Survival of lactic acid bacteria in sea water. A factorial study

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Abstract

A feasibility study of lactic bacteria as potential probiotics in larval cultures of marine fish was performed by investigating the survival of five strains of lactic bacteria in sea water by readily standardised procedures at different temperatures and salinities. These conditions were chosen in such a way that their combinations define a complete first order factorial design. Depending on the strain and the ambient conditions, the survival adhered to first order kinetics in some cases and to the Gompertz equation in others. The half lives ($t_{0.5}$) calculated from these models were subsequently introduced as responses to the factorial designs, estimating the coefficients of empirical equations which describe the group effect of temperature and salinity on $t_{0.5}$. Simply additive effects were found in two cases, a negative first order interaction in another case, whilst another two required second order models.

Introduction

One of the most serious problems regarding the culture of marine fish, in particular turbot (*Scophthalmus maximus*), relates to the high mortality in the critical phases of larval development [1]. This mortality is associated with opportunistic bacteria [2, 3] which readily

develop when live food and microalgae are used [4, 5]. Moreover, in the 3-5 days following exogenous larval feeding, strong decreases in ingestion due to bacterial surplus are frequent, leading to a total rejection of food and massive mortalities. Application of antibiotics significantly improves survival [6], but weakens the intestinal flora and induces the selection of resistant micro-organisms with unpredictable long-term effects to the environment and human health.

Probiotics are a promising alternative to antibiotics, *i.e.*, food supplements consisting of live micro-organisms with beneficial effects to the host organism by improvement of the intestinal microbial balance [7]. Probiotic effects have been related to increases in disease resistance by stimulation of natural defences or immune responses; competition with pathogenic micro-organisms for limiting nutrients or adhesion points to the mucous; and the production of inhibitory substances. Despite the fact that the evidence for these effects remains inconclusive, in the most clearest cases it has been shown that (1) the micro-organisms involved are usually lactic bacteria such as *Lactococcus*, *Lactobacillus*, *Bifidobacillus* [8], *Leuconostoc* or *Pediococcus*, (2) the probiotic activity is often conserved in the cell-free medium of the probiont cultures, which alludes to the role of specific metabolites, such as anti-microbial peptides produced by the lactic bacteria (bacteriocins).

Reproducible and quantitative work with probiotics and their possible application to other micro-organisms requires an understanding of the survival of potential probiotics in the marine medium. However, in spite of studies regarding survival in sea water of bacterial indicators of environmental quality, such as faecal [9, 10] or, specifically, *Escherichia coli* [11, 12, 13], there are no data of this type for lactic bacteria (LAB).

In this work the survival curves of different species of LAB in sea water are determined. The effects of salinity, temperature and their interactions on bacterial half-lives are studied by readily standardised procedures to evaluate feasibility as probiotics for turbot larval cultures.

Materials and Methods

Microbiological methods

The micro-organisms used included *Lactococcus lactis* CECT 539 (abbreviated key Lc 1.04), *Lactobacillus brevis* CECT 216 (Lb 2.01), *Lactobacillus casei ssp. casei* CECT 4040 (Lb 3.03), *Lactobacillus casei ssp. casei* CECT 4043 (Lb 3.04), and *Pediococcus acidilactici* NRRL B-5627 (Pc 1.02). Stock cultures were stored at –50ºC in powdered skimmed milk suspension with 25% glycerol [14]. Micro-organisms were grown in 300 ml Erlenmeyer flasks with 100 ml of MRS medium (DIFCO) at 30ºC with 200 rpm orbital shaking. Inocula (0.5% vol/vol) consisted of cellular suspensions from 20 h aged cultures on the same medium and under the same conditions, adjusted to an OD (700 nm) of 0.900.

For the survival tests the biomass of 10 h cultures were used, collected by centrifugation at 10,000 g for 10 min. From a previous calibration of the relationship between dry weight and optic density at 700 nm the sediments were resuspended in 0.9% KCl and the suspensions used (<0.5% vol/vol) to supply initial populations of 1 g l^{-1} to the experimental units. These units consisted of 300 ml Erlenmeyer flasks with 100 ml of sea water of various salinities maintained in orbital stirring (200 rpm) at different temperatures (Table 1).

At predetermined times, viable cells were quantified by means of a plate count technique on MRS agar. Serial 10-fold dilutions were prepared in peptone buffered solutions and 0.1 ml samples were plated in quadruplicate, incubated at 30ºC overnight and manually counted. Results were expressed in colony forming units per ml (CFU ml^{-1}). In tests involving starvation, the counting is usually performed in dilute media, although here this precaution can be ignored here. Accordingly, the results obtained represent the most severe operating conditions.

Experimental design and statistics

The experimental plan was organised to permit two approximations. Firstly, the temporal variation of the population was adjusted to adequate functional forms for determining the half-life of the micro-organisms. Secondly, the combinations of salinity and temperature were chosen to define a complete first order orthogonal design [15] in which half-lives can be introduced as responses. In the first case, the calculation was carried out by means of a non-linear least squares test (quasi-Newton). Results of the factorial designs (orthogonal least-squares calculation) were used to obtain empirical equations which describe half-lives as a function of temperature and salinity. Statistical significance of the coefficients was verified by means of the student t-test (α=0.05), and model consistency by means of the Fisher F test (α=0.05) applied to following means squares ratios:

Model / Total error

(Model + Lack of fitting) / Model

Total error / Experimental error

Lack of fitting / Experimental error.

Results and Discussion

Survival kinetic analysis

To describe the kinetics of survival the experimental data were normalised (the number of cfu/ml corresponding to the initial load of 1 g l^{-1}) and adjusted to two equations from which the times necessary for the population to be reduced to 50% ($t_{0.5}$ or half life, parameter affected by the minimal error) and 10% of the initial ($t_{0.1}$ which may be a more intuitive vision of survival) were deduced.

The first of the equations corresponded to a first order decay process:

$$\frac{dN}{dt} = -\mu N$$

which, resolved for initial conditions t=0 and N=N_0, produces the explicit form:

$$N = N_0 \exp(-\mu t) \ , \ \text{where:} \qquad (1)$$

N: number (normalized) of CFU, with N_0 as initial value
μ: specific mortality (dimensions t^{-1})

and where $t_{0.5}$ and $t_{0.1}$ values can be obtained by replacing in (2) N by $N_0/2$ and $N_0/10$:

$$\frac{N_0}{2} = N_0 \exp(-\mu t_{0.5}) \ ; \ t_{0.5} = \frac{\ln(2)}{\mu} \qquad (2)$$

$$\frac{N_0}{10} = N_0 \exp(-\mu t_{0.1}) \ ; \ t_{0.1} = \frac{\ln(10)}{\mu} \qquad (3)$$

The second equation applied was that of Gompertz (1825):

$$\frac{dN}{dt} = cN(\ln a - \ln N)$$

which, similarly resolved for initial conditions t=0 and N=N_0 :

$$N = a\exp\left[-b\exp(ct)\right], \text{ where:} \qquad (4)$$

N: number (normalized) of CFU

a: fitting parameter (dimensions: N)

b: fitting parameter (dimensionless), related with N_0 through: b= ln(a/N_o)

c: fitting parameter (dimensions: t^{-1})

The constants $t_{0.5}$ and $t_{0.1}$ are obtained as in the preceding case, but adjusting N_0/2 and N_0/10 to the limit of the function when time tends to zero:

$$N_0 = \lim_{t\to 0} N = \lim_{t\to 0} ae^{-be^0} = ae^{-b} \text{ ; then:}$$

$$N = \frac{N_o}{2} = \frac{ae^{-b}}{2} \text{ ; } \frac{ae^{-b}}{2} = ae^{-}be^{ct_{0.5}} \text{ ; and, finally: } t_{0.5} = \frac{1}{c}\ln\left[1+\frac{\ln 2}{b}\right] \qquad (5)$$

Similarly, when N=N_0/10 : $t_{0.1} = \frac{1}{c}\ln\left[1+\frac{\ln 10}{b}\right]$ (6)

The fits of the experimental series to these two equations are shown in Figures 1 to 2 (Pc 1.02 and Lb 3.04 like example), and the corresponding values of $t_{0.5}$ and $t_{0.1}$ are shown in Table 2. It is clear that the most adequate model for describing the results depends on the

strain and test conditions, with no other meaning than that directly obtained from the curves. For certain species or conditions mortality can be assimilated to a first order kinetic process, similar to sterilisation at high temperatures [17, 18]. In other cases, however, the decrease of the bacterial population demonstrates an initial period of reduced mortality and suggests resistance mechanisms. In these cases the survival profile is better adjusted to the equation of Gompertz and is readily paralleled to those found from sterilisation by gentle thermal process [19, 20, 21, 22, 23].

Temperature-salinity interactions

Introducing the half lives as responses (Y), the temperature (T) and salinity (S) combinations which define the orthogonal design specified in Table 1 were determined (see Methods), and fitting each group to a model of the type:

$$Y = b_0 + b_1 S + b_2 T + b_{12} ST \qquad (7)$$

For the species Lb 3.04, Lc 1.04 and Pc 1.02 all the significant criteria specified above validated the half-life descriptions as a function of T and S with equation (7) with the coefficients given in Table 3 (Table 4 shows, for example, the statistical analyses corresponding to Lb 3.04). In the other two species tested (Lb 2.01 and Lb 3.03) the Fisher test applied to the relationship between experimental error and lack of model fitting demonstrated that the functional form (7) was not adequate for the description of the results. In addition, the distribution of the deviations suggested the need for second order terms. In routine applications, it is advisable to change the orthogonal for the rotatable design for deriving quantitative empirical models in these cases of complex response [15].

The response surfaces corresponding to the three first order cases are shown in Figure 3. Survival increases with temperature and decreases with salinity. In addition, within the experimental domain both effects are simply additive in Lc 1.04 and Pc 1.02, whereas in Lb 3.04 a negative interaction (shortening of the half-life) is demonstrated.

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Table 1: Experimental domain of the orthogonal design and codification of the independent variables.

Codified values	Natural values	
	T: Temperature (ºC)	S: Salinity (g.l^{-1})
1 ; 1	30	35
1; -1	30	18
-1 ; 1	20	35
-1; -1	20	18
0;0 (4 replicates)	25	26.5

Table 2: Values of $t_{0.5}$ (half life) and $t_{0.1}$ obtained by means the negative exponential and Gompertz models in all the species and conditions assayed.

			Negative exponential $N = N_0 \exp(-\mu t)$ $t_{0.5} = \ln(2)/\mu$ $t_{0.1} = \ln(10)/\mu$		Gompertz $N = a \exp[-b \exp(c\,t)]$ $t_{0.5} = 1/c\,\{\ln[(1 + \ln 2)/b]\}$ $t_{0.1} = 1/c\,\{\ln[(1 + \ln 10)/b]\}$	
	S (g l^{-1})	T (ºC)	$t_{0.5}$ (h)	$t_{0.1}$ (h)	$t_{0.5}$ (h)	$t_{0.1}$ (h)
Lb 2.01	18	20	16.1	53.3	16.7	36.4
	18	30	19.9	66.2		
	35	20	13.9	46.1	17.6	22.9
	35	30	14.0	46.5		
	26.5	25	22.7	75.5		
Lb 3.03	18	20	10.8	35.9		
	18	30	15.2	50.4		
	35	20	16.9	56.2		
	35	30	13.9	46.2		
	26.5	25	18.9	62.9		
Lb 3.04	18	20	3.4	11.3		
	18	30	8.4	27.9		
	35	20	2.7	8.9		
	35	30	3.3	11.0		
	26.5	25	3.8	12.5		
Lc 1.04	18	20			21.7	43.2
	18	30			23.5	34.8
	35	20			10.8	15.9
	35	30			16.6	23.4
	26.5	25	16.6	55.2	22.6	64.2
Pc 1.02	18	20			15.4	26.8
	18	30			22.1	35.3
	35	20			12.2	14.8
	35	30			20.5	34.6
	26.5	25	18.5	61.6	21.3	60.2

Table 3: Parameters fitted to the equation 7

	Lb 3.04	Lc 1.04	Pc 1.02
b_0	4.18	17.25	18.52
b_1 *(T)*	1.41	1.90	3.74 T
b_2 *(S)*	–1.44	–4.49	–1.19 S
b_{12} *(TS)*	–1.09	NS	NOT TS
r^2	0.972	0.878	0.870
r^2 (corrected for number of variables)	0.961	0.851	0.841

Table 4: Results of factorial design for Lb 3.04 and significance analysis for model (7). *Y*: response ($t_{0.5}$ in hours); $\hat{Y}$: expected response; NS: non significant coefficient; SS: sum of squares; υ: degrees of freedom; MS: mean squares; MSE: mean squares for total error; MSEe: mean squares for experimental error; MSLF: mean squares for lack of fitting; MSM: mean squares for model; MSMLF: mean squares for (model + lack of fitting).

T	*S*	*Y*	$\hat{Y}$	Coefficients	t	Model
-1	-1	3.40	3.13	4.18	103.98	4.18
1	-1	8.39	8.12	1.41	24.76	1.41 *T*
-1	1	2.69	2.42	-1.44	25.41	-1.44 *S*
1	1	3.32	3.05	-1.09	19.17	-1.09 *TS*
0	0	3.78	4.18	Average value =	4.18	
0	0	4.05	4.18	Expected average value =	3.91	
0	0	3.91	4.18	Var (Ee) =	0.0129	
0	0	3.91	4.18	t(α<0.05; υ=3) =	3.1824	

	SS	υ	MS		
				MSM / MSE = 91.4	F_8^3 (α=0,05) = 4.066
Model	21.02	3	7.007	MSMLF / MSM = 0.385	F_3^8 (α=0,05) = 8.845
Error	0.61	8	0.077	MSE / MSEe = 5.927	F_3^8 (α=0,05) = 8.845
Experim. error	0.04	3	0.013	MSLF / MSEe = 8.883	F_3^5 (α=0,05) = 9.013
Lack of fitting	0.57	5	0.115	r^2 =	0.972
Total	21.63	11		corrected r^2 =	0.961

FIGURE CAPTIONS

Figure 1: Survival of *Pediococcus acidilactici* (Pc 1.02) under temperature (*T*) and salinity (*S*) conditions required by the orthogonal design. Experimental values (points) were adjusted to the negative exponential (solid line) and Gompertz equations (dotted line).

Figure 2: Survival of *Lactobacillus casei* (Lb 3.04). Conditions and keys as in figure 1.

Figure 3: Response surfaces corresponding to the joint effect of temperature (*T*) and salinity (*S*) on the half life ($t_{0.5}$) of Lb 3.04, Lc 1.04 and Pc 1.02. Independent variables in codified values.

FIGURE 1

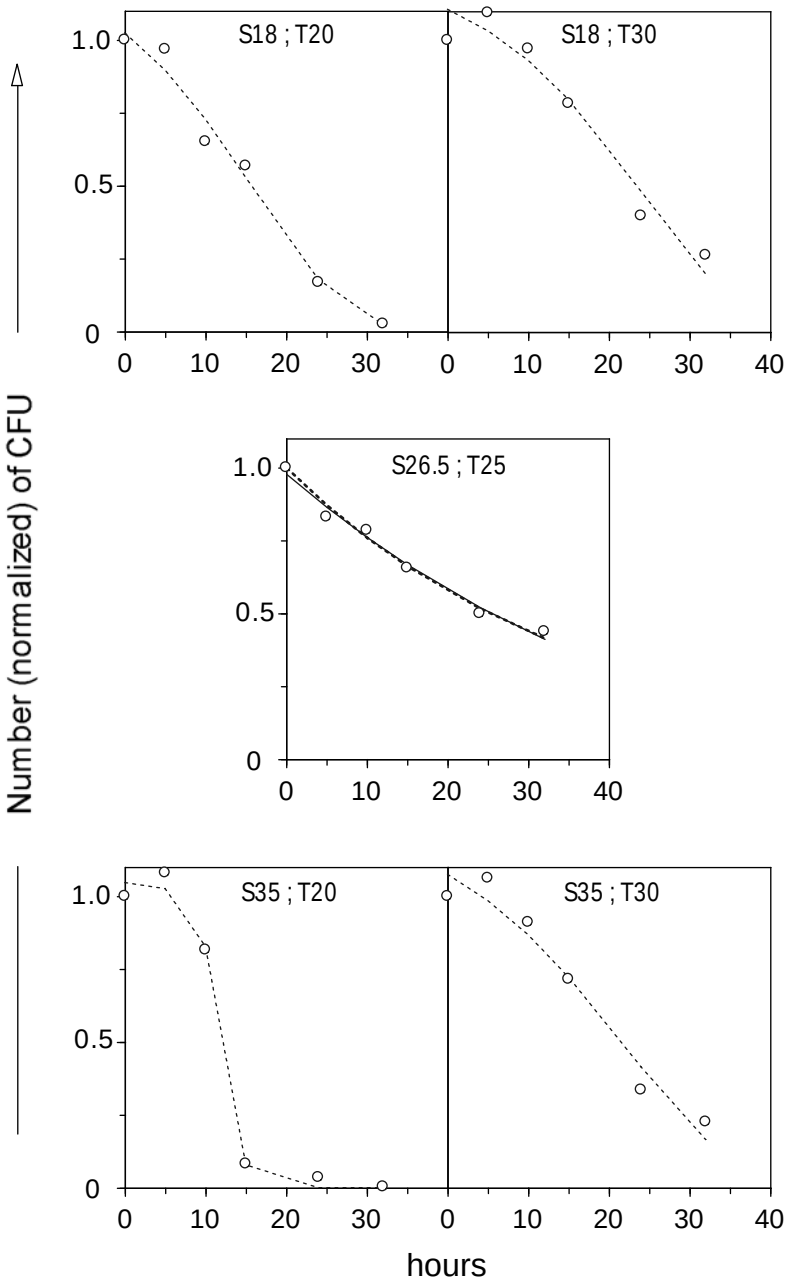

S18 ; T20
S18 ; T30
S26.5 ; T25
S35 ; T20
S35 ; T30
Number (normalized) of CFU
hours
1.0
0.5
0
0
10
20
30
40

FIGURE 2

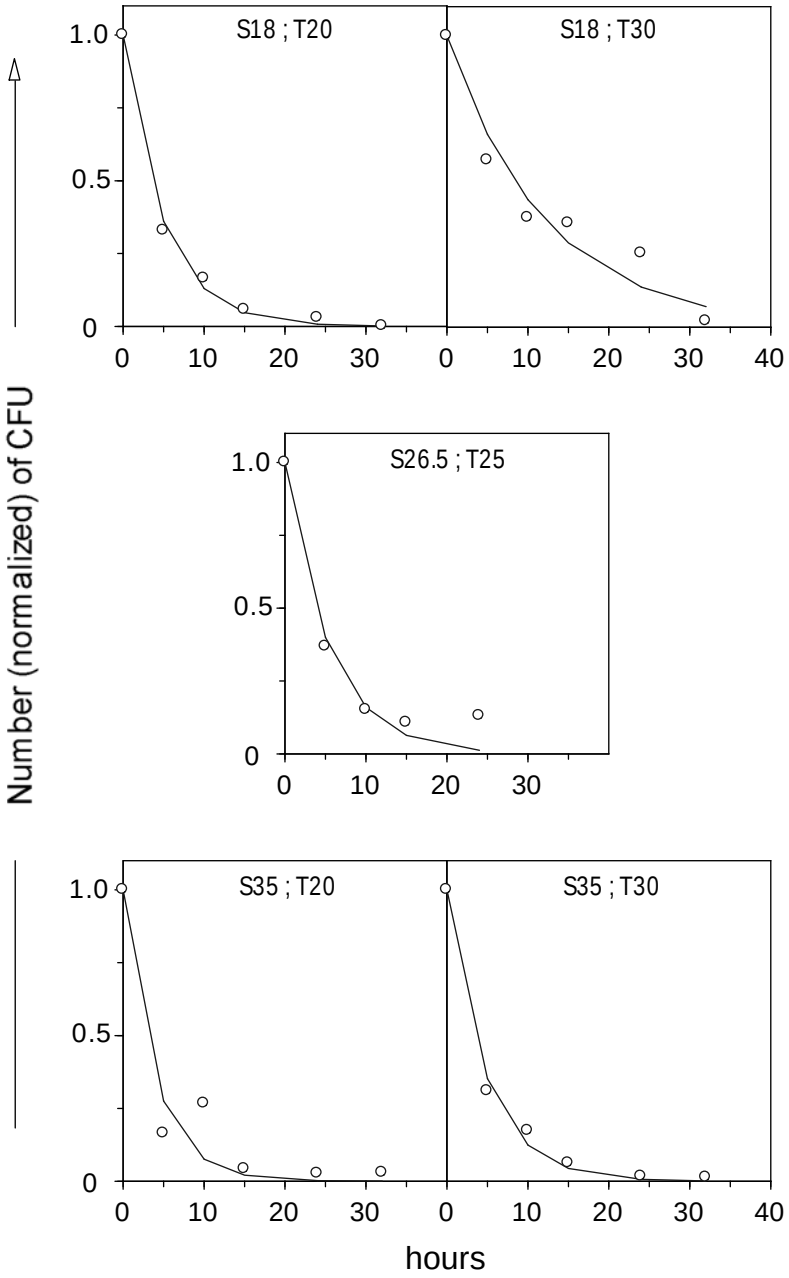
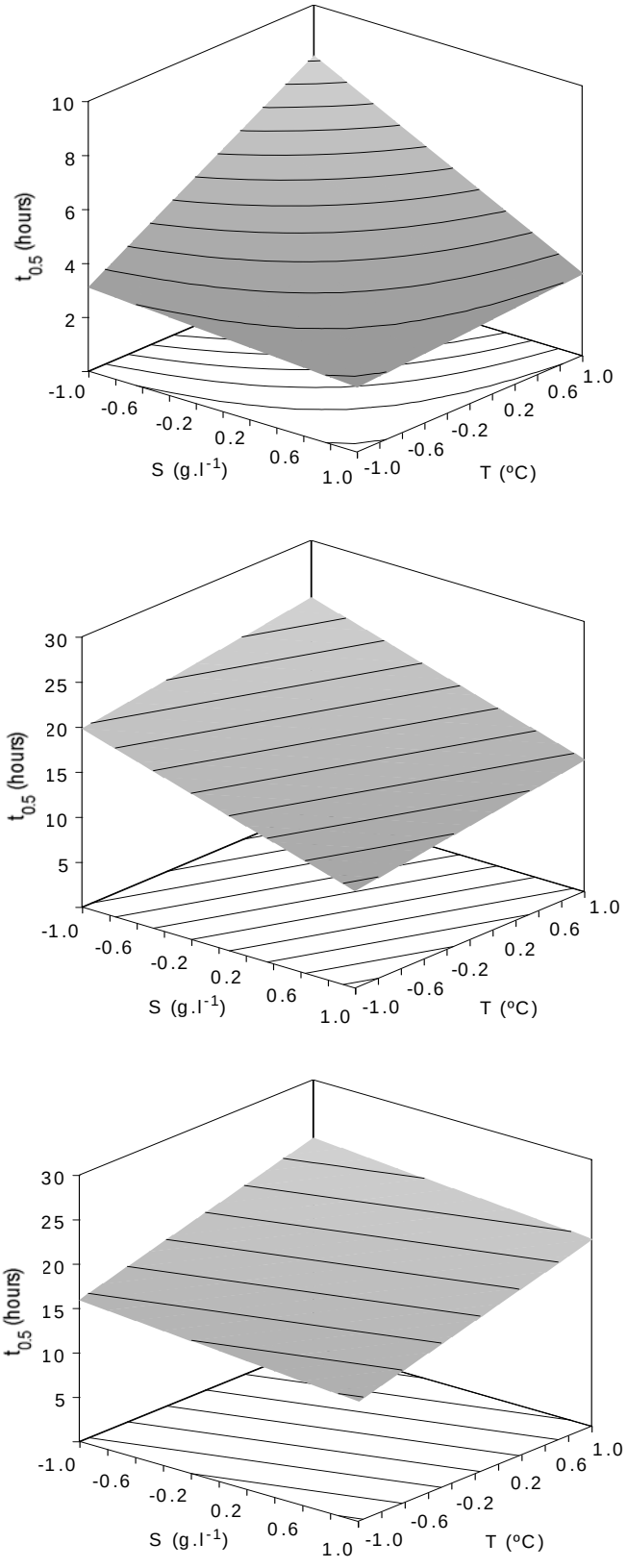

S18 ; T20
S18 ; T30
S26.5 ; T25
S35 ; T20
S35 ; T30
Number (normalized) of CFU
hours
1.0
0.5
0
0
10
20
30
40

FIGURE 3


10
8
6
4
2
$t_{0.5}$ (hours)
-1.0
-0.6
-0.2
0.2
0.6
1.0
S (g.l^{-1})
-1.0
-0.6
-0.2
0.2
0.6
1.0
T (ºC)
30
25
20
15
10
5
$t_{0.5}$ (hours)
S (g.l^{-1})
T (ºC)
30
25
20
15
10
5
$t_{0.5}$ (hours)
S (g.l^{-1})
T (ºC)