, the effectiveness of sodium benzoate and ethyl-paraben (para-hydroxybenzoic acid ethylester) against 18 osmotolerant yeast strains was investigated at different water activity values ($a_w$). The influence of pH, acidulant, humectant as well as inoculum level on the tolerance limits for growth of selected strains has also been determined. The tolerance limits for growth of all 18 tested strains were only slightly affected by the $a_w$ of the substrate, provided that the preservative concentration reflects only the amount of water and not the whole volume of the substrate. At $a_w$ <0.900 and pH<4.0 1500 ppm Na-benzoate was necessary to inhibit growth of all 18 tested osmotolerant yeast strains for 30 d, while in a similar medium but at higher pH-values Na-benzoate was less effective. Similarly, at $a_w$ <0.900 and pH 3.0 a 30-day-free shelf life was guaranteed by addition of 400 ppm ethyl-paraben, while a higher concentration of ethyl-paraben (900 ppm) was necessary if the medium was acidulated to pH 4.8 only. The activity of Na-benzoate or ethyl-paraben against osmotolerant yeasts was usually poor if the initial count of contaminants was high. *Zygosaccharomyces bailii* was the most preservative-resistant osmotolerant yeast among the tested genera and species.

Spoilage of Intermediate Moisture Foods (IMFs) by osmotolerant yeasts is a known problem which is aggravated by the appearance of preservative-resistant species or strains, especially *Zygosaccharomyces bailii (48),* together with the present consumer as well as industry trend for moderate processing and reduced chemical preservation.

The choice of antimycotics is restricted mainly to benzoic acid and its sodium salt, sorbic acid and its potassium salt, propionic acid as well as the esters of para-hydroxy-benzoic acid. The tolerance of several yeast species to propionic, acetic and lactic acid has already been investigated *(30,31,47).* These acids showed low activity against osmotolerant yeasts and organoleptic alterations of the preserved products were also noted. Hence their use as food preservative is not appropriate.

The uses of potassium sorbate and sorbic acid as antimycotics are well established *(4,5,8,16,27,36,37,46).* Works dealing with the physical and chemical properties of benzoate as well as with its mechanism of action, antimicrobial activity, application and other characteristics have been recently reviewed by Chipley *(10).* The activity of sodium benzoate and benzoic acid against yeasts is well recognized but most of the previous works are without statements on its interaction with the water activity value of the substrate (e.g. *14,21,27,28).* The antimycotic effectiveness of low pH, low $a_w$ and benzoic acid was interrelated against the growth of various yeast strains in a soft drink and preservative model system by Baird-Parker and Kooiman *(3).* However, these data give only an indication of the preservative levels necessary for microbial stability; their confirmation in an actual high sugar product using the appropriate yeasts is necessary.

The methyl-, ethyl-, and propyl-esters of para-hydroxybenzoic acid (methyl-, ethyl-, and propyl-paraben) were introduced as preservatives by Sabalitschka et al. *(42).* Their effectiveness is less pH-dependent than that of benzoic or sorbic acid and in addition they are stated to be less injurious to health than benzoic acid *(1,2,7,10,20,41).* For these reasons they represent a good alternative for the chemical preservation of neutral or slightly acid foods.

The purpose of the present work was to evaluate the antimycotic effects of sodium benzoate and ethyl-paraben, in conjunction with different acidulants and humectants, on the growth of eighteen osmotolerant yeast strains under $a_w$ and pH conditions usually found in IMFs.

## MATERIAL AND METHODS

*Preparation of media and solutions.*

*Enrichment broth and diluent.* Yeast extract glucose 30 (YEG30) broth and diluent DS30 were prepared and sterilized as previously described *(24).*

*Yeast extract solution, standard preservative solutions and acidulant solutions.* A solution of yeast extract was prepared by dissolving 0.5% (w/w) yeast extract in distilled demineralized water. Five, 10, 15, 20, 25, 30, 35, 40, 45 and 50 g of Na-benzoate (Fluka No. 71300) were dissolved each

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TABLE 1. *Composition, $a_w$- and pH-values of different test systems containing sodium benzoate.*

| | Composision, $a_w$- and pH-values of test solutions with Na-benzoate | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| System No. ⟶ | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| $a_w$ (30°C) | 0.980 | 0.900 | 0.837 | 0.795 | 0.980 | 0.980 | 0.909 | 0.909 | 0.900 | 0.900 | 0.900 | 0.900 |
| Fructose (g) | 35 | 200 | 300 | 371.5 | 0 | 0 | 0 | 0 | 200 | 200 | 200 | 200 |
| Glucose (g) | 0 | 0 | 0 | 0 | 45 | 45 | 180 | 180 | 0 | 0 | 0 | 0 |
| Yeast extract solution (g) | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 |
| Na-benzoate | with each system; a series of 10 concentrations in the range 500 ppm-5000 ppm | | | | | | | | | | | |
| Acidulant | citric acid | citric acid | citric acid | citric acid | citric acid | phosp. acid | citric acid | phosp. acid | citric acid | citric acid | lactic acid | lactic acid |
| pH | 4.8 | 4.8 | 4.8 | 4.8 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 3.0 | 4.0 | 3.0 |

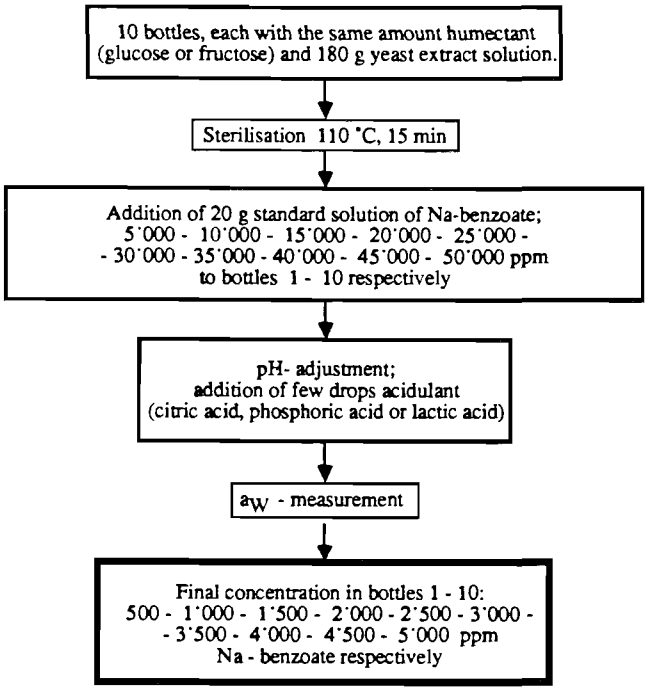


Figure 1. *Procedure adopted to prepare test solutions with sodium benzoate.*

in 1000 g of double distilled water under moderate warming (45°C) to produce a series of 10 standard solutions in the range 5,000-50,000 ppm. Similarly 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8 g of ethyl-paraben were dissolved each in 1000 g of double distilled water to create a series of 8 standard solutions in the range 400-1,800 ppm. All preservative standard solutions were filter sterilized. Phosphoric acid (p.A.) and pure lactic acid were used as acidulant in the concentration supplied by the manufacturer (85% and 90%, respectively), while a 1.5 *M* citric acid solution (22.37%) was prepared by dissolving 288.2 g of crystalline citric acid in 1000 g of double distilled water.

*Final test solutions with Na-benzoate.* Twelve different systems, each characterized by a different combination of $a_w$, pH and acidulant were prepared. A single flow diagram (Fig. 1) applicable to all the 12 systems and a table (Table 1), summarizing the variables of each of them, are presented. As Fig. 1 illustrates, 10 bottles (500 ml) were used with each system. An equal amount of humectant (glucose or fructose) and 180 g of yeast extract solution were mixed in each of them, dissolved in a steam-boiler at 80°C and autoclaved at 110°C for 15 min to avoid excessive browning and formation of toxic compounds. After cooling at room temperature, 20 g of a different standard solution of Na-benzoate at concentration 5,000, 10,000, 15,000, 20,000, 25,000, 30,000, 35,000, 40,000, 45,000, and 50,000 ppm were poured aseptically into bottles 1 to 10, respectively. The solutions obtained were adjusted to the desired pH (4.8, 4.0 or 3.0) with a few drops of one of the acidulants (citric, lactic or phosphoric acid) and their $a_w$-values were measured. By mixing 20 g of standard solution with 180 g of yeast extract solution a 1:10 dilution of the preservative, thus a series of 10 Na-benzoate concentrations in the range 500-5,000 ppm was finally obtained. Controls without preservative were also created. The concentrations of preservative are calculated as

amount preservative added (g)/amount water (g)

*Final test solutions with ethyl-paraben.* The procedure adopted to prepare the test solutions with ethyl-paraben was analogous to that used with Na-benzoate, however modified to permit a 1:2 dilution of the preservative. For this purpose the humectant (glucose or fructose) was dissolved in 100 g of yeast extract solution and sterilized. After cooling 100 g of a different standard solution of ethyl-paraben at concentration 400, 600, 800, 1,000, 1,200, 1,400, 1,600 and 1,800 ppm were poured into bottles 1 to 8, respectively. Citric acid or lactic acid was used as acidulant to adjust the solutions to pH 4.8 or 3.0. By mixing 100 g of standard solution of ethyl-paraben with 100 g of yeast extract solution a 1:2 dilution of the preservative, thus a series of 8 ethyl-paraben concentrations in the range 200-900 ppm was finally obtained. The concentrations are calculated as previously described. The type and the amount of humectant as well as the $a_w$, the pH and the acidulant used to prepare each of the 9 test systems are listed in Table 2. Controls without preservative were also created.

*Yeast taxa*

A total of 18 strains, i.e. *Zygosaccharomyces rouxii* LMZ 104, LMZ 105, LMZ 106, LMZ 107, LMZ 111, LMZ 112, LMZ 113, LMZ 114, LMZ 117, LMZ 120, LMZ 126, *Z. bailii* LMZ 108, LMZ 109, *Debaryomyces hansenii* LMZ 1902 and *Torulaspora delbrueckii* LMZ 1901, isolated from various high sugar products and maintained on agar slants for 2 years as described in Jermini et al. *(24),* as well as the reference strains *Z. rouxii* CBS 732, CBS 736 and *Z. bisporus* CBS 702, were used throughout this study. In some series of experiments all 18 strains listed above were investigated, while a smaller

TABLE 2. *Composition, $a_w$- and pH-values of different test systems containing ethyl-paraben.*

| | Composision, $a_w$- and pH-values of test solutions with ethyl-paraben | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| System No. ⟶ | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| $a_w$ (30°C) | 0.980 | 0.900 | 0.837 | 0.795 | 0.900 | 0.900 | 0.837 | 0.837 | 0.980 |
| Fructose (g) | 35 | 200 | 300 | 371.5 | 200 | 200 | 300 | 300 | 0 |
| Glucose (g) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 45 |
| Yeast extract solution (g) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Ethyl-paraben | with each system; a series of 8 concentrations in the range 200-900 ppm | | | | | | | | |
| Acidulant | citric acid | citric acid | citric acid | citric acid | citric acid | lactic acid | citric acid | lactic acid | citric acid |
| pH | 4.8 | 4.8 | 4.8 | 4.8 | 3.0 | 3.0 | 3.0 | 3.0 | 4.8 |

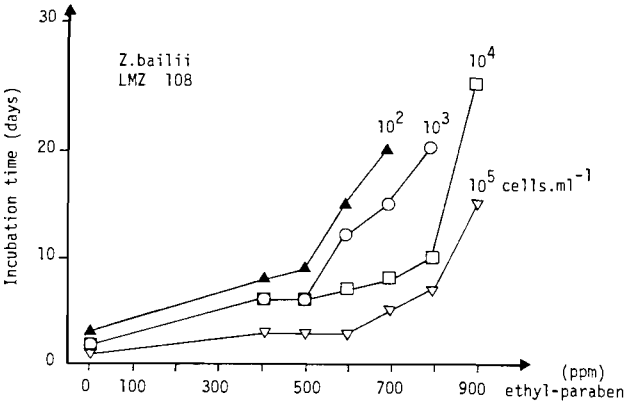


Figure 2. *Incubation time (d) necessary to observe growth of* Z. bailii *LMZ 108 at different concentrations of ethyl-paraben dependent on the inoculum level (pH 4.8, controlled with citric acid; $a_w$ 0.900).*

number of strains, randomly selected to represent at least three yeast species, were used in other series.

*Microbiological methods*

*Preparation of the inocula, inoculation and incubation of the test solutions.* YEG30 broth was inoculated from a stock culture on agar slant and incubated for 60 h. The cells were then harvested by centrifugation [Heraeus Centrifuge, Osterode, GFR; Relative Centrifugal Field (RCF) 2060 × *g;* 5 min] and resuspended in diluent DS30. Inoculation suspensions with $10^5$ cells/ml were prepared by counting with a Helber chamber and appropriate dilution with DS30. Portions of 9.9 ml of each final test solution were then aseptically dispensed from the bottles into reagent tubes and inoculated with a 0.1 ml inoculation suspension to create an initial level of $10^3$ cells/ml, unless otherwise stated. The cultures in reagent tubes were incubated at 25±0.5°C for 30 d. To avoid excessive loss of moisture, the tubes were sealed with a thin layer of parafilm. Twice a day the cultures were vigorously shaken.

*Determination of the tolerance.* The tolerance limit was defined as "the highest concentration of preservative at which yeast growth could occur at 25°C within 30 d." The appearance of growth was determined by placing the culture tubes, which had been vigorously shaken to disperse all yeast growth, against a white card bearing lines, drawn with black India ink, approximately 0.75 mm wide. Growth was considered to be "positive" if the lines appeared as diffuse bands or were distinguishable but had indistinct edges. A microscopic control of all the cultures rated "positive" was also carried out. Test solutions without evidence of growth were tested for viability on YEG30 agar (YEG30 broth supplemented with 1.5% agar) and found to have less than 1 CFU/ml. All experiments were done in duplicate.

## RESULTS

*Influence of the inoculum level on the effectiveness of chemical preservatives*

The incubation times necessary to observe growth with initial levels of $10^2$, $10^3$, $10^4$ and $10^5$ cells per ml of culture were investigated using *Z. rouxii* LMZ 104, *Z. bailii* LMZ 108 and *T. delbrueckii* LMZ 1901. The $a_w$ and the pH of the solutions were 0.900 and 4.8, respectively. Since all the tested strains exhibited a similar trend, only the results connected with the experiment carried out with *Z. bailii* LMZ 108 are presented (Fig. 2). *Z. bailii* LMZ 108 was chosen because of its higher preservative-resistance. If high counts of yeast cells, e.g. $10^4$ or $10^5$ per ml, were initially present, much more preservative was needed to inhibit their growth. In other words the lower the contamination, the lower the preservative concentration needed to inhibit growth of the contaminant. Second, the lower the contamination, the longer the growth-free shelf life of the test solutions.

*Influence of $a_w$ on the antimycotic effectiveness of Na-benzoate and ethyl-paraben*

Four different $a_w$ (0.980, 0.900, 0.837 and 0.795) values at a constant pH (4.8) and acidulant (citric acid) were investigated using all 18 strains previously listed as test organisms. Figure 3 and Fig. 4 illustrate the interactions on growth between $a_w$ and levels of benzoate and ethyl-paraben with *Z. rouxii* LMZ 112 and *Z. bailii* LMZ 108, respectively. Similar plots obtained with all other 16 strains tested are not shown. Two distinct interactions of $a_w$ and preservative were observed: *(a)* lowering the $a_w$ of the test solutions was more effective in delaying the appearance of their growth than an increase in the preservative concentration; *(b)* if the preservative concentration is calculated on the basis of the amount of water present in the substrate, the parameter $a_w$ has little or

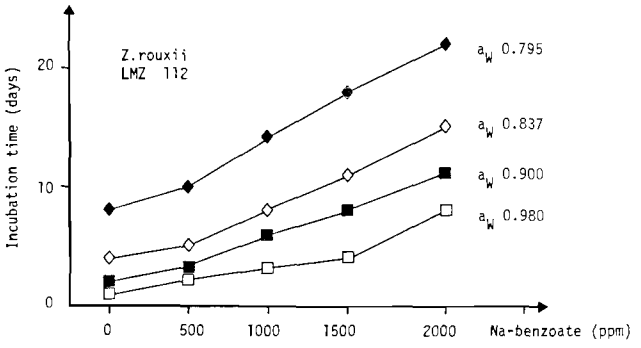


Figure 3. *Incubation time (d) necessary to observe growth of* Z. rouxii *LMZ 112 at different concentrations of Na-benzoate dependent on the $a_w$ of the test solution (pH 4.8 - controlled with citric acid; inoculum level $10^3$ cells/ml).*

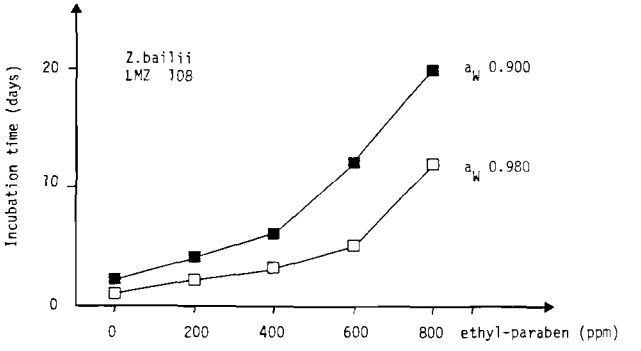


Figure 4. *Incubation time (d) necessary to observe growth of* Z. bailii *LMZ 108 at different concentrations of ethyl-paraben dependent on the $a_w$ of the test solution (pH 4.8 - controlled with citric acid; inoculum level $10^3$ cells/ml).*

no influence on the tolerance limits growth (Fig. 5 and Fig. 6).

*Z. bailii* was found to be an extremely preservative-resistant species. At $a_w$ 0.900, pH 4.8 and 25°C 5,000 ppm Na-benzoate and 900 ppm ethyl-paraben were necessary to inhibit growth of the strains LMZ 108 and LMZ 109 within 30 d. *T. delbrueckii* was the most tolerant among the other tested species, the growth of strain LMZ 1901 being inhibited by 3,500 ppm Na-benzoate and 700 ppm ethyl-paraben. *Z. rouxii* (3,000 ppm Na-benzoate and 700 ppm ethyl-paraben), *Z. bisporus* (2,000 ppm and 400 ppm, respectively) and *D. hansenii* (1,000 ppm and 400 ppm, respectively) followed in the order indicated. Moreover, reference strains of *Z. rouxii* were more sensitive than freshly isolated strains of the same species.

*Influence of pH and acidulant on the antimycotic effectiveness of Na-benzoate and ethyl-paraben*

*Na-benzoate.* Two different $a_w$ (0.980 and 0.909) values and two acidulants (citric acid and phosphoric acid) at constant pH (4.0) were adopted as experimental conditions to investigate interactions between $a_w$ and acidulant on growth of strains *Z. rouxii* CBS 732, LMZ 104, LMZ 105, LMZ 117, LMZ 120, *Z. bailii* LMZ 108, LMZ 109, *Z. bisporus* CBS 702 as well as *T. delbrueckii*

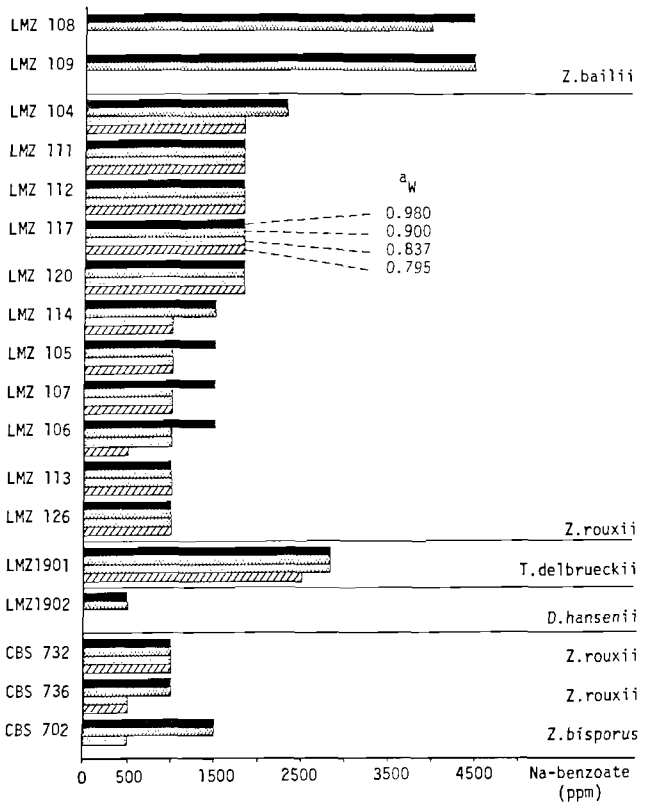


Figure 5. *Sodium benzoate tolerance limits (ppm) for growth of osmotolerant yeasts at pH 4.8 (controlled with citric acid) dependent on the $a_w$ of the test solution.*

LMZ 1901. In a separate series of trials, two different pH (4.0 and 3.0) values each controlled with citric acid or lactic acid were investigated at constant $a_w$ (0.900) using strains *Z. rouxii* LMZ 104, *Z. bailii* LMZ 108 and *Z. bisporus* CBS 702 as test organisms. As Table 3 shows, the antimycotic effectiveness of Na-benzoate is strongly pH-dependent, e.g. *Z. rouxii* LMZ 104 tolerated 2,500 ppm in slightly acid substrate but was not able to growth in the presence of 500 ppm at pH 3.0. The type of acidulant used to control the pH did not play an important role. At $a_w$ 0.900 no difference was recorded between test solutions acidulated with citric acid and those acidulated with lactic acid. However, in solutions of $a_w$ in the range 0.909-0.980 the combination of the preservative with phosphoric acid was slightly more effective than that with citric acid in inhibiting the growth of *Z. bailii* LM 109, *Z. bisporus* CBS 702 and *T. delbrueckii* LMZ 1901.

*Ethyl-paraben.* Different combinations of $a_w$ (0.900 and 0.837), acidulants (citric and lactic acid) and pH (4.8 and 3.0) were adopted as experimental conditions to investigate interactions between $a_w$, pH, acidulant and preservative on growth of the strains *Z. rouxii* LMZ 104, *Z. bailii* LMZ 108 and *Z. bisporus* CBS 702. As Table 4 illustrates, the antimycotic effectiveness of ethyl-paraben is slightly pH-dependent. An increase in acidity from pH 4.8 to pH 3.0 caused a reduction of the tolerance limits for growth by approximately 50-75%. The combi-

TABLE 3. *Sodium benzoate tolerance limits (ppm) for growth of nine osmotolerant yeast strains, dependent on the $a_w$ and pH value and acidulant of the test solution.*

| | Na-benzoate tolerance limits at different $a_w$- and pH-values | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $a_w$ 0.900/fructose | | | | | $a_w$ 0.909/glucose | | $a_w$ 0.980/glucose | |
| | pH 4.8 | pH 4.0 | | pH 3.0 | | pH 4.0 | | pH 4.0 | |
| Strains | Citric acid | Citric acid | Lactic acid | Citric acid | Lactic acid | Citric acid | Phosp. acid | Citric acid | Phosp. acid |
| *Z. rouxii* LMZ 104 | 2500 | 1000 | 1000 | <500 | <500 | 1000 | 1000 | 500 | 500 |
| *Z. rouxii* LMZ 105 | --[a] | -- | -- | -- | -- | 500 | 500 | 500 | 500 |
| *Z. rouxii* LMZ 117 | -- | -- | -- | -- | -- | 1000 | 1000 | 1000 | 1000 |
| *Z. rouxii* LMZ 120 | -- | -- | -- | -- | -- | 1000 | 1000 | 500 | 500 |
| *Z. rouxii* CBS 732 | -- | -- | -- | -- | -- | 500 | 500 | 500 | 500 |
| *Z. bailii* LMZ 108 | 4000 | 1500 | 1500 | 1000 | 1000 | 2000 | 2000 | 2000 | 2000 |
| *Z. bailii* LMZ 109 | -- | -- | -- | -- | -- | 2000 | 1500 | 2000 | 1500 |
| *T. delbrueckii* LMZ 1901 | -- | -- | -- | -- | -- | 500 | 500 | 500 | <500 |
| *Z. bisporus* CBS 702 | 1500 | 1000 | 1000 | 500 | 500 | 1000 | <500 | 500 | <500 |

[a]-- = not tested

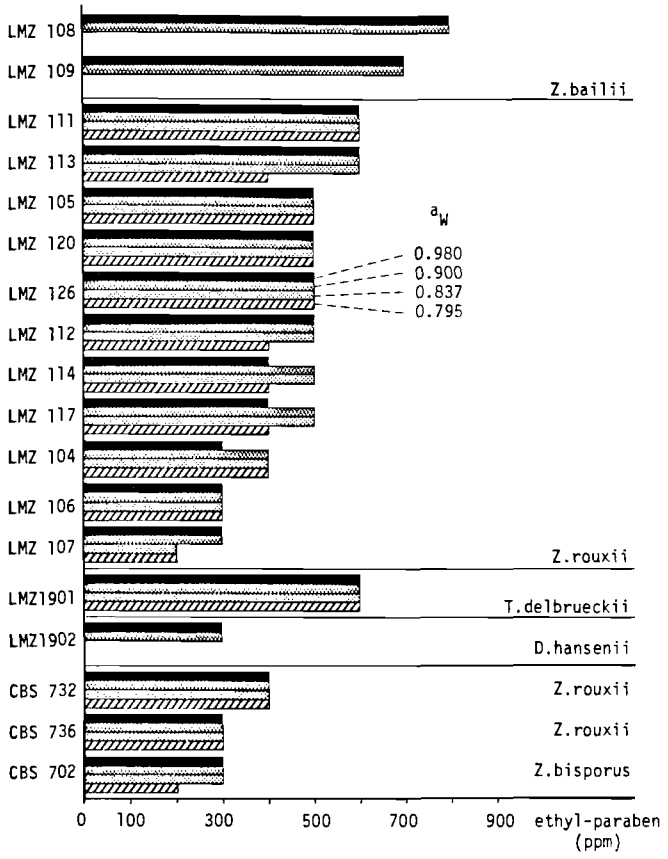


Figure 6. *Ethyl-paraben tolerance limits (ppm) for the growth of osmotolerant yeasts at pH 4.8 (controlled with citric acid) dependent on the $a_w$ of the test solution.*

nations of ethyl-paraben with citric acid and with lactic acid were absolutely equivalent.

*Influence of the humectant on the antimycotic effectiveness of ethyl-paraben*

Two humectants (glucose and fructose) were tested at constant $a_w$ (0.980), pH (4.8) and acidulant (citric acid) using strains *Z. rouxii* LMZ 104, LMZ 105, LMZ 117, LMZ 120 and CBS 732, *Z. bailii* LMZ 108 and LMZ 109, *Z. bisporus* CBS 702 and *T. delbrueckii* LMZ 1901 as test organisms. With 4 strains no difference was noted between the ethyl-paraben tolerance limits for growth in test solutions containing glucose and in those containing fructose as the humectant to control the $a_w$-value at 0.980. In both instances, the limits for the growth of *Z. rouxii* LMZ 104 (300 ppm ethyl-paraben), *Z. rouxii* LMZ 105 (500 ppm), *Z. rouxii* LMZ 117 (400 ppm) and *Z. bisporus* CBS 702 (300 ppm) were identical. With the remaining 5 strains only slight differences were stated. In test solutions with glucose *Z. rouxii* LMZ 120, *Z. rouxii* CBS 732, *Z. bailii* LMZ 108, *Z. bailii* LMZ 109 as well as *T. delbrueckii* LMZ 1901 showed tolerance limits of 400, 400, 700, 600 and 500 ppm, respectively. If fructose was used as humectant these limits were by 100 ppm higher.

## DISCUSSION

In the present work only the sensitivity of vegetative cells was investigated. The resistance of spores to heat, alcohol and diethylether is well recognized *(15,18,*

TABLE 4. *Ethyl-paraben tolerance limits (ppm) for growth of three osmotolerant yeast strains, dependent on the $a_w$ and pH value and acidulant of the test solution.*

| Strains | Ethyl-paraben tolerance limits at different $a_w$- and pH-values | | | | | |
|---|---|---|---|---|---|---|
| | $a_w$ 0.900/fructose | | | $a_w$ 0.837/fructose | | |
| | pH 4.8 | pH 3.0 | | pH 4.8 | pH 3.0 | |
| | Citric acid | Citric acid | Lactic acid | Citric acid | Citric acid | Lactic acid |
| *Z. rouxii* LMZ 104 | 400 | 200 | 200 | 400 | 100 | 100 |
| *Z. bailii* LMZ 108 | 800 | 300 | 300 | 600 | 300 | 300 |
| *Z. bisporus* CBS 702 | 300 | 100 | 100 | 300 | 100 | 100 |

*34,35,39,40).* Moreover, Romano and Suzzi *(38)* tested $SO_2$, benzoic acid, potassium sorbate, salicylic acid, nystatin, actidione and pimaricin against ascospores and vegetative cells of *Saccharomyces cerevisiae*. The *S. cerevisiae* ascospores were more resistant than the corresponding vegetative cells. This resistance varied, however, with the antimicrobial compound used. All compounds tested were selective and killed vegetative cells and ascospores at different rates, with the exception of potassium sorbate. Therefore, based on previous investigations, higher concentrations of Na-benzoate or ethyl-paraben than those recorded in the present work are probably necessary if ascospores of osmotolerant yeasts must be inhibited.

*Influence of $a_w$ and humectant on the antimycotic effectiveness of the tested preservatives*

The results of the present investigations, showing that the $a_w$ of the substrate has little or no influence on the preservative tolerance limits for the growth of osmotolerant yeasts, do not completely agree with previous literature. A possible explanation of such disagreement might be found, however, in the different ways adopted by previous researchers for calculating the preservative concentrations in the substrates. In the recent investigations of Baird-Parker and Kooiman *(3),* Bills et al. *(5)* and Restaino et al. *(37)* as well as in older works *(6,14,27,28)* the preservative concentration was always calculated as:

amount preservative added (g)/total substrate volume (ml) [1]

This might have led some authors to erroneous interpretations of their results. In fact, if similar amounts of preservative are added to similar volumes of substrate but at different water activity values, higher preservative concentrations are expected at lower $a_w$ where less water is available as solvent. Following this statement, in the present work the preservative concentrations have been calculated as:

amount preservative added (g)/amount water (g) [2]

Oka *(32),* Freese et al. *(19),* Macris *(29)* and Cramer and Prestegard *(11)* demonstrated that the only form of preservative taken up by yeast cells through their permeable membrane is the undissociated acid molecule present in the aqueous phase of the substrate. Moreover, Bosund *(9)* showed that the amount of benzoic acid absorbed by yeast cells was roughly proportional to the concentration of molecular benzoic acid in the substrate. Since a part of the substrate-water is already bound by other molecules, e.g. sugars and proteins, the preservative concentration in the substrate should be expressed as:

amount preservative added (g)/amount "free" water (g) [3]

However, a formula to calculate with accuracy how much water is available as solvent in a substrate of defined $a_w$ has not yet been devised. One could suggest to express it as:

"free" water (g) = water content (g) × water activity [3a]

Although incomplete, the formula [2] adopted in the present work represents an improved alternative to preceeding methods and it is convenient for practical purposes. Further theoretical and practical studies are necessary to find a correct way of predicting the amount of "free" water in a substrate at given $a_w$.

In the present work the activity of ethyl-paraben against osmotolerant yeasts in simulated Intermediate Moisture Foods (IMFs) has also been investigated using different sugars as humectant. Some of the tested strains were able to tolerate higher concentrations of ethyl-paraben when grown in media containing fructose rather than glucose as humectant. This is likely due to the fructophilic attitude of osmotolerant yeasts *(49)* rather than to a synergistic inhibitory effect of glucose and preservatives. Humectants are usually assumed to control microbial growth in IMFs by their ability to lower the water activity value of the substrate. Sugar and salts are traditional humectants but their use is limited by their solubility and organoleptic acceptance. These disadvantages have stimulated the search for humectants other than sugars (too sweet for some applications), salts (too salty), polymers (too viscous) and glycerol (off-flavor). Ideally such compounds should not affect the flavor of the prod-

ucts and exhibit antimicrobial properties in addition to their $a_w$-lowering role. However, all presently available new humectants such as aliphatic diols and propyleneglycols are limited in their use by a lack of assumed safety.

*Influence of acidity and acidulant on the antimycotic effectiveness of the tested preservatives*

The undissociated molecules of the organic acids and their esters are responsible for their antimicrobial activity *(22,29)*. It is an old observation that weak acids among the food preservatives are more active in an acidic than in a neutral environment *(12,13,14,21,29,44,45)*. Lowering the pH of a food increases the proportion of molecules of an organic acid that are undissociated and thus increases its antimicrobial or antimycotic effectiveness. Therefore, use of weak acids as antimicrobials is limited principally to foods with pH <5.5. If the data presented in Table 3 are expressed as concentration of undissociated acid (for example for *Z. bailii* LMZ 108: 788, 910 and 940 ppm benzoate at pH 4.8, 4.0 and 3.0, respectively), the slight differences in the tolerance limits so obtained are not statistically significant and are within experimental errors. Results of the present work are therefore in agreement with previous literature.

In accordance with Ecklund *(17)*, the present investigation with ethyl-paraben showed an increase in the tolerance limits with decreasing pH, and this cannot be due to different amounts of undissociated ester, since ethyl-paraben stays completely undissociated at the pH-values tested. High "per-se" effectiveness of the pH and/or an antimycotic activity of the used acidulant (citric or lactic acid) are possible explanations for these results. Differences in the antimicrobial activity of the acids used as acidulants have also been recorded here, confirming earlier reports *(25,26,31,36)*. Although no difference was stated between citric acid and lactic acid, the growth of some strains in media acidulated to pH 4.0 with phosphoric acid was inhibited at lower benzoate concentrations than in media adjusted with citric acid.

It must, however, be said that without a test to determine and separate the effects of the individual components, it is not possible to state with certitude to which extent the reduced concentrations of antimycotic necessary to cause inhibition of yeast growth at lower pH values is due to the undissociated antimycotic, the hydrogen ion concentration, the undissociated acidulant or its anion or, more than likely, all of the aforementioned.

*Other factors influencing the preservative resistance*

Whether or not particular organisms will be inhibited by a given concentration of preservative depends on the factors considered previously, i.e. $a_w$, pH, acidulant, humectant. However, there are several other limitations to the use of organic acids as microbial inhibitors in foods; *(a)* when the contamination is too high, chemical preservatives are usually less effective *(2)*; *(b)* the conditions of the organisms, i.e. whether damaged by exposure to adverse physical or chemical conditions *(23)*; *(c)* some microorganisms metabolize organic acids as a carbon source *(23)*; *(d)* resistant species and individual strains with variable resistance have been selected *(21,48)*.

In agreement with Baird-Parker *(2)*, chemical preservatives were found to be ineffective when the initial levels of microorganisms were high ($10^4$-$10^5$ cells/ml). No preservative should be added to cover up the use of spoiled or spoiling foods, and in most instances where a preservative failed, it was because of the use of inappropriate ingredients or of unhygienic food processing conditions *(23)*. Thus use of hygienic equipment and strict attention to factory hygiene reduced the risk of failure *(3)*.

It is well known that some spoilage yeasts develop tolerance to organic acid preservatives *(33,48,50,51)*. In accordance with these previous authors and with Sand *(43)*, *Z. bailii* was found to be the most preservative-resistant osmotolerant yeast species. Present results were obtained investigating strains isolated from high sugar products; no information was available on the chemical preservation of these products. Since the yeasts were maintained for 2 years on preservative-free agar slants, they were, however, assumed to be preservative non-adapted. The risk of adaptation of resistant types can be reduced by avoiding raw materials that have been treated with preservatives, because they may introduce resistant-adapted yeast into the factory. A profile of characteristics and spoilage activities of *Z. bailii* was made by Thomas and Davenport *(48)*, while Warth *(50)* discussed its mechanisms of resistance to benzoic, sorbic and other weak acid used as food preservatives, proposing a mechanism of resistance involving an inducible energy-requiring system which transported the preservative out of the cell.

*Practical implications of the results*

Table 5 summarizes results of the present research, indicating the preservative concentrations necessary to obtain a growth-free shelf life of 30 d with products having an $a_w$ in the range 0.980-0.795, depending on the pH-value. A contamination level of $10^3$ cells per ml or g as well as a storage temperature of 25°C were considered. Results of the present work have the following practical implications: *(a)* at pH 4.0 or below [such as in fruit concentrates or in some fitness drinks *(24)*] both sodium benzoate and ethyl-paraben are effective against osmotolerant yeasts in concentrations, which do not exceed levels required by most world-wide Food Administrations *(10)*; *(b)* in slightly acidic to neutral foods [such as marzipan, fondant, fudge, nut pastes a.s.on *(24)*] the chemical preservation with benzoic acid or Na-benzoate is not suitable, requiring concentrations exceeding the above mentioned levels; *(c)* no preservative should be used to cover up the use of spoiled or spoiling foods. Preservatives are not effective if the contamination levels exceed $10^3$-$10^4$ cells per ml or g. The initial infection can be minimized by Good Manufacturing Practice (GMP); *(d)* GMP also helps to exclude problems arising from the appearance

TABLE 5. *Sodium benzoate and ethyl-paraben preservation of food products with $a_w$ and pH values in the range of 0.980 to 0.795 and 4.8 to 3.0, respectively, against osmotolerant yeasts.*

| ppm Preservative necessary to obtain a fermentation-free shelf life of 30 days | | | |
|---|---|---|---|
| Temperature: 25°C<br>$a_w$-Range: 0.980-0.795<br>Contamination: $10^3$ cells/g | | | |
| Preservative | pH 4.8 | pH 4.0 | pH 3.0 |
| Na-benzoate | 4500 | 2500 | 1500 |
| ethyl-paraben | 900 | 400 | 400 |

of preservative-resistant species such as *Z. bailii*. Use of good quality, not-preserved raw materials is recommended; *(e)* the preservative concentrations should be calculated on the basis of the amount of water present in the substrate and not on the whole substrate volume. In this way the amount of preservative added can be minimized.

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