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Weak-acid preservatives: pH and proton movements in the yeast *Saccharomyces cerevisiae*

Malcolm Stratford [a], Gerhard Nebe-von-Caron [b], Hazel Steels [b], Michaela Novodvorska [a], Joerg Ueckert [c], David B. Archer [a,*]

[a] School of Biology, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom
[b] Mologic Ltd., Bedford Technology Park, Thurleigh, Bedford MK44 2YP, United Kingdom
[c] Unilever Research and Development Vlaardingen, Biosciences, The Netherlands

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ABSTRACT

Weak-acid preservatives commonly used to prevent fungal spoilage of low pH foods include sorbic and acetic acids. The "classical weak-acid theory" proposes that weak acids inhibit spoilage organisms by diffusion of undissociated acids through the membrane, dissociation within the cell to protons and anions, and consequent acidification of the cytoplasm. Results from 25 strains of *Saccharomyces cerevisiae* confirmed inhibition by acetic acid at a molar concentration 42 times higher than sorbic acid, in contradiction of the weak-acid theory where all acids of equal pK_a should inhibit at equimolar concentrations. Flow cytometry showed that the intracellular pH fell to pH 4.7 at the growth-inhibitory concentration of acetic acid, whereas at the inhibitory concentration of sorbic acid, the pH only fell to pH 6.3. The plasma membrane H^+-ATPase proton pump (Pma1p) was strongly inhibited by sorbic acid at the growth-inhibitory concentration, but was stimulated by acetic acid. The H^+-ATPase was also inhibited by lower sorbic acid concentrations, but later showed recovery and elevated activity if the sorbic acid was removed. Levels of *PMA1* transcripts increased briefly following sorbic acid addition, but soon returned to normal levels. It was concluded that acetic acid inhibition of *S. cerevisiae* was due to intracellular acidification, in accord with the "classical weak-acid theory". Sorbic acid, however, appeared to be a membrane-active antimicrobial compound, with the plasma membrane H^+-ATPase proton pump being a primary target of inhibition. Understanding the mechanism of action of sorbic acid will hopefully lead to improved methods of food preservation.

1. Introduction

Many yeast species grow in media of high osmotic strength and low pH, enabling growth and spoilage on a variety of food products. Such foods include confectionary, preserved/dried fruits, pickles, butter and cheese, salted, dried and smoked meats, and stored fruit and vegetables. Yeast growth on foods is regarded as a cause of spoilage rather than a safety issue. The symptoms of yeast spoilage are formation of clouds, particulates, or white colonies, alteration of flavour and odour (ethanolic taint being common), and causing "blown" containers due to excess gas formation (Stratford, 2006). The vast majority of yeast species rarely cause food spoilage. It has been estimated that only 10–12 yeast species are responsible for the great majority of spoilage of foods that have been processed and packaged according to good manufacturing practice (Pitt and Hocking, 1997). Several weak acids have been approved for use in foods within the EC, some of which are legally designated as preservatives (Anon, 1995). These include sorbic acid (2,4-hexadienoic acid), benzoic acid, propionic acid, and sulphites. These, together with acetic acid used as an acidulant in pickles, dressings and mayonnaise, are commonly termed the weak-acid preservatives. Research into preservative resistance has largely been carried out on the yeast *Saccharomyces cerevisiae*, recently reviewed by Piper (2011).

The "classical weak-acid theory" of inhibition of microbes by preservatives is defined largely by the parameters of physical chemistry. Weak acids in aqueous solution partially dissociate leading to a dynamic equilibrium between molecular acids and charged anions/protons. At low pH, the equilibrium increasingly favours molecular acids while at neutral pH, charged anions predominate. Molecular weak-acids of preservatives are lipid-soluble, unlike the charged anions, and at low pH, are able to penetrate cells by simple diffusion through the lipids of the plasma membrane into the cytoplasm. Diffusion is rapid, being fully complete within 1–3 min (acetic acid, Conway and Downey, 1950; benzoic acid, Macris, 1975; sulphite, Stratford and Rose, 1986). The apparent cessation of transport after 3 min is really a highly dynamic exchange of weak acids, effluxing

* Corresponding author. Tel.: +44 115 951 3313; fax: +44 115 951 3251.
E-mail addresses: malcolm.stratford@nottingham.ac.uk (M. Stratford), g.nebe-von-caron@alere.com (G. Nebe-von-Caron), hazel.steels@mologic.co.uk (H. Steels), michaela.novodvorska@nottingham.co.uk (M. Novodvorska), joerg.ueckert@unilever.com (J. Ueckert), david.archer@nottingham.ac.uk (D.B. Archer).

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and influxing through the membrane (Stratford and Rose, 1986). Low concentrations of preservatives are concentrated many-fold within the cytoplasm due to ionization, leading to early claims that weak-acid permeation was active transport. In reality, weak-acid molecules entering the neutral pH of the cytoplasm, dissociate into charged anions, which are not able to diffuse out of the cell. The neutral cytoplasm acts, in effect, as a pH-mediated sink for preservatives. Dissociation of weak acids also releases protons in equimolar proportions, and high concentrations of preservatives release sufficient protons to cause substantial cytoplasmic acidification. Cellular metabolism is then inhibited through acidification (Krebs et al., 1983; Pearce et al., 2001). Removal of cells from preservatives causes an immediate diffusive efflux of weak acids, and consequent rise in cytoplasmic pH. The classic weak-acid theory was independently proposed, and cytoplasmic acidification was verified for acetic acid (Neal et al., 1965), benzoic acid (Krebs et al., 1983) and sulphite (Pilkington and Rose, 1988; Stratford, 1983).

Resistance to weak-acid preservatives in yeast has been reported to involve ejection of protons via the plasma membrane H^+-ATPase proton pump, encoded by *PMA1* (Carmelo et al., 1997; Cole and Keenan, 1987; Holyoak et al., 1996). This removes protons from the cytoplasm with a normal stoichiometry of 1 proton ejected/ATP (Cid et al., 1987) although this may decline to 0.1 proton/ATP in starved cells (Venema and Palmgren, 1995). In addition, it has been demonstrated in *S. cerevisiae* that Pdr12p has a major effect on weak-acid resistance (Hatzixanthis et al., 2003; Piper et al., 1998). It has been proposed that this plasma-membrane pleiotropic drug resistance pump causes ejection of preservative anions from the cytoplasm into the external media.

The assumption that all weak-acid preservatives act identically in causing inhibition has recently been questioned from several perspectives. Examination of genetic and transcriptional responses to a variety of weak-acid preservatives showed extensive specific responses (Abbott et al., 2007) but very limited consistent up-regulation and the authors concluded that weak acids did not have a common generic response in *S. cerevisiae*. Theoretical calculations of the proton release from *S. cerevisiae* by weak-acid preservatives showed that while acetic acid and sulphite released high cytoplasmic concentrations of protons; sorbic acid did not (Stratford and Anslow, 1997). These calculations were confirmed by direct measurement of intracellular pH drop in germinating spores of the mould *Aspergillus niger* (Stratford et al., 2009). It was concluded that in mould spores, acetic acid inhibition was due to intracellular acidification, but that inhibition by sorbic acid was not. In the present paper, these studies are extended to *S. cerevisiae* to determine the mechanism of sorbic acid inhibition of this spoilage yeast.

2. Materials and methods

2.1. Yeast strains

The yeast strains used in this study are listed in Table 1 together with their source of isolation. The identity of all strains was confirmed by sequencing of the D1/D2 region of the 26S rDNA using the method described by Kurtzman (2003). The plasma-membrane H^+-ATPase proton pump encoded by *PMA1* is essential in *S. cerevisiae* and *PMA1* deletion is lethal. However, the DAmP-*PMA1* strain has reduced PMA1p activity caused by diminished mRNA abundance due to insertion of a kanamycin-resistance cassette into the 3′ untranslated region (UTR) immediately following the *PMA1* ORF (Breslow et al., 2008). DAmP-*PMA1* strain YSC5094-99851795, clone ID YGL008C (Decreased Abundance by mRNA Perturbation), was obtained from Open Biosystems (https://www.openbiosystems.com/Query/?i=0&q=YGL008C). The presence of the disruption cassette was verified by PCR using specific primers (Breslow et al., 2008). Deletion of subunits of the vacuolar H^+-ATPase proton pump causes misdirection of Pma1p away from the plasma membrane and results in reduced activity of Pma1p (Kane, 2006; Tarsio et al., 2011). Δ*vma2* strains lack V-ATPase activity and Pma1p activity is 65–75% lower (Martinez-Munoz and Kane, 2006). Mutant Δ*vma2* strain Y03266 (Euroscarf gene reference YBR127c) was obtained from Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/). Yeast strains were stored in glycerol on ceramic beads at −80 °C (Microbank™), and maintained short term on MEA (malt extract agar, Oxoid) slopes at 4 °C.

Table 1
Strains of *Saccharomyces cerevisiae* used in this study and their origins. NCYC strains are available from the National Collection of Yeast Cultures, Norwich, UK. Others were from a collection (Mologic strain number) assimilated over several years from the food industry. All strains were confirmed in identity by D1/D2 rDNA sequencing. Weak-acid preservative resistance, sorbic acid and acetic acid, was measured in YEPD pH 4.0 at 10^3 cells/ml and incubated at 25 °C for 2 weeks at pH 4.0. Data are the lowest concentration of weak acids (mM) to completely inhibit growth, and the concentration ratio (acetic:sorbic).

Strain	Origin	Acetic acid	Sorbic acid	Ratio
22	Spoilage, carbonated soft drink, UK	145	4	36.25
47	Spoilage, apple concentrate, UK	140	3.9	35.90
48	Spoilage, fruit drink, UK	140	3.7	37.84
49	NCYC 366, ale strain	165	3.6	45.83
51	NCYC X2180-1B, lab strain	140	3.5	40.00
56	Spoilage, mayonnaise, UK	110	3.7	29.73
61	NCYC 87, distillery strain	115	3.1	37.10
62	Wine strain	155	4.3	36.05
63	Wine strain	110	2.3	47.83
65	Baker's yeast	140	3.9	35.90
125	Spoilage, soft drink, France	180	3.9	46.15
174	Raspberry juice	145	3.6	40.28
176	Euroscarf BY4741	120	3	40.00
244	Soy sauce, Netherlands	125	2.8	44.64
253	Soy sauce, Netherlands	170	2.6	65.38
282	Spoilage, soft drink, Netherlands	165	2.8	58.93
291	NCYC 3253, spoiled soft drink, UK	145	3.5	41.43
292	Spoilage, sugar syrup, UK	155	3.4	45.59
308	Spoilage, soft drink, Belgium	170	3.3	51.52
359	Factory isolate, Turkey	150	3.3	45.45
632	Spoilage, natural yoghurt, UK	115	3	38.33
633	Spoilage, Greek yoghurt, UK	110	3.1	35.48
634	Spoilage, Greek yoghurt, UK	115	3	38.33
635	Spoilage, fruit yoghurt, UK	110	3	36.67
636	Spoilage, fruit yoghurt, UK	115	2.9	39.66

2.2. Growth medium and conditions

The growth medium used in this study was YEPD, containing glucose 20 g/l, bacteriological peptone (Oxoid) 20 g/l, and yeast extract (Oxoid) 10 g/l, adjusted to pH 4.0 with 10 M HCl prior to heat sterilization. Starter cultures comprised 10 ml YEPD pH 4.0 in 28 ml McCartney bottles, inoculated with yeast from MEA slopes, and incubated for 48 h at 25 °C. Experimental cultures comprised either 10 ml YEPD pH 4.0 in 28 ml McCartney bottles, or 40 ml YEPD pH 4.0 in 100 ml conical flasks shaken at 130 rpm at 25 °C.

2.3. Measurement of weak-acid resistance (MIC tests)

Resistance of yeast strains to weak-acid preservatives was investigated by determination of the minimum inhibitory concentration (MIC) of each acid to completely prevent growth. Series of McCartney bottles were prepared with 10 ml aliquots of YEPD, each containing a progressively higher concentration of preservative. The pH of all media was back-titrated to pH 4.0 following acid addition. Bottles were inoculated with yeast at a final concentration of 10^3 cells/ml and incubated for 14 days at 25 °C. The MIC was the lowest concentration of preservative at pH 4.0, at which no growth was detectable at 14 days.