ORIGINAL PAPER

Use of a D-optimal design with constrains to quantify the effects of the mixture of sodium, potassium, calcium and magnesium chloride salts on the growth parameters of *Saccharomyces cerevisiae*

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Abstract The combined effect of NaCl, KCl, CaCl$_2$, and MgCl$_2$ on the water activity (a_w) and the growth parameters of *Saccharomyces cerevisiae* was studied by means of a D-optimal mixture design with constrains (total salt concentrations ≤ 9.0%, w/v). The a_w was linearly related to the concentrations of the diverse salts; its decrease, by similar concentrations of salts, followed the order NaCl > CaCl$_2$ > KCl > MgCl$_2$, regardless of the reference concentrations used (total absence of salts or 5% NaCl). The equations that expressed the maximum specific growth (μ_{max}), lag phase duration (λ), and maximum population reached (N_{max}) showed that the values of these parameters depended on linear effects and two-way interactions of the studied chloride salts. The μ_{max} decreased as NaCl and CaCl$_2$ increased (regardless of the presence or not of previous NaCl); however, in the presence of a 5% NaCl, a further addition of KCl and MgCl$_2$ markedly increased μ_{max}. The λ was mainly affected by MgCl$_2$ and the interactions NaCl × CaCl$_2$ and CaCl$_2$ × MgCl$_2$. The further addition of NaCl and CaCl$_2$ to a 5% NaCl medium increased the lag phase while KCl and MgCl$_2$ had negligible or slightly negative effect, respectively. N_{max} was mainly affected by MgCl$_2$ and its interactions with NaCl, KCl, and CaCl$_2$; MgCl$_2$ stimulated N_{max} in the presence of 5% NaCl while KCl, NaCl, and CaCl$_2$ had a progressive decreasing effect. These results can be of interest for the fermentation and preservation of vegetable products, and foods in general, in which this yeast could be present.

Keywords Sodium chloride · Magnesium chloride · Potassium chloride · Calcium chloride · Mixture design · Food fermentation · Food preservation · *Saccharomyces cerevisiae*

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Introduction

Salt is already mentioned in the Old Testament as a food additive with a ritual character. Its use as a food preservative was known not only in Ancient Egypt and the Middle East but also in Ancient Rome [14]. Many foods are prepared containing common salt as a main ingredient and employed to flavour and preserve foods of various kinds, especially meat, fish and vegetables. Common salt, principally constituted by sodium chloride, lowers the water activity (a_w) of a system [24] and thus renders conditions less favourable to microbial growth. Also, this additive can reduce the solubility of oxygen in water. Hence, the quantity of oxygen available to aerobic microorganisms in products containing high concentrations of sodium chloride is lower [14]. Many of the key systems used by the microorganisms to respond to either the loss or the gain of water due to changes in the osmolarity of the environment are now beginning to be understood, but the specific details of how each organism deals with these problems are different and are not completely known yet [21].

Increasing concern about hypertension in humans, which is correlated with cardiovascular diseases, has prompted food processors to develop low-sodium products. Sodium chloride is contraindicated for certain heart diseases, the

circulatory system and the kidneys. The negative effects related to high sodium chloride concentration can be reduced by replacing NaCl with an equivalent amount of other salts like potassium or calcium chloride. Tsapatsaris and Kotzekidou [25] studied the effects of the substitution of NaCl by 50% KCl on *Lactobacillus plantarum* and *Debaryomyces hansenii* growth. Naewbanij et al. [19] also showed the influence of various chloride salts on *L. plantarum* growth in cucumber extract. However, a detailed study of the effects of different chloride salts and their combinations on microbial growth is not available.

In this context, predictive microbiology could be an efficient tool. The effects of environmental factors on growth parameters (μ_{max}, λ, and N_{max}) can be described by predictive models [16]. The coefficients of response surface (RS) on the growth parameters, fitted to the environmental factors, can give estimations of the changes induced by each variable [18]. In the area of food research, RS methodology has been extensively applied to the treatment of data obtained by physicochemical and sensory analysis or nutritional parameters [6] and, of course, to study microbial growth [2, 25]. The D-optimal design process for mixture designs works exactly the same way as for RS. The design space is defined by the low- and high-level constrains on each factor and any multifactor constraints. The D-optimal selection chooses points from the candidate point set that are spread throughout the design region. The points selected are model dependent and are selected to minimize the variance associated with the coefficients. Therefore, the design should be augmented to provide for estimates of pure error by replication and for determining the lack of fit using excess design points. The primary difference between a standard RS experiment and a mixture experiment is that a special type of design must be used and the form of the mixture polynomial is slightly different from those used in RS. The coordinate system for mixture proportions is a simple coordinate system. Each of the vertices corresponds to a pure blend and each of the sides represents a mixture of two components and none of the others. Interior points in the triangle are mixtures in which all ingredients are present. The centroid of the triangle corresponds to mixtures with equal proportions of all ingredients [18].

Saccharomyces cerevisiae is a microorganism with a general application food manufacturing. It is very important in many food fermentations such as bread, wine, beer and table olives, but it also causes spoilage by refermentation after packaging regarding table olives, bottling of wines, beverages, and fruit concentrates and preserves [10, 23]. López-Malo et al. [15] and Arroyo López et al. [3] studied the influence of water activity and NaCl concentrations on the growth–no growth interface of *S. cerevisiae* in laboratory media. Almagro et al. [1] compared the effects of Na$^+$ and K$^+$ on growth and thermal death of *D.*

hansenii and *S. cerevisiae* under stress conditions, using individual concentrations of NaCl and KCl. However, there are no detailed studies on the influence of different chloride salts in the growth response of this microorganism.

The purpose of this work was to investigate the effects of different concentrations of sodium, potassium, calcium, and magnesium chloride salts on the growth parameters of *S. cerevisiae*, using a D-optimal mixture design with constrains, and quantifying such effects by polynomial equations. The information obtained by this means can be used to control vegetable fermentations and as hurdles for packed foods in which this yeast could be present.

Materials and methods

Origin and identification of the yeast specie

The yeast used in this study was *S. cerevisiae* (reference IGAL01), previously isolated from table olives fermentations. It was identified using API 20C AUX (bioMérieux, Marcy l'Etiole, France) together with the restriction pattern generated with endonucleases *Cfo*I, *Hae*III, *Hinf*I and *ScrF*I (Roche Molecular Biochemicals, Germany) from the polymerase chain reaction (PCR) to amplified 5.8.S rRNA gene and the two internal transcribed spacers (ITS$_1$ and ITS$_2$) [8].

Inoculum preparation and growth medium

One single colony from pure cultures of *S. cerevisiae* was inoculated into 5 ml of yeast–malt–peptone–glucose broth medium (YM, Difco^{TM}, Becton and Dickinson Company, Sparks, USA). After 48 h of incubation at 30 °C, the tubes were centrifuged, and the pellet was washed with sterile peptone water (0.1%, w/v), centrifuged and re-suspended again in a sterile peptone water to obtain a concentration of 7.40 ± 0.20 log$_{10}$ CFU/ml.

The basal growth medium selected for all experiments was YM broth, modified with different proportions of chloride salts (Table 1). The salts used were NaCl, KCl, CaCl$_2$ and MgCl$_2$ (assay of 99.9%) supplied by Panreac Química SA (Castellar del Vallés, Barcelona, Spain). After the addition of salts, 100-ml aliquots of medium were dispensed into 250-ml bottles and sterilized at 115 °C for 10 min. Values of pH after sterilization was ≈ 5.5. Subsequently, the bottles were inoculated with 10 μl of the suspension of *S. cerevisiae*, reaching an initial concentration of inoculum of 3.59 ± 0.03 log$_{10}$ CFU/ml. Water activity (a_w) was measured by triplicate in each medium after the sterilization with an Aqua Lab metre (Decagon Devices Inc., Pullmant, USA) at 25 °C. Average values resulting from the combination of salts in each run (±standard deviation) are shown in Table 1.

Table 1 Treatments included in the D-optimal experimental mixture design, using the diverse mixture of sodium, potassium, calcium and magnesium chloride salts with their respective water activities (a_w)

Run	NaCl (%)	KCl (%)	CaCl$_2$ (%)	MgCl$_2$ (%)	a_w
1	3.00	3.00	0.00	3.00	0.970 (0.004)
2	9.00	0.00	0.00	0.00	0.953 (0.003)
3	2.25	2.25	2.25	2.25	0.967 (0.000)
4[a]	4.50	0.00	0.00	0.00	0.975 (0.002)
5	3.00	0.00	3.00	3.00	0.967 (0.005)
6	0.00	0.00	4.50	4.50	0.977 (0.001)
7	0.00	9.00	0.00	0.00	0.969 (0.000)
8[a]	0.00	0.00	4.50	0.00	0.983 (0.002)
9	0.00	4.50	0.00	0.00	0.980 (0.001)
10	0.00	3.00	3.00	3.00	0.973 (0.006)
11	0.00	0.00	9.00	0.00	0.956 (0.005)
12	4.50	0.00	0.00	4.50	0.955 (0.001)
13[a]	0.00	0.00	4.50	0.00	0.980 (0.000)
14	0.00	0.00	0.00	9.00	0.978 (0.003)
15	4.50	4.50	0.00	0.00	0.960 (0.002)
16	0.00	4.50	4.50	0.00	0.962 (0.015)
17	4.50	0.00	4.50	0.00	0.957 (0.006)
18[a]	0.00	0.00	0.00	0.00	0.999 (0.000)
19[a]	0.00	0.00	0.00	0.00	0.997 (0.001)
20[a]	4.50	0.00	0.00	0.00	0.982 (0.003)
21	0.00	4.50	0.00	4.50	0.966 (0.001)
22	0.00	0.00	0.00	4.50	0.988 (0.002)
23	3.00	3.00	3.00	0.00	0.964 (0.004)
24[a]	0.00	0.00	0.00	0.00	0.992 (0.000)

Standard deviation from triplicate measurements for a_w in parentheses. % (equivalent to g per 100 ml)

[a] Duplicate treatments

Experimental design

The plan was generated by Design-Expert v.6 software (Stat-Easy Inc., Minneapolis, USA), according to a D-optimal experimental mixture design with constraint (NaCl + KCl + CaCl$_2$ + MgCl$_2$ ≤ 9.0%, w/v), which, in general, aims at the minimization of uncertainty in the global parameter estimation. A detailed discussion of the D-optimal designs can be found in [18] and application of its use in [2]. The design consisted of 20 selected level combinations of the quantitative variables plus four replicates, chosen also by the software to provide estimation of the pure error; then, the total number of treatments was 24 (Table 1). The units habitually used at industrial scale (%, equivalent to g per 100 ml) have been intentionally chosen to facilitate the possible application of results directly to production. The experiments were carried out at 30 °C.

Samples were taken from the bottles of the experiments at predetermined time intervals, established according to the yeast growth conditions, and plated onto YM agar plates. The total number of samples ranged from 8 to 15, distributed throughout the different phases of yeast growth (lag, exponential and stationary) and the experiments were monitorized at least for 10 days. Cells were diluted in sterile peptone water and surface spread using a spiral plate maker model dwScientific (Don Whitley Scientific Limited, England). All the plates were incubated aerobically at 30 °C for 48 h and counted using a CounterMat v.3.10 (IUL, Barcelona, Spain) imagine analysis system.

Effects on the growth parameters

The growth data from plate counts were enumerated as $\log_{10}$ values. The model of [4] was fitted to the growth curves by using the MICROFIT 1.0 software (Institute of Food Research, Norwich, UK) and the growth parameters final population cell density (N_{max}), maximum specific growth rate (μ_{max}), and lag time (λ) estimated (primary model).

To describe the combined effect of environmental factors (chloride salts) on growth parameters, a multiple quadratic regression models was applied to the design and responses (secondary model). It is represented in the following equation, expressed in the canonical (Sheffé) form:

$$P = \sum_1^4 \beta_i x_i + \sum \sum_{i<j}^4 \beta_{ij} x_i x_j + \sum_1^4 \beta_{ii} x_{ii}^2 \tag{1}$$

where x_1, x_2, x_3, and x_4 stand for NaCl, KCl, CaCl$_2$, and MgCl$_2$, respectively, P is the growth parameter (μ_{max}, λ, and N_{max}) or its transformation, and the βs are the coefficients to be estimated. An analysis of variance was produced for each of the response variables, testing the value of the applied model and determining if a more complex model could have a better fit. If the F test for the model was significant ($P < 0.05$) there was evidence that the model could explain the variations in the responses. The backward option (alpha to exit = 0.05) was used to retain the significant coefficients. In the final model, coefficients required to support hierarchy were also maintained. This was necessary because only hierarchical models are scale-independent and can be translated into actual units. Statistical analysis was carried out using the value of each replicate, in order to test the lack of fit test. Fit adequacy was checked by different diagnostic tests, which included the presence of outliers, Cook's distance, leverage, graphs of residual versus predicted values, and the graphic representation of predicted versus experimental values.

The terms in the canonical mixture polynomial have simple interpretations. Geometrically, the parameter β_i represents the expected response to the pure mixture $x_i = 1$, $x_j = 0$, and is the height of the mixture surface at the vertex $x_i = 1$. Usually, the portion of each polynomial

given by $\sum_{1}^{q} \beta_i x_i (q = $ number of variables) is called the linear blending portion. The two factor interaction $\left(\sum \sum_{i<j}^{4} \beta_{ij} x_i x_j\right)$ represents the excess response over the linear one and is often called the synergism (or antagonism, depending on the sign) due to non-linear blending. Quadratic terms were not additionally significant while cubic terms were always aliased. The equations in terms of actual values of the variables included also the intercept.

The effect of each factor was further assessed by the use of the trace (perturbation) plot to show how the response changes as each factor moves from chosen reference points, with all other factors held constant at reference values [22]. The response model was defined as:

$$\hat{Y} = f(x_1, x_2, x_3, x_4) \qquad (2)$$

with x_1^*, x_2^*, x_3^*, and x_4^* at chosen specific values of the factors (0, 0, 0, 0) and (5, 0, 0, 0).

The perturbation effect of x_1 was defined as follows:

$$\hat{Y}(x_1) = f(x_1, x_2^*, x_3^*, x_4^*) \qquad (3)$$

The perturbation effects of x_2, x_3 and x_4 were defined similarly.

Trace curves were produced with the vertical axis representing $\hat{Y}(x_i)$, in actual values, and the horizontal axis representing x_i (in coded values). By overlaying all the response trace plots, the joined trace plot was obtained [13].

Model validation

For the model validation, a new series of independent experiments were carried out. Levels were chosen at random within the range of the values used in the design. Validation treatments were: (1) 4% NaCl, 2% KCl, 2% CaCl$_2$, 1% MgCl$_2$; (2) 1% NaCl, 4% CaCl$_2$; (3) 1% KCl, 1% MgCl$_2$; (4) 5% NaCl, 1% KCl, 2% CaCl$_2$; and (5) 1% NaCl, 1% CaCl$_2$, 1% MgCl$_2$. Salts not mentioned in the respective treatments were absent from them. Growth parameters of *S. cerevisiae* were directly estimated from these experiments, and compared to those predicted by the model using the accuracy (A) and bias (B) factors as described by Baranyi and Pin [5].

Results

Contribution of each salt and their mixture to the water activity of the media

The experimental design and responses, expressed as a_w, μ_{max}, λ, and N_{max}, are shown in Tables 1 and 2. The highest

a_w was observed in the culture media (average 0.996) and the lowest (0.953) at 9.0% NaCl concentration (Table 1). The a_w for 9.0% KCl, CaCl$_2$, and MgCl$_2$, were 0.969, 0.956, and 0.978, respectively. Then, the addition of CaCl$_2$ showed an a_w decrease close to that of NaCl, followed by KCl and MgCl$_2$. The rest of the mixtures had a_w that ranged between these extreme values.

The contribution of each salt to the total a_w was estimated by means of the polynomial equation adjusted to a_w as a function of the NaCl, KCl, CaCl$_2$ and MgCl$_2$, concentrations. Only linear terms were significant. The model F value ($42.88_{4,4}$) gave a $P < 0.0001$, which implied that the model was significant. The lack of fit was insignificant ($F_{15,4} = 1.64$, $P = 0.3376$).

The equation which related a_w with the content of the diverse salts, in terms of the actual units (% each salt), was the following:

Table 2 Growth parameters for *S. cerevisiae* obtained by applying the primary model of [4] to the growth curves in laboratory media containing the diverse mixture of sodium, potassium, calcium and magnesium chloride salts

Run	μ_{max} (h^{-1})	λ (h)	N_{max} (log$_{10}$ CFU/ml)
1	0.48 (0.02)	8.31 (0.88)	7.54 (0.04)
2	0.08 (0.01)	34.59 (8.49)	6.60 (0.10)
3	0.51 (0.02)	8.86 (0.57)	7.50 (0.02)
4	0.29 (0.04)	11.11 (2.76)	7.33 (0.08)
5	0.55 (0.06)	7.51 (1.48)	7.47 (0.08)
6	0.56 (0.03)	6.64 (0.61)	7.55 (0.03)
7	0.53 (0.05)	8.52 (1.28)	7.18 (0.07)
8	0.34 (0.04)	13.36 (1.94)	7.15 (0.06)
9	0.63 (0.03)	6.52 (0.45)	7.51 (0.03)
10	0.59 (0.05)	8.77 (0.40)	7.44 (0.08)
11	0.23 (0.09)	149.78 (7.90)	6.46 (0.12)
12	0.51 (0.03)	8.95 (0.65)	7.33 (0.04)
13	0.48 (0.03)	16.43 (0.78)	7.06 (0.04)
14	0.47 (0.01)	5.90 (0.46)	7.52 (0.03)
15	0.55 (0.07)	10.56 (1.49)	7.17 (0.08)
16	0.23 (0.02)	73.63 (1.31)	6.94 (0.06)
17	0.19 (0.02)	17.62 (2.50)	6.66 (0.08)
18	0.56 (0.02)	4.14 (0.48)	7.61 (0.02)
19	0.59 (0.03)	4.92 (0.73)	7.59 (0.02)
20	0.24 (0.02)	9.24 (1.20)	7.26 (0.09)
21	0.59 (0.04)	6.31 (0.77)	7.64 (0.04)
22	0.65 (0.07)	6.48 (0.85)	7.60 (0.05)
23	0.32 (0.03)	19.94 (1.08)	6.72 (0.04)
24	0.53 (0.02)	5.78 (0.58)	7.57 (0.04)

Standard deviation from non-linear estimation in parentheses. μ_{max} (maximum specific growth rate), λ (lag phase duration), N_{max} (maximum population reached)

$$a_w = 0.9973 - 0.00485NaCl - 0.00335KCl$$
$$- 0.00385CaCl_2 - 0.00221MgCl_2 \qquad (4)$$

The negative sign of NaCl, CaCl$_2$, KCl, and MgCl$_2$ in the equation represents that all of them contribute to decrease a_w with respect to that obtained in the absence of salts. This equation permits the calculation of a_w in a laboratory medium containing any combination of the studied salts within the experimental region (restricted to NaCl + KCl + CaCl$_2$ + MgCl$_2 \leq 9.0\%$). The equation may also be of guidance for estimating a_w in brines containing mixtures of these salts.

As the equation only contains linear terms, the contribution of each salt to the a_w is proportional to the corresponding coefficients in coded values. The highest contribution to an a_w decrease was due to NaCl (A) (coefficient -0.022 for x_1 in coded values), followed by CaCl$_2$ (C) (-0.017, x_3), KCl (B) (-0.015, x_2), and MgCl$_2$ (D) (-0.0099, x_4), at the same concentration (%) of each salt.

When the effect was measured as the a_w deviation from the reference point containing 5% NaCl (5% NaCl, 0% KCl, 0% CaCl$_2$, and 0% MgCl$_2$), there was always a further decrease of a_w as the NaCl, CaCl$_2$, KCl or MgCl$_2$ were progressively added (Fig. 1, upper left). Then, any initial brine composed of NaCl may be complemented with any other salt with an additional a_w decrease that will depend on the type of salt added, following the effect in the order: NaCl > CaCl$_2$ > KCl > MgCl$_2$.

Effect of each salt and their mixture on the growth parameters of S. cerevisiae

Effect on maximum specific growth rate (μ_{max})

The model suggested by the sequential sums of square analysis included linear and two-way interactions (Table 3). Applying the backward option to it, the chosen model had a non-significant lack of fit ($P > 0.3351$) and explained a high proportion of the total variance ($R^2 = 0.8812$) while retaining five coefficients plus those required to maintain hierarchy (essential to estimate the equation in actual values). The information related to the statistical analysis of the model and their selected terms, in coded terms, are shown in Table 4.

The effect of each salt individually on μ_{max} can be deduced from the slopes of the variables in the coded equation when all others were absent. When a moderate proportion of NaCl (A) was present (reference point 5%, 0, 0, 0), the addition of a further amount of NaCl (A) and CaCl$_2$ (C) had a decreasing effect on μ_{max} but increasing proportions of MgCl$_2$ (D) and KCl (B) had a stimulating effect on μ_{max} (Fig. 1, upper right).

The equation of μ_{max} as a function of the actual values of the variables is convenient because it leads to a more intuitive interpretation of their terms. It was:

$$\mu_{max} = 0.5696 - 0.0563NaCl + 0.00079KCl$$
$$- 0.03588CaCl_2 - 0.00433MgCl_2 + 0.000966NaCl$$
$$\times KCl + 0.01034NaCl \times MgCl_2 - 0.00673KCl$$
$$\times CaCl_2 + 0.01222CaCl_2 \times MgCl_2 \qquad (5)$$

The plots of the significant interactions in the final model (Fig. 2) can help for its interpretation. The presence of increasing amounts of NaCl or CaCl$_2$ decreased μ_{max} but the addition of either KCl (Fig. 2, left) or MgCl$_2$ (Fig. 2, centre and right) always increased μ_{max} particularly at low levels of NaCl and CaCl$_2$. In both cases, μ_{max} reached a plateau around 0.54–0.57 h^{-1}. The addition of KCl to the solution already containing CaCl$_2$ had only slight increasing effect on μ_{max} (data not shown).

Effect on lag phase duration (λ)

In the analysis of this response, the Box–Cox plot for power transformation yielded a lambda $= -0.5$, which suggested an inverse square root transformation for variance stabilization. The model suggested by the sequential sums of square analysis of the transformed data included linear and two-way interactions (Table 3). The ANOVA corresponding to the reduced model obtained after application of the backward option, details of the fit, the coefficients retained, their standard errors and probabilities as well as the complete equation in coded terms (at the bottom), are shown in Table 5.

The lag phase was mainly influenced by MgCl$_2$ and NaCl and the interactions NaCl $\times$ CaCl$_2$, CaCl$_2$ $\times$ MgCl$_2$, and KCl $\times$ CaCl$_2$ but the interactions NaCl $\times$ KCl, Na $\times$ MgCl$_2$, and KCl $\times$ MgCl$_2$ were also significant. The linear effects of KCl and CaCl$_2$ were removed by the reduction procedure but were introduced again in application of the hierarchical principle. The individual effects of other salts in presence of an initial 5% NaCl (reference point 5%, 0, 0, 0), obtained as already described for μ_{max}, is shown in Fig. 1 (lower, left). The further addition of NaCl (A) and CaCl$_2$ (C) increased λ, with a more pronounced slope for NaCl. The lower effect of CaCl$_2$ than that due to NaCl when there is already a 5% NaCl can be due to the previous content of NaCl and the global constrain (NaCl + CaCl$_2 \leq 9.0\%$), which limits the additional CaCl$_2$ concentration that can be added and therefore its influence. KCl had a negligible effect and MgCl$_2$ decrease λ.

The equation of the lag phase as a function of the diverse salt contents, in terms of the actual values, was the following:

Fig. 1 Trace plot of a_w, μ_{max} (h^{-1}), $1/\sqrt{\lambda}$, and N_{max} ($\log_{10}$ CFU/ml), in actual values, as a function of the NaCl (A), KCl (B), CaCl$_2$ (C), and MgCl$_2$ (D) concentrations (in coded values) for the (5, 0, 0, 0), reference points. The effects are expressed as deviations from the reference point
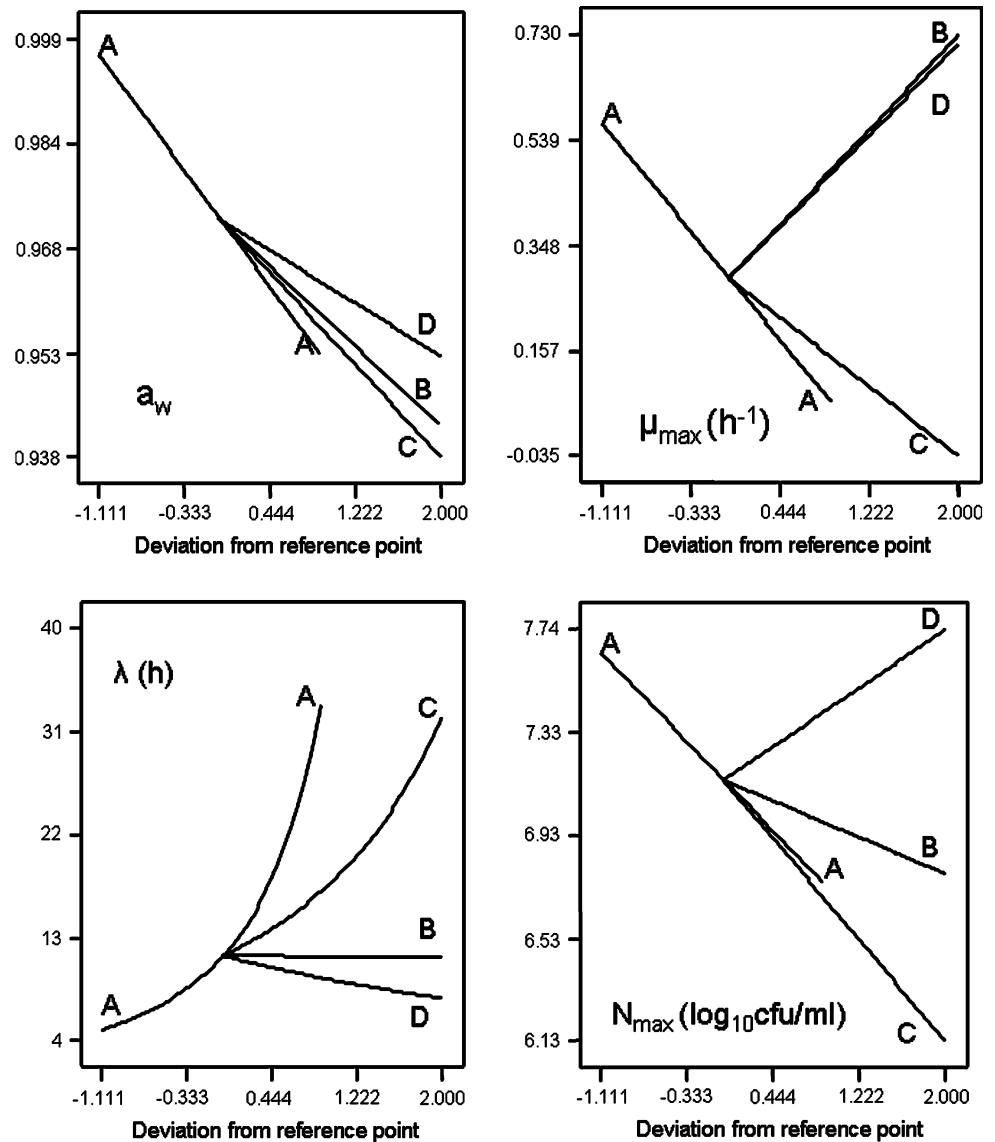
$$\frac{1}{\sqrt{\lambda}} = 0.449 - 0.031\text{NaCl} - 0.012\text{KCl} - 0.041\text{CaCl}_2$$
$$- 0.006\text{MgCl}_2 + 0.002\text{NaCl} \times \text{KCl} + 0.006\text{NaCl}$$
$$\times \text{CaCl}_2 + 0.003\text{NaCl} \times \text{MgCl}_2 - 0.004\text{KCl} \times \text{CaCl}_2$$
$$+ 0.002\text{KCl} \times \text{MgCl}_2 + 0.008\text{CaCl}_2 \times \text{MgCl}_2 \quad (6)$$

Interpretation of the equation, expressed in transformed response is difficult. Furthermore, the equation of $1/\sqrt{\lambda}$ cannot be visualized as a function of all the design's variables. The effect of the most significant ($P < 0.05$) interaction can visualize the outstanding features (Fig. 3). The interaction NaCl × CaCl$_2$ was marked and, for the same NaCl, there was always an increase in λ period as the CaCl$_2$ increased. Addition of CaCl$_2$ to one solution already containing KCl led always to an increase in λ but the incorporation of MgCl$_2$ to solutions of CaCl$_2$ decrease λ.

Effect on maximum population reached (N_{max})

N_{max} in the diverse runs ranged from 6.46 to 7.64 $\log_{10}$ CFU/ml. Thus, the final populations were less influenced by the presence of the diverse salts than μ_{max} and λ. The model suggested by the sequential sum of square analysis included linear and two-way interactions (Table 3). By applying the backward option to the full equation, it was possible to obtain a reduced significant model (Table 6), although with a non-significant lack of fit. A non-significant lack of fit would have been preferred, but the model chosen may be considered as a reasonable option in this case because the alternative model, special cubic, which showed a non-significant lack of fit included aliased variables and had negative predicted R^2. The coefficient retained (NaCl, KCl, and CaCl$_2$ linear effects were included in application of the hierarchy principle), their standard

Table 3 Sequential model sums of squares for μ_{max}, λ, N_{max}

Source	Sum of squares	Degree of freedom	Mean square	F value	P value prob > F	Decision
μ_{max}						
Mean versus total	4.77	1	4.77			
Linear versus mean	0.3740	4	0.0934	7.94	0.0006	
2FI versus Linear	0.1600	6	0.0266	5.43	0.0052	Suggested
Quadratic versus 2FI	0.0248	4	0.00620	1.43	0.300	
λ						
Mean versus total	2.48	1	2.48			
Linear versus mean	0.1810	4	0.04580	15.7	<0.0001	
2FI versus linear	0.0496	6	0.00827	20.9	<0.0001	Suggested
Quadratic versus 2FI	0.000817	4	0.00204	0.424	0.788	
N_{max}						
Mean versus total	0.0013	1	0.0013			
Linear versus mean	2.200	4	0.5500	14.5	<0.0001	
2FI versus linear	0.552	6	0.0921	7.13	0.00157	Suggested
Quadratic versus 2FI	0.0628	4	0.0157	1.35	0.326	

All cubic versus quadratic were aliased and not considered in the model

Table 4 ANOVA of the model and coefficient estimates, using backward elimination, for the response factor Y^a (μ_{max}, h^{-1}), using the diverse mixture of sodium, potassium, calcium and magnesium chloride salts

Source of variation or factor	Sum of squares or coefficient estimate	df	Mean square or standard error	F or t values	P value
Source of variation					
Model[b]	0.530	8	0.066000	13.91	<0.0001[d]
Residual	0.071	15	0.004728		
Lack of fit	0.058	11	0.005297	1.64	0.3351
Pure error	0.013	4	0.003212		
Total	0.600	23			
Factor[c]					
Intercept	$a = 0.660$	1	0.110		
x_1	$b_1 = 0.150^e$	1	0.089	2.93	0.1075
x_2	$b_2 = 0.063^e$	1	0.088	0.51	0.4859
x_3	$b_3 = -0.050^e$	1	0.089	0.32	0.5790
x_4	$b_4 = 0.440$	1	0.088	24.58	0.0002[d]
$x_1 \times x_2$	$b_{12} = 0.200$	1	0.070	7.75	0.0139[d]
$x_1 \times x_4$	$b_{14} = 0.210$	1	0.070	8.87	0.0094[d]
$x_2 \times x_3$	$b_{23} = -0.140^e$	1	0.070	3.76	0.0716
$x_3 \times x_4$	$b_{34} = 0.250$	1	0.070	12.39	0.0031[d]

[a] $Y = 0.66 + 0.150x_1 + 0.063x_2 - 0.050x_3 + 0.440x_4 + 0.200x_1 \times x_2 + 0.210x_1 \times x_4 - 0.140x_2 \times x_3 + 0.250x_3 \times x_4$ (in coded variables)

[b] $R^2 = 0.8812$

[c] Only coefficients retained. x_1, NaCl; x_2, KCl; x_3, CaCl$_2$; x_4, MgCl$_2$

[d] Significant at an alpha value of 0.05

[e] Introduced in application of the principle of hierarchy

errors, F values, probability and the equation in coded values are shown in Table 6.

The effect of each other salt in presence of an initial 5% NaCl (reference point: 5% NaCl, 0, 0, 0) is shown also in Fig. 1 (lower right). The population size of *S. cerevisiae* could only be increased by the addition of MgCl$_2$ while the incorporation of any of the other salts always decreased the population size; the effect was higher (lower N_{max}) for

Fig. 2 Surface and contour plots of μ_{max} (h^{-1}) as a function of the binary mixture of the significant effects of the equation showed in Table 4, expressed in terms of actual value concentrations. Concentrations of the salts not in each of the graphs were set a 0%
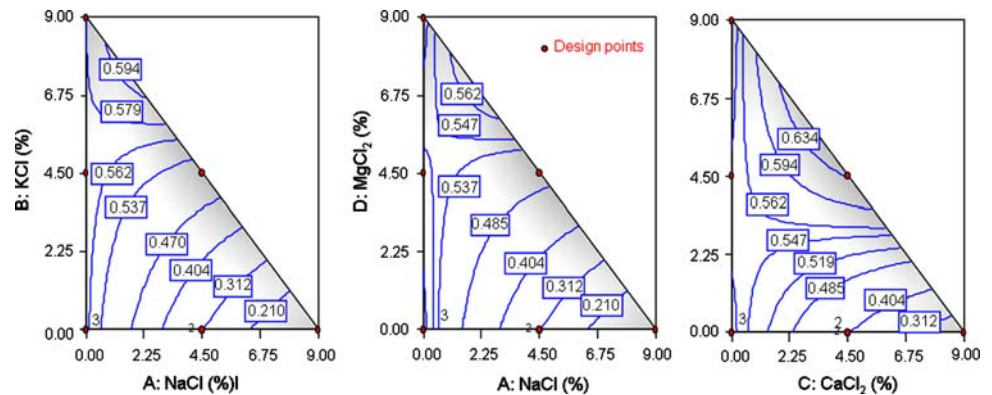

Table 5 ANOVA of the model and coefficient estimates, using backward elimination, for the response factor Y^a ($1/\sqrt{\lambda}$), using the diverse mixture of sodium, potassium, calcium and magnesium chloride salts

Source of variation or factor	Sum of squares or coefficient estimate	df	Mean square or standard error	F or t values	P value
Source of variation					
Model[b]	0.23	10	0.023	58.33	<0.0001[d]
Residual	0.0051	13	0.0004		
Lack of fit	0.0002	9	0.0002	0.18	0.9832
Pure error	0.0036	4	0.00091		
Total	0.2400	23			
Factor[c]					
Intercept	$a = 0.370$	1	0.040		
x_1	$b_1 = 0.077^e$	1	0.031	6.23	0.0268
x_2	$b_2 = -0.053^e$	1	0.031	2.82	0.1172
x_3	$b_3 = 0.004^e$	1	0.031	0.013	0.9124
x_4	$b_4 = 0.230$	1	0.031	51.69	<0.0001
$x_1 \times x_2$	$b_{12} = 0.050$	1	0.020	5.91	0.0303[d]
$x_1 \times x_3$	$b_{13} = 0.110$	1	0.020	31.63	<0.0001[d]
$x_1 \times x_4$	$b_{13} = 0.052$	1	0.020	6.55	0.0237[d]
$x_2 \times x_3$	$b_{23} = -0.086$	1	0.020	17.79	0.0010[d]
$x_2 \times x_4$	$b_{24} = 0.038$	1	0.020	3.45	0.0860[f]
$x_3 \times x_4$	$b_{34} = 0.160$	1	0.020	63.08	<0.0001[d]

[a] $Y = 0.370 + 0.077x_1 - 0.053x_2 + 0.004x_3 + 0.230x_4 + 0.0500x_1 \times x_2 + 0.110x_1 \times x_3 + 0.052x_1 \times x_4 - 0.086x_2 \times x_3 + 0.038x_2 \times x_4 + 0.160x_3x_4$ (in coded variables)

[b] $R^2 = 0.8095$

[c] Only coefficients retained. x_1, NaCl; x_2, KCl; x_3, CaCl$_2$; x_4, MgCl$_2$

[d] Significant at an alpha value of 0.05

[e] Introduced in application of the principle of hierarchy

[f] Significant before introducing the hierarchical coefficients

CaCl$_2$, followed by NaCl, and KCl, which induced a less marked N_{max} decrease. The order of effects was not the same as when each salt was studied individually due, possibly, to the disturbance created by the NaCl content already in the solution.

The equation of the polynomial for N_{max} in actual values was:

$$N_{max} = 7.64 - 0.097\text{NaCl} - 0.040\text{KCl} - 0.126\text{CaCl}_2 - 0.016\text{MgCl}_2 + 0.0162\text{NaCl} \times \text{MgCl}_2 + 0.015\text{KCl} \times \text{MgCl}_2 + 0.031\text{CaCl}_2 \times \text{MgCl}_2 \tag{7}$$

The graphical study of the three significant interactions is shown in Fig. 4. It was always observed that the addition of MgCl$_2$ salt to a solution already containing any other salt

Fig. 3 Contour plots of λ (h) as a function of the NaCl, KCl, and MgCl$_2$ interactions with CaCl$_2$ from the equation showed in Table 5. Concentrations and original response are expressed in terms of actual values. Concentrations of the salts not in the graph were set a 0%
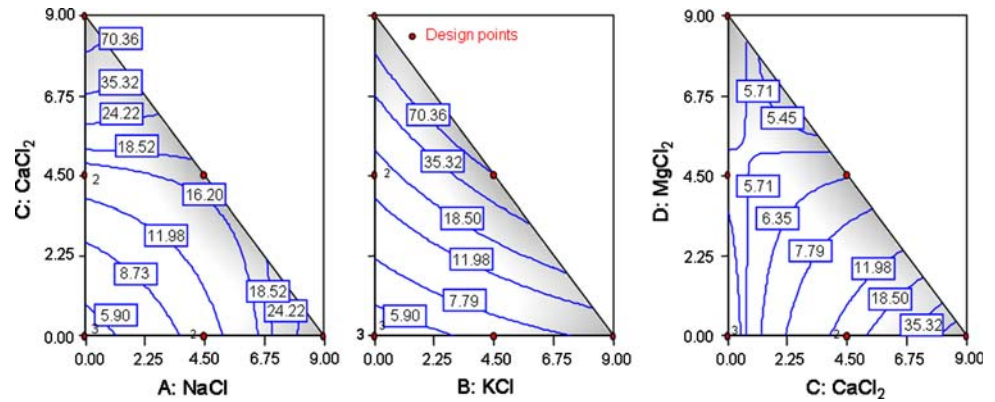
Table 6 ANOVA of the model and coefficient estimates, using backward elimination, for the response factor Y^a (N_{max}, log$_{10}$ CFU/ml), using the diverse mixture of sodium, potassium, calcium and magnesium chloride salts

Source of variation or factor	Sum of squares or coefficient estimate	df	Mean square or standard error	F or t values	P value
Source of variation					
Model[b]	2.720	7	0.3900	31.06	<0.0001[d]
Residual	0.200	16	0.0130		
Lack of fit	0.1900	12	0.0170	8.80	0.0247
Pure error	0.007	4	0.0016		
Total	2.920	23			
Factor[c]					
Intercept	$a = 7.630$	1	0.17		
x_1	$b_1 = -0.110^e$	1	0.11	0.14	0.7134
x_2	$b_2 = -0.120^e$	1	0.11	1.19	0.2929
x_3	$b_3 = 0.050^e$	1	0.15	0.49	0.4950
x_4	$b_4 = 1.180$	1	0.18	42.47	<0.0001[d]
$x_1 \times x_4$	$b_{34} = 0.330$	1	0.11	7.93	0.0130[d]
$x_2 \times x_4$	$b_{24} = 0.300$	1	0.12	6.56	0.0217[d]
$x_3 \times x_4$	$b_{34} = 0.620$	1	0.11	27.97	<0.0001[d]

[a] $Y = 7.630 - 0.110x_1 - 0.120x_2 - 0.055x_3 + 1.800x_4 + 0.330x_1 \times x_4 + 0.300x_2 \times x_4 + 0.62x_3 \times x_4$ (in coded variables)

[b] $R^2 = 0.9327$

[c] Only coefficients retained: x_1, NaCl; x_2, KCl; x_3, CaCl$_2$; x_4, MgCl$_2$

[d] Significant at an alpha value of 0.05

[e] Introduced in application of the principle of hierarchy

increased N_{max}. High N_{max} depended on the respective salt concentrations, but always required relatively low concentrations of NaCl ($\approx 2.0\%$), KCl (≈ 2.25–4.60%), and CaCl$_2$ ($\approx 2.25\%$) and relatively high concentrations of MgCl$_2$.

Validation results

The accuracy (A) and bias (B) factors, obtained for μ_{max}, λ, and N_{max} from independent experiments are shown in Table 7. The best results were obtained for N_{max} (A and B ≈ 1), while the worst predictions were for the bias parameter of μ_m (1.154) but, in general, values can be

considered acceptable. The models obtained could then be used to navigate throughout the experimental region and make predictions with respect to the probable values for the growth parameters of *S. cerevisiae* in the presence of NaCl, KCl, CaCl$_2$, and MgCl$_2$ salt or their mixtures.

Discussion

The impact on growth of some spoilage bacteria and pathogenic organisms has been investigated in model systems of either broth or meat emulsions where Na$^+$ from sodium chloride was substituted with other cations, i.e. K$^+$,

Fig. 4 Contour plots of N_{max} ($\log_{10}$ CFU/ml) as a function of the binary mixtures of the significant effects of the equation showed in Table 6, expressed in terms of actual values. Concentrations of the salts not in each of the graphs were set a 0%
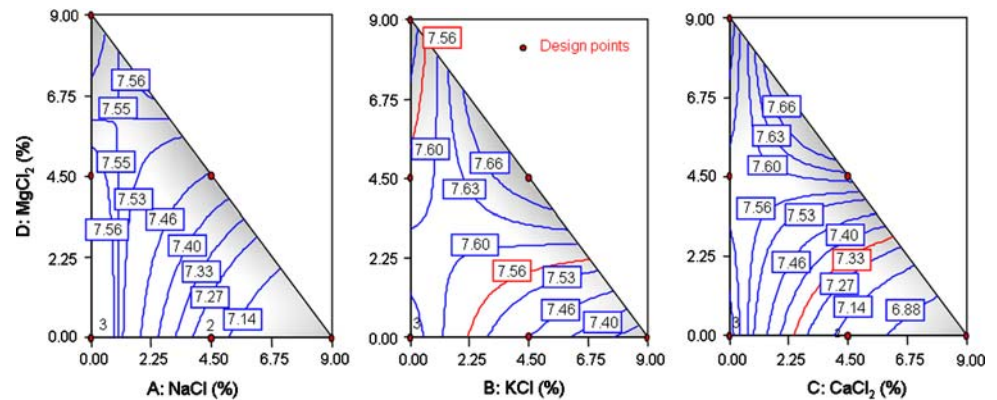

Table 7 Accuracy (A) and bias (B) factors [5] obtained for μ_{max}, λ, and N_{max} from independent experiments within experimental region (NaCl + KCl + CaCl$_2$ + MgCl$_2$ ≤ 9.0%)

Index	μ_m (h^{-1})	λ (h)	N_{max} ($\log_{10}$ CFU/ml)
A	1.030	1.054	1.00
B	1.154	0.934	0.900

Mg^{++} and Ca^{++}, but maintaining similar a_w and pH. The strongest inhibition was observed with divalent cations [20] but the effect of replacing half or all of the Na$^+$ content with K$^+$ in meat slurries was either rather small or negligible [20]. The growth of *Listeria innocua* in broth and the surface of gelatine were controlled by a_w in the range 0.91–0.99 and 0.92–0.99, respectively, by the addition of solutes such as NaCl, KCl, glucose, sucrose, and glycerol. The shortest generation time was observed in broth with NaCl followed by gelatine with NaCl, but removal of water was more stressful than the addition of NaCl [12].

NaCl in vegetable fermentations could also be substituted with other chloride salts, especially those with favourable effects on nutritional value of the final products. The most studied compounds have been KCl and CaCl$_2$, but MgCl$_2$ was also included in this research. Viander et al. [26] studied the impact of low NaCl, in combination with different proportions of KCl, on the spontaneous large-scale fermentation process of white cabbage into sauerkraut juice. The changes did not influence the pH decrease and acetic acid production but the lactic acid yield in the presence of KCl was clearly lower. The presence of calcium affected the microbial quality and consistency of chile pepper (*Capsicum annum*) mash during fermentation. The addition of a concentration at 8 or 15 g/100 g released more soluble sugars, increased alcohol production and reduced pH faster than the control or any other treatment. The CaCl$_2$ was also effective in controlling the microbial growth [9]. The natural content of calcium in olives is fairly high [10] but its effect on a_w of fermentation brines had not been studied yet.

The estimation of a_w can be achieved by considering the solutes in solution (1975), or these and the non-solutes [7]. In this work, it was preferred to directly measure a_w in the diverse treatments and then find the function which linked it with the salt concentrations. The relationship can be used as an approach to explore their effects on other fermented foods. The presence of the habitual NaCl concentration in the brines of fermented or stored olives (8–9%) produces only a limited decrease in the a_w and a complete stabilization would require higher concentrations incompatible with the organoleptic and nutritional characteristics [10]. The antimicrobial properties of NaCl alone, used for the preservation of natural casings were sufficient to reduce the bacterial contamination below acceptable levels (except for *Clostridium* spores) at a_w below 0.850 [27]. Other chloride salts could have similar trend. The effect of supplementing a moderate level of NaCl (5%) with CaCl$_2$ caused a further decrease of μ_{max} but the incorporation of KCl or MgCl$_2$ stimulated growth. Furthermore, MgCl$_2$ stimulated μ_{max} in the presence of either NaCl, or CaCl$_2$. As a result, the use of magnesium chloride, in addition to NaCl, could favour the growth of *S. cerevisiae* and, in consequence, the fermentation or spoilage rate, depending on the processing step. However, this yeast usually coexists with lactic acid bacteria and other micro-organisms and the behaviour in real fermentations cannot be extrapolated straightforward. Yeasts are a part of the natural micro flora of olive fermentation but the effect of a higher population of *S. cerevisiae* on physicochemical and organoleptic characteristics is not yet known [10]. As the presence of KCl and CaCl$_2$ in olive fermentations is fairly high, some of the empirical practices to elaborate this product at industrial scale may be based on the above-mentioned relationships [10].

According to the results found in this work, the addition of NaCl and CaCl$_2$ to a brine already containing NaCl, increased the λ period. Therefore, the addition of both salts could eventually be used alone or in combination to prevent a rapid development of *S. cerevisiae* not only in

laboratory media but also in foods in general. This practice could improve stabilization of some foods like olives. The presence of $CaCl_2$ may not only lead to a longer lag phase but may also be used to control *S. cerevisiae* final population due to its effect decreasing the N_{max}. On the contrary, addition of $MgCl_2$ can stimulate the growth and N_{max} in brines already containing NaCl or other salts.

Mulé et al. [17], used different types of brines when processing green table olives (lavorazione "al naturale") to obtain a product with lower sodium content, suitable for those who suffer from hypertension, by partially substituting Na^+ with Ca^{++} and K^+ in the fermentation brines.

Regarding pickles on the effects of NaCl, KCl, $CaCl_2$, and $MaCl_2$ and their combinations could be useful. Calcium chloride and potassium sorbate (KS) reduced the NaCl used during natural cucumber fermentation and storage but the presence of $CaCl_2$ helped to maintain cucumber firmness. A synergistic action between NaCl, $CaCl_2$, and KS allowed good quality pickles using moderate amounts (5% NaCl, 0.2% $CaCl_2$, and 0.2% KS) of the three components [11]. The effect of including KCl, $CaCl_2$, and $MgCl_2$ as partial substitutes for NaCl in the fermentation in cucumber extracts showed that the salts that gave the highest percentage of total lactic population, provided the most selective environment, were NaCl and KCl in the filtered, raw cucumber extract and KCl in the heated and filtered extract, while $CaCl_2$ was more selective than $MgCl_2$ in either medium [19]. Results from this work open the possibility to use diverse salt combinations.

Conclusions

Results from this work showed that the effects of similar proportions of diverse salts in laboratory media on a_w and on the growth parameters of *S. cerevisiae* were diverse and could be quantified by polynomial equations as a function of their concentrations. The order of contribution to a_w decrease was NaCl > $CaCl_2$ > KCl > $MgCl_2$. When the salts were added to a solution of NaCl (5%), further additions of any other salt followed a similar trend. The contribution of each salt to the μ_{max} decrease followed the similar trend for NaCl and $CaCl_2$; but, in the presence of a 5% NaCl solution, a further addition of KCl and $MgCl_2$ markedly increased μ_{max}. $MgCl_2$ had a stimulating effect on μ_{max} when used in combination with NaCl or $CaCl_2$, while KCl followed the same trend in the presence of NaCl. The lag phase of *S. cerevisiae* was mainly affected by $MgCl_2$ and the interactions NaCl × $CaCl_2$ and $CaCl_2$ × $MgCl_2$. The further adding of NaCl and $CaCl_2$ to a solution already containing 5% NaCl increased the length of the lag phase but KCl had negligible effect and $MgCl_2$ slightly decreased it. The most outstanding effects on N_{max}

were due to $MgCl_2$ and the interactions of $MgCl_2$ with NaCl, KCl and $CaCl_2$. When the salts were added to an initial solution already containing 5% NaCl, $CaCl_2$ (strongly), NaCl and KCl decreased the N_{max} but $MgCl_2$ increased it; the incorporation of $MgCl_2$ to NaCl, $CaCl_2$ or KCl produced a stimulating effect on N_{max}. These results can be applied to fermentation, storage and preservation of pickles or to any other fermented food in which *S. cerevisiae* is present.

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