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A study of the Gamma hypothesis: Predictive modelling of the growth and inhibition of *Enterobacter sakazakii*

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Abstract

Although the temperature growth profile of the opportunistic pathogen *Enterobacter sakazakii* is known, few other environmental factors affecting growth have been analysed. Using a model based on the Gamma hypothesis – that antimicrobial factors in mixtures exert independent effects – a range of weak acids (lactic, acetic, propionic, citric, sorbic and benzoic), pH, salt and temperature and some of their combinations were examined. The weak acids examined inhibited principally with the acid-form of the weak acid, however, benzoic, sorbic and propionic acids also displayed an inhibitory contribution from their respective anionic forms. In all cases pH could be considered an independent inhibiting factor. The minimum pH and maximum salt concentration for growth were calculated to be 3.89 and 9.1% respectively. In combination, there was no suggestion of any interactive effect between them. Studies performed on combinations of Na acetate/pH between 25 and 41 °C showed that temperature did not affect the relative inhibitory effects of the weak acid/pH mixtures.

The results of this study support the Gamma hypothesis suggesting that there are no synergistic interactions between inhibitory factors and that growth can be predicted from a library of known effects. More importantly to the food industry, the results can be used to design good quality shelf-life challenge tests by reducing the number of studies required.

Keywords: Predictive modelling; Response surfaces; Synergy; Gamma hypothesis; *Enterobacter sakazakii*

1. Introduction

Enterobacter sakazakii is considered an opportunistic pathogen and has been implicated in outbreaks causing meningitis or bacteraemia, especially in neonates and infants, (Urmenyi and Franklin, 1961; Lehner and Stephan, 2004; Kaufman and Fairchild, 2004), with mortality rates of 20 to 50%. Although *E. sakazakii* has been detected in various types of food, only powdered infant formula has been linked to outbreaks of disease.

E. sakazakii can grow at refrigeration temperatures (Iversen et al., 2004). Nazarowec-White and Farber (1997) reported an average generation time of 40 min at 23 °C and 5 h at 10 °C. Knowledge, however, of other inhibitory factors is limited. Breeuwer et al. (2003) demonstrated that *E. sakazakii* cells are more resistant to osmotic and desiccation stress than are other species of the Enterobacteriacae; Nair et al. (2004) inoculated high levels of *E. sakazakii* into reconstituted infant formula followed by up to 1% of monocaprylin (monoglyceride ester of caprylic acid). Although the treatments containing monocaprylin were shown to significantly reduce the population of *E. sakazakii* compared with the controls even at refrigeration temperatures, at the levels needed to control the organism the adverse sensory effects outweigh any advantage.

The hurdle concept developed by Leistner (1995) requires the aggregation of various preservation processes — chemical, physical and biological, to control the growth of spoilage or pathogenic organisms in foods. The hypothetical basis of hurdle technology is that the combination of several inhibitory processes or events (hurdles) is better, or can achieve equivalent results, than a single inhibitory action. The goal of an appropriate hurdle technology is to increase the acceptability of a product to the consumer whilst ensuring a maximum shelf-life with respect to safety and spoilage.

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Predictive microbiology, or "the quantitative microbial ecology of foods" (McMeekin et al., 1997; McMeekin and Ross, 2002) attempts to provide mathematical models of microbial growth under a variety of environmental conditions — e.g. temperature, pH, a_w and the effect of preservatives. Predictive modelling can be seen, therefore, as the quantification of hurdle technology.

Given that there were very few growth models for *E. sakazakii* and given the organism's importance, especially to those businesses producing infant formula, the development of a greater knowledge of its growth characteristics under a variety of environmental conditions appeared appropriate. Moreover, a more general study of its growth characteristics would allow us to investigate further the Gamma hypothesis which states that combined antimicrobial effects act independently (Zwietering et al., 1992).

2. Materials and methods

2.1. Culture preparation

Five strains of *E. sakazakii*, isolated from the environments of factories producing infant formula, were used in this study. *E. sakazakii* (FSM263) was grown overnight in a flask containing 80 ml tryptone soya broth (TSB; Oxoid CM 129) shaking at 30 °C. The cells were harvested, centrifuged to a pellet, washed and re-suspended in peptone water. A standard inoculum was produced by diluting the culture to an optical density (OD) of 0.5 at 600 nm. This standardized culture was then further diluted to produce the starting inoculum of approximately 1×10^5 cfu ml^{-1}.

Table 1
Modelled parameters for inhibitors challenged with *E. sakazakii*

Inhibitory system	Effect	Parameter	Value	St. Err	CL-1	CL-2	RMSE/*DF*
pH	RTD ref	P_0	3.76E−03	2.75E−05	3.71E−03	3.82E−03	9.85E−05 72
	pH	P_1	3.42E−05	8.10E−07	3.26E−05	3.58E−05	
		P_2	0.7564	0.0199	0.7187	0.7939	
Salt	RTD ref	P_0	3.58E−03	3.27E−06	3.57E−03	3.61E−03	2.71E−05 95
	Salt	P_1	47961	190	46604	49308	
		P_2	1.5711	0.0187	1.5377	1.7138	
Na lactate/pH (pK_a 3.86)	RTD ref	P_0	4.60E−03	1.05E−04	4.42E−03	4.81E−03	1.69E−04 68
	pH	P_1	1.33E−05	7.52E−07	1.19E−05	1.47E−05	
		P_2	0.6408	0.0349	0.5750	0.7090	
	Lactic	P_3	1191	465	657	3319	
		P_4	0.6200	0.1053	0.4380	0.8569	
Na acetate/pH pK_a 4.76	RTD ref	P_0	3.82E−03	5.37E−05	3.71E−03	3.93E−03	1.22E−04 64
	pH	P_1	3.08E−05	1.43E−06	2.81E−05	3.38E−05	
		P_2	0.7224	0.0397	0.6626	0.8068	
	Acetic	P_3	146.9	5.0	137.1	157.2	
		P_4	0.8857	0.0390	0.8129	0.9682	
Na propionate/pH pK_a 4.86	RTD ref	P_0	4.21E−03	1.71E−04	3.93E−03	4.56E−03	1.97E−04 61
	pH	P_1	2.45E−05	3.21E−06	1.93E−05	3.01E−05	
		P_2	0.6335	0.0631	0.5294	0.7538	
	Propionic	P_3	349.0	63.01	250.9	U/d	
		P_4	0.8951	0.1945	0.6749	1.2685	
	Propionate	P_5	936.3	82.3	795.5	1129	
		P_6	1.086	0.128	0.886	1.373	
Na_2 citrate/pH (log model) pK_a 3.15, 4.77, 6.4	Ln (TTD) ref	P_0	5.665	0.0177	5.630	5.699	4.78E−02 72
	pH	P_1	1.56E−05	8.34E−07	1.4E−05	1.73E−05	
		P_2	0.5583	0.0149	0.5301	0.5876	
	Citric	P_3	729.8	150.0	510.2	1193	
		P_4	0.4829	0.0516	0.3885	0.6027	
K sorbate/pH pK_a 4.67	RTD ref	P_0	3.84E−03	8.83E−05	3.68E−03	4.05E−03	1.89E−04 107
	pH	P_1	3.92E−05	3.07E−06	3.27E−05	4.5E−05	
		P_2	0.5854	0.0430	0.5088	0.6687	
	Sorbic	P_3	155.0	7.979	139.2	169.5	
		P_4	1.702	0.148	1.431	2.478	
	Sorbate	P_5	3851	256.9	3384	4446	
		P_6	1.550	0.200	1.185	2.017	
Na benzoate/pH pK_a 4.19	RTD ref	P_0	3.66E−03	6.51E−05	3.55E−03	3.79E−03	1.13E−04 48
	pH	P_1	2.64E−05	1.35E−06	2.39E−05	2.92E−05	
		P_2	0.7818	0.0423	0.7029	0.8668	
	Benzoic	P_3	58.45	2.840	53.18	64.85	
		P_4	1.765	0.1857	1.445	2.198	
	Benzoate	P_5	1553	61.7	1436	1688	
		P_6	1.409	0.0999	1.222	1.629	

Parameters obtained using the reciprocal model except for Na_2 citrate/pH; St. Err, standard error; CL-1 and CL-2, lower and upper 95% confidence intervals; RMSE, root mean square error; *DF*, degrees of freedom.