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FEMS Yeast Research 2 (2002) 113–121

FEMS Yeast Research

www.fems-microbiology.org

Zygosaccharomyces kombuchaensis: the physiology of a new species related to the spoilage yeasts *Zygosaccharomyces lentus* and *Zygosaccharomyces bailii*

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Received 5 September 2001; received in revised form 7 January 2002; accepted 9 March 2002

First published online 9 April 2002

Abstract

Zygosaccharomyces kombuchaensis was recently discovered in the 'tea fungus' used to make fermented tea. *Z. kombuchaensis* was shown by ribosomal DNA sequencing to be a novel species, and a close relative of *Zygosaccharomyces lentus*, from which it could not be distinguished by conventional physiological tests. *Z. lentus* was originally established as a new taxon by growth at 4°C, sensitivity for heat and oxidative stress, and lack of growth in aerobic shaken culture at temperatures above 25°C. Subsequent analysis of *Z. kombuchaensis* reveals that this species shares these unusual characteristics, confirming its close genealogical relationship to *Z. lentus*. Detailed physiological data from a number of *Z. kombuchaensis* and *Z. lentus* strains clearly demonstrate that these two species can in fact be distinguished from one another based on their differing resistance/sensitivity to the food preservatives benzoic acid and sorbic acid. The spoilage yeasts *Zygosaccharomyces bailii* and *Z. lentus* are resistant to both acetic acid and sorbic acid, whereas *Z. kombuchaensis* is resistant to acetic acid but sensitive to sorbic acid. This would indicate that *Z. kombuchaensis* strains lack the mechanism for resistance to sorbic acid, but possess the means of resistance to acetic acid. This observation would therefore suggest that these two resistance mechanisms are different, and that in all probability acetic and sorbic acids inhibit yeast growth by different modes of action. *Z. kombuchaensis* strains were also sensitive to benzoic acid, again suggesting inhibition dissimilar from that to acetic acid.

Keywords: Sorbic acid; Benzoic acid; Acetic acid; Preservative resistance; *Zygosaccharomyces kombuchaensis*; *Zygosaccharomyces lentus*

1. Introduction

In western cultures, tea is most commonly prepared as a hot water infusion of black tea leaves. Black tea manufacture involves a process described as 'fermentation' following the plucking of tea leaves. This so-called 'fermentation' does not involve microbes, but action of enzymes derived from the tea-plant tissues. However there do exist many locally produced beverages where tea is genuinely fermented by microbial action, forming ethanol. The microbes involved are primarily acid-tolerant bacteria and yeasts, but fermented teas may include moulds in the culture, ishizuchi-kurocha [1].

Kombucha is one of a number of tea-based beverages of Asian origin, fermented by mixed cultures of bacteria and yeasts, together forming a surface mat or pellicle described as 'the tea fungus'. Other synonyms of kombucha and similar beverages include haipao [2], kocha kinoko [3], hongo [4], suancha or takezutsu-sancha [5]; the suffix 'cha' relating to the tea component of the beverage. Typically, black tea, 1.5–5 g l^{-1} [6–8] is infused with boiling water and sucrose, 50–100 g l^{-1} added before inoculation with a portion of 'the tea fungus'. The taste of kombucha changes during fermentation from sour, fruity and lightly sparkling after a few days, to a mild vinegary taste with prolonged incubation [6,7,9]. Kombucha is becoming increasingly popular in Europe and the USA accompanied

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1567-1356/02/$22.00
PII: S1567-1356(02)00080-6

by, as yet, poorly substantiated claims of health benefits and longevity.

It is probable that kombucha-type beverages have been home-fermented and consumed across Eastern Asia for several millennia, which may account for the variation reported in the microorganisms comprising 'the tea fungus', and consequently for the variations in names, final composition and taste. These beverages are generally acidic, pH 2.0–3.6, [6,7,9] and consequently the bacterial species present are acid-tolerant, primarily acetic acid bacteria, *Acetobacter* spp. or lactic acid bacteria, *Lactobacillus* spp. [2,5,7]. Yeast species reported in tea fungus include *Brettanomyces bruxellensis*, *Brettanomyces lambicus* and *Brettanomyces custersii* (all now recognized as *Dekkera bruxellensis* [10]), *Candida guilliermondii*, *Candida obtusa* (*Clavispora lusitaniae* [10]), *Kloekera apiculata*, *Pichia membranifaciens*, *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*, *Saccharomyces cerevisiae*, *Torulopsis famata* (*Debaryomyces hansenii* [10]), *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* [2,7,11,12]. An examination of two commercial tea fungi and 32 cultures from private households in Germany [13] showed very variable composition of yeasts and no defined symbiosis of yeasts and *Acetobacter* spp. The predominant yeast species were *Brettanomyces*, *Zygosaccharomyces* or *Saccharomyces* spp. In kombucha the role of yeasts is to invert sucrose and form ethanol, which *Acetobacter* spp. then convert to acetic acid [14].

A recent study [15], using 26S rDNA D1/D2 sequencing, of the yeasts isolated by Hesseltine [11] from kombucha originating in Russia revealed one strain as *Pichia fluxuum*, and two strains of a hitherto unknown species, named *Zygosaccharomyces kombuchaensis*. Two further strains from this taxon were isolated in kombucha from the USA. 18S and 26S rDNA D1/D2 sequences both showed *Z. kombuchaensis* to be most closely related to *Zygosaccharomyces lentus*, and more distantly to *Z. bailii* and *Zygosaccharomyces bisporus*; all three being noted spoilage yeasts [16]. Other related species, *Z. rouxii* and *Zygosaccharomyces mellis* (Fig. 1) are remarkable for extreme osmotolerance and spoilage of high-sugar foods and honey. It was reported that *Z. kombuchaensis* could be distinguished from *Z. lentus* using RFLP (random fragment length polymorphism) but these species could not be distinguished by standard taxonomic physiological tests [15]. In the current paper, the physiological and spoilage characteristics of all four strains of *Z. kombuchaensis* are examined, and compared with the 10 *Z. lentus* strains described to date, and six strains of *Z. bailii* as controls. The identity of *Z. kombuchaensis* as a distinct new taxon was confirmed by physiological tests.

2. Materials and methods

2.1. Yeast strains

The yeast strains used in this work are listed in Table 1. *Z. kombuchaensis* strains were a gift from Professor Kurtzman, National Center for Agricultural Utilization Research, Peoria, IL, USA.

2.2. Media and growth conditions

Yeasts were maintained at 4°C on slopes of malt extract agar, MEA, subcultured annually. YEPD, used as standard broth medium, contained per l of water: glucose, 20 g; bacteriological peptone (Oxoid) 20 g; yeast extract (Oxoid) 10 g. YEPD broth was corrected to pH 4.0 using HCl, 10 M. Starter cultures comprised 10 ml YEPD broth in 30 ml capped McCartney bottles, cultured at 25°C for 48 h. Experimental cultures, similarly 10 ml YEPD in 30 ml bottles, were inoculated at 1×10^3 cells ml^{-1}, cultured without shaking at 25°C for 14 days, unless otherwise stated. Yeast growth was assessed visually and by measurement of optical density at 600 nm, after subtraction of media blanks. In certain experiments, yeasts were cultured aerobically in shaken conical flasks; 125 ml flasks

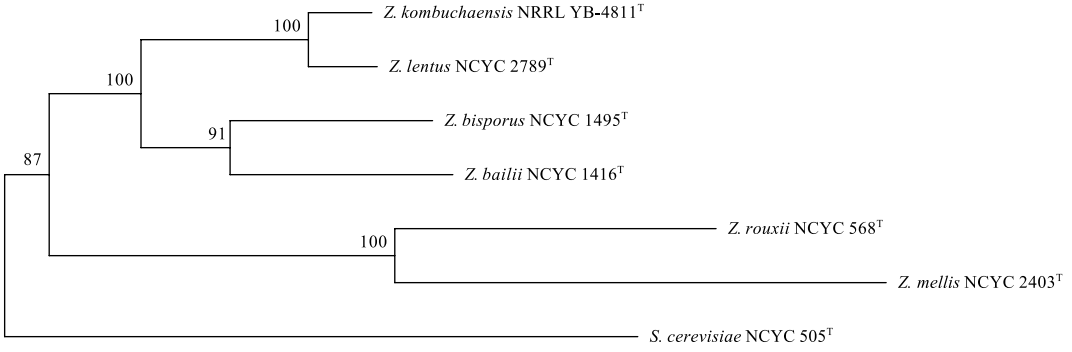


Fig. 1. The taxonomic relationships of the *Zygosaccharomyces* sensu stricto clade. This phylogenetic tree is based on 26S rDNA D1/D2 sequences with *S. cerevisiae* used as the outgroup species. The scale bar represents 1 base substitution per 100 nucleotides.

Table 1
Yeast strains used in this study

Yeast species	Strain	Source
Z. bailii	CMCC 2589	spoiled soft drink, lemon
Z. bailii	CMCC 2959	spoiled herring in tomato sauce
Z. bailii	CMCC 2960	spoiled herring in tomato sauce
Z. bailii	CMCC 2968	spoiled concentrated orange juice
Z. bailii	CMCC 3298	acetic acid-tolerant yeast, Netherlands
Z. bailii	NCYC 1766	spoiled blackcurrant and grape juice
Z. lentus	CMCC 3628	spoiled tomato product, UK
Z. lentus	IGC 5207	spoiled orange beverage, UK
Z. lentus	IGC 5316	wine, France
Z. lentus	NCYC 1601	spoiled orange squash
Z. lentus	NCYC 2406	spoiled tomato ketchup, UK
Z. lentus	NCYC 2789	spoiled whole orange juice
Z. lentus	TNO 0566	
Z. lentus	TNO 0567	
Z. lentus	TNO 0569	
Z. lentus	TNO 0572	
Z. kombuchaensis	NRRL YB4810	kombucha tea fungus, Russia
Z. kombuchaensis	NRRL YB4811	kombucha tea fungus, Russia
Z. kombuchaensis	NRRL Y27163	kombucha tea fungus, USA
Z. kombuchaensis	NRRL Y27162	kombucha tea fungus, USA

NCYC strains are available from the National Collection of Yeast Cultures, Institute of Food Research, Norwich NR4 7UA, UK (Web site: www.ncyc.co.uk); IGC strains from the Portuguese Yeast Culture Collection, Monte de Caparica, Portugal and TNO strains from TNO Voeding, Zeist, The Netherlands. NRRL refers to the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA, and CMCC to the Colworth Microbiology Culture Collection, Unilever R&D, Colworth House, Bedford, UK.

containing 50 ml YEPD broth medium were orbitally shaken at 140 rpm at 27°C.

2.3. Addition of inhibitors

As indicated in the text, yeasts were characterized by resistance to preservatives or inhibitors, osmotolerance and low pH. Degree of resistance was determined by the lowest concentration of inhibitor required to prevent growth, the minimum inhibitory concentration (MIC). All tests were carried out in YEPD medium at pH 4.0. Sorbic acid and benzoic acid were added from 10% w/v

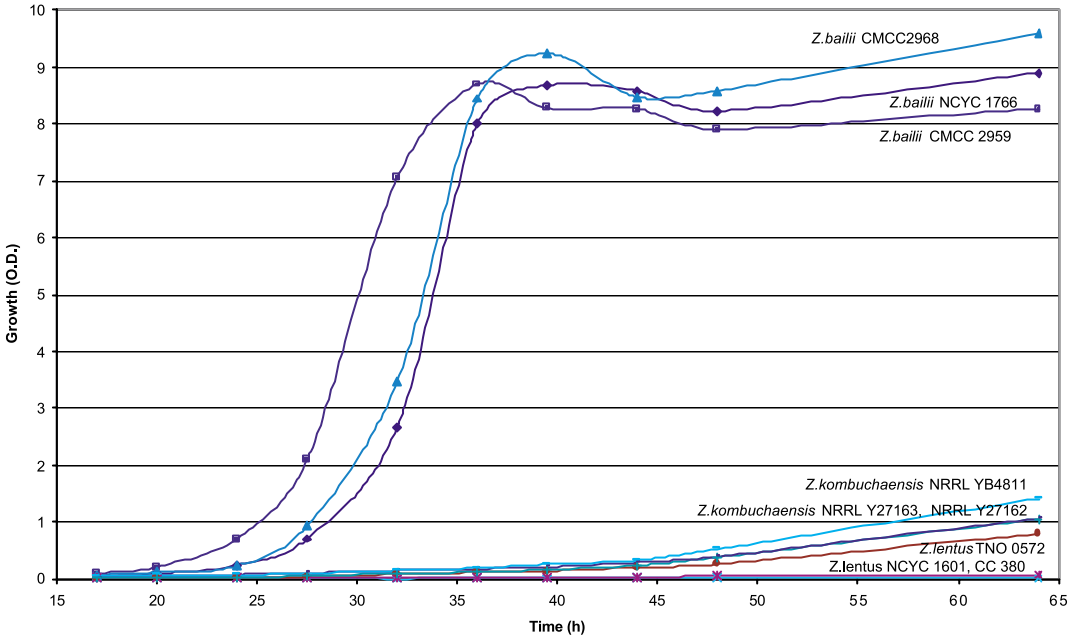


Fig. 2. Poor growth in three strains of *Z. kombuchaensis*, NRRL YB4811, NRRL Y27162, NRRL Y27163, in aerobic shake flasks, 50 ml YEPD pH 4.0, orbitally shaken at 140 rpm at 27°C. Three *Z. bailii* control strains, NCYC 1766, CMCC 2959, CMCC 2968 grew rapidly. *Z. lentus* strain TNO 0572 grew poorly and growth of *Z. lentus* strains NCYC 1601 and CC 380 (NCYC 2406) was not detectable over the course of the experiment.

stock solutions of acids in ethanol. Acetic acid, ethanol, salt (NaCl), EDTA and HCl (pH minimum) were added directly and pH corrected before sterilization. Nickel and copper resistances were assessed by addition of filter-sterilized nickel chloride or copper sulfate to autoclaved media. Dimethyl dicarbonate, DMDC, and H_2O_2, were added to cultures immediately following inoculation, to avoid inhibitor decomposition. To prevent caramelization of sugars in solutions containing high concentrations of glucose, media for determination of osmotolerance were autoclaved in two parts, glucose at $\times 1.1$ final concentration, and YEP, yeast extract+peptone at $\times 10$ concentration. 1 ml aliquots of $\times 10$ YEP were added to 9 ml aliquots of glucose solution after autoclaving. Heat resistance of yeast strains was assessed by immersing inoculated tube cultures into water at 37.5°C, 40°C, 42.5°C, 45°C, 47.5°C, 50°C, 52.5°C, 55°C, 57.5°C and 60°C for 25 min. Controls showed that the tube contents reached temperature within 5 min. Heat resistance was determined as the temperature at which the yeast inoculum declined by 2 log in 20 min. Antibiotic resistance of strains was determined using Etest antibiotic strips (Cambridge Diagnostic Services Ltd., UK) containing fluconazole, ketoconazole, itraconazole, amphotericin B, or flucytosine. Etest strips were laid onto yeast lawns on yeast nitrogen base agar (Difco) supplemented with 0.1 g l^{-1} yeast extract and buffered at pH 7.1 using MOPS buffer (100 mM). MIC values were read directly from zones of inhibition, after growth at 25°C for 2–5 days.

3. Results

3.1. Unusual attributes of Z. lentus, shared by Z. kombuchaensis

Strains of *Z. lentus* were first noticed as unusual yeasts and distinct from *Z. bailii* and other *Zygosaccharomyces* spp. by their lack of growth in shaking culture at temperatures greater than 25°C, and by their ability to grow at 4°C [16,17]. When similar tests were carried out on the four strains of *Z. kombuchaensis*, it was found that while these yeasts grow in static culture up to 30°C, growth in shaking flasks at temperatures above 25°C was very poor. Fig. 2 shows rapid growth of three strains of *Z. bailii* in shaken culture at 27°C, whereas growth by three strains each of *Z. lentus* and *Z. kombuchaensis* was slight or lacking over the period of the experiment. Estimations of the growth rate of *Z. kombuchaensis* strains by linear regression of semi-log growth plots showed the doubling times to range between 8.5 and 10.5 h, whereas *Z. bailii* strains doubled between 2.3 and 2.5 h, and *Z. lentus* strains between 10.2 h and infinity. The slow growth of *Z. lentus* strains was reflected in the naming of the species (*lentus*, Latin adj., slow, lingering, apathetic) [16], a characteristic apparently shared by *Z. kombuchaensis*.

Lack of growth of *Z. lentus* strains in shaken flasks was attributed to sensitivity to oxidative stress in combination with a temperature close to the upper limit for growth of this species [17]. Tests were therefore carried out on the

Table 2
Comparison of H_2O_2 sensitivity, growth at low temperature and heat kill, of six strains of *Z. bailii*, *Z. lentus* (10 strains) and *Z. kombuchaensis* (four strains)

Species	Strain	H_2O_2 (mM)	Growth at 4°C	2 log kill temp.
Z. bailii	CMCC 2589	8	absent at 4 weeks	49.5°C
Z. bailii	CMCC 2959	8.5	absent at 4 weeks	51.3°C
Z. bailii	CMCC 2960	7.5	absent at 4 weeks	50.2°C
Z. bailii	CMCC 2968	7	absent at 4 weeks	49.7°C
Z. bailii	CMCC 3298	10	absent at 4 weeks	50.3°C
Z. bailii	NCYC 1766	9	absent at 4 weeks	49.2°C
Z. lentus	CMCC 3628	4	present at 2 weeks	45.5°C
Z. lentus	IGC 5207	4.5	present at 2 weeks	45.0°C
Z. lentus	IGC 5316	4	present at 2 weeks	45.2°C
Z. lentus	NCYC 1601	6	present at 2 weeks	45.7°C
Z. lentus	NCYC 2406	4.5	present at 2 weeks	46.2°C
Z. lentus	NCYC 2789	4	present at 2 weeks	46.3°C
Z. lentus	TNO 0566	5.5	present at 2 weeks	46.3°C
Z. lentus	TNO 0567	5	present at 2 weeks	46.2°C
Z. lentus	TNO 0569	4	present at 2 weeks	45.5°C
Z. lentus	TNO 0572	4.5	present at 2 weeks	45.8°C
Z. kombuchaensis	NRRL YB4810	5	present at 2 weeks	45.3°C
Z. kombuchaensis	NRRL YB4811	5	present at 2 weeks	44.0°C
Z. kombuchaensis	NRRL Y27163	4	present at 2 weeks	45.7°C
Z. kombuchaensis	NRRL Y27162	4.5	present at 2 weeks	44.8°C
Z. bailii	mean of 6	8.33	negative at 4 weeks	50.03°C
Z. lentus	mean of 10	4.60	positive at 2 weeks	45.77°C
Z. kombuchaensis	mean of 4	4.62	positive at 2 weeks	44.95°C

H_2O_2 sensitivity was determined by the MIC at which no growth occurred after 2 weeks. Heat kill was determined as the temperature at which the yeast inoculum, 10^4 cells, declined by 2 log in 20 min. Values are the means of at least two determinations for each strain.

Table 3
Mean and standard deviation (σ_{n-1}) values of MIC concentrations of inhibitors applied to *Z. kombuchaensis*, four strains, *Z. lentus*, 10 strains and *Z. bailii*, six strains

	Z. kombuchaensis	*Z. lentus*	*Z. bailii*
Acetic acid (M)	0.300 ± 0.02	0.276 ± 0.088	0.438 ± 0.099
Ethanol (M)	1.34 ± 0.36	1.25 ± 0.41	1.8 ± 0.13
Glucose (M)	3.18 ± 0.25	3.66 ± 0.19	4.17 ± 0.68
Salt (NaCl) (M)	2.04 ± 0.05	2.34 ± 0.25	2.72 ± 0.22
Decanoic acid (mM)	0.14 ± 0.03	0.32 ± 0.13	0.25 ± 0.07
EDTA (mM)	8.3 ± 1.7	22.4 ± 10.7	26.2 ± 8.5
DMDC (mM)	2.15 ± 0.57	1.29 ± 0.32	1.92 ± 0.49
Nickel ($NiCl_2$) (mM)	0.71 ± 0.27	0.98 ± 0.32	1.50 ± 0.17
Copper ($CuSO_4$) (mM)	3.25 ± 0.29	3.51 ± 0.47	2.80 ± 0.54
Fluconazole (ppm)	> 256 ± 0	243 ± 40	243 ± 29
Ketoconazole (ppm)	1.12 ± 0.52	1.18 ± 1.23	1.58 ± 2.19
Itraconazole (ppm)	24.4 ± 15.2	14.1 ± 15.5	5.7 ± 7.3
Amphotericin B (ppm)	0.33 ± 0.25	0.20 ± 0.12	0.64 ± 0.58
Flucytosine (ppm)	0.18 ± 0.08	0.05 ± 0.08	0.47 ± 0.20
Growth pH minimum	1.54 ± 0.18	1.87 ± 0.15	2.03 ± 0.08

Experiments were all carried out at pH 4.0 at 25°C and measured at 14 days, unless otherwise stated.

ability of *Z. kombuchaensis* strains to grow at low temperature, death of cells at high temperature, and H_2O_2 sensitivity. The results are summarized in Table 2. These results show that *Z. kombuchaensis* strains share the unusual characteristics of *Z. lentus* of sensitivity to oxidative stress (H_2O_2), the ability to grow at low temperature, and sensitivity to heat killing at high temperature.

3.2. Non-discriminating attributes of Z. kombuchaensis

Due to strain variation, some tests of inhibition did not show clear-cut differences between yeast species (Table 3). Inhibition by acetic acid or ethanol showed a high degree of similarity between *Z. kombuchaensis* and *Z. lentus* strains. But, while the mean MIC of each species were similar for *Z. kombuchaensis* and *Z. lentus*, the large variation in individual strain results shows considerable overlap with other species. Other results, e.g. tests on decanoic acid, EDTA or minimum pH for growth, showed a trend distinguishing between *Z. kombuchaensis* and *Z. lentus*, but again a trend partially obscured by strain variation (Table 3). These results, therefore, cannot be regarded as firm or direct evidence for, or against, a close taxonomic link between *Z. kombuchaensis* and *Z. lentus*.

3.3. Attributes distinguishing Z. kombuchaensis from Z. lentus

Yeast inhibition by acidic preservatives, sorbic or benzoic acids, showed clear distinction between strains of *Z. kombuchaensis* and *Z. lentus*, despite individual strain variation. These results are shown in Table 4. Detailed examination of the sensitivity of *Z. kombuchaensis*, and resistance of *Z. lentus* and *Z. bailii* to sorbic acid is shown in Fig. 3. It was noted that sorbic acid-resistant species, *Z. bailii* and *Z. lentus*, grew at high concentrations of sorbic acid, but only as a 'tail' of poor growth (Fig. 3). In contrast, *Z. kombuchaensis* cultures were inhibited at low concentration of sorbic acid; growth ceased abruptly, without the poorly growing tail (Fig. 3c). Acetic acid-treated cultures of these same yeasts all showed broadly similar resistance to acetic acid, and similar tails of poorly growing cells at high concentrations of acetic acid (Fig. 4).

4. Discussion

4.1. Separation of Z. lentus and Z. kombuchaensis

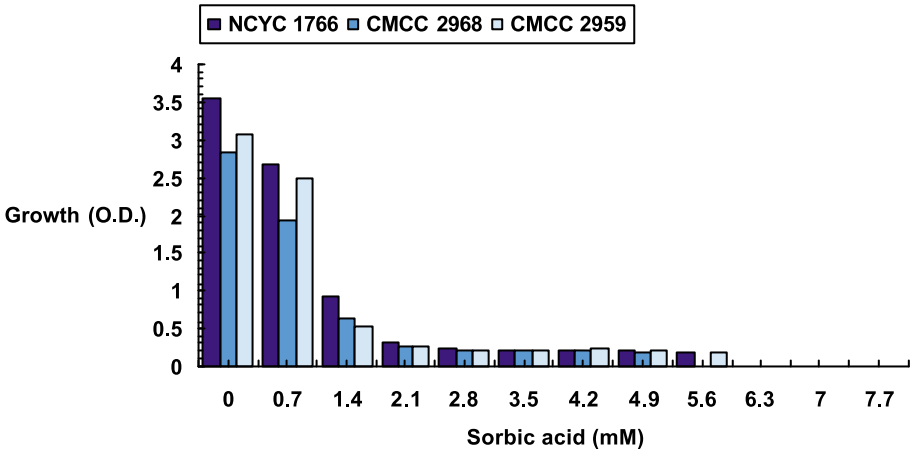
In ideal circumstances, to distinguish between closely related species, simple tests must be devised that show clear separation between the properties of all strains in each species, despite individual strain variation. Of the tests described in this paper, many indicate difference in physiological behavior between *Z. kombuchaensis* and *Z. lentus*, as assessed by mean inhibition of species (Table 3). *Z. kombuchaensis* was more sensitive to high glucose, salt, nickel and copper, and notably more sensitive to EDTA

Table 4
Comparison of sorbic acid and benzoic acid resistance in *Z. bailii*, six strains, *Z. lentus,*, 10 strains and *Z. kombuchaensis*, four strains

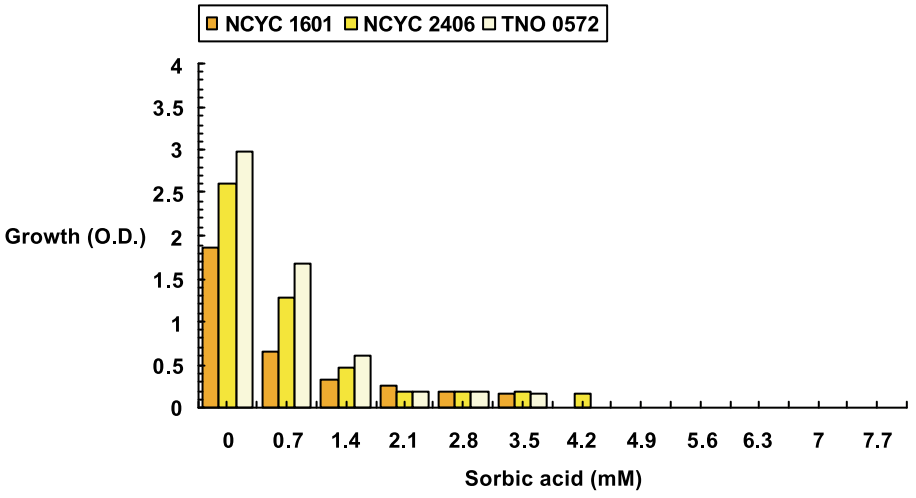
	Z. kombuchaensis	*Z. lentus*	*Z. bailii*
Sorbic acid (mM)	0.808 ± 0.059	3.910 ± 1.012	6.028 ± 0.982
Benzoic acid (mM)	2.105 ± 0.225	6.381 ± 1.340	8.153 ± 1.097

Values are the mean and standard deviation (σ_{n-1}) of the MIC, measured after 2 weeks incubation at 25°C. Tests were carried out at pH 4.0 with an inoculum of 10^4 cells/bottle. Values, mM, are means of at least two determinations for each strain.

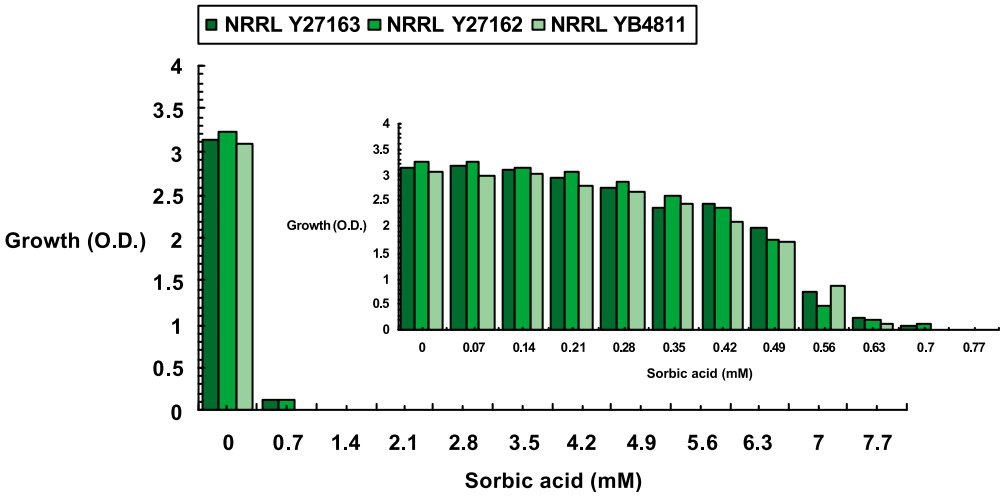
a. *Zygosaccharomyces bailii*



b. *Zygosaccharomyces lentus*



c. *Zygosaccharomyces kombuchaensis*



and decanoic acid than *Z. lentus*. *Z. kombuchaensis* appeared more resistant to DMDC and low pH of the growth media. Regrettably, the individual strain variation, particularly in *Z. lentus*, was sufficiently great as to cause overlap of the standard distributions of the two species (Table 3). This effectively prevents any of these inhibitors being used as tests to definitively separate the two species.

However tests on the food preservatives, sorbic acid and benzoic acid, showed that not only were *Z. kombuchaensis* strains very sensitive to preservatives but also that there was a clear separation between the standard distributions of the species means of *Z. kombuchaensis* and *Z. lentus* (Table 4). The following tests are therefore proposed to distinguish between these species:

4.1.1. Sorbic acid

Positive growth in 14 days in 2 mM sorbic acid in pH 4.0 YEPD broth = *Z. lentus*.

Negative growth in 14 days in 2 mM sorbic acid in pH 4.0 YEPD broth = *Z. kombuchaensis*.

4.1.2. Benzoic acid

Positive growth in 14 days in 4 mM benzoic acid in pH 4.0 YEPD broth = *Z. lentus*.

Negative growth in 14 days in 4 mM benzoic acid in pH 4.0 YEPD broth = *Z. kombuchaensis*.

Other results (Fig. 2, Table 2) show that *Z. kombuchaensis* strains share the unusual characteristics of *Z. lentus*, namely, sensitivity to H_2O_2, growth at low temperature, sensitivity to heat, low growth rate and poor growth in shaken flasks at temperatures greater than 25°C [16,17]. Overall, these physiological data support the 18S and 26S D1/D2 sequencing data [15], indicating a close taxonomic relationship between *Z. lentus* and *Z. kombuchaensis* but the existence of two distinct taxa.

4.2. The physiology and growth environment of *Z. kombuchaensis*

The physiological strengths of *Z. kombuchaensis*, low pH tolerance and acetic acid resistance, appear well matched to the unusual conditions present in fermenting tea. It is not widely appreciated that acids such as acetic acid inhibit yeasts in their own right, an action distinct from inhibition by low pH per se. Yeasts, such as *Z. bailii*, most resistant to acidic preservatives are relatively sensitive to low pH [18]. Kombucha has been reported to contain substantial concentrations of gluconic acid (19.7–30 g l^{-1}), acetic acid (5.6–28 g l^{-1}), more rarely lactic acid, with the pH falling during fermentation to pH 3.6–2.0 [6,7,9]. Antimicrobial action by kombucha was shown by Hesseltine [11] albeit using very high tea concentrations (34 g l^{-1}). While the tea present in kombucha may have a role in the inhibition of viruses or certain bacteria [19,20], it has been demonstrated that the antimicrobial action of kombucha can be entirely attributed to the acetic acid content [8]. Lipophobic acids such as gluconic acid or lactic acid have slight effects on yeasts [21,22] while acetic acid inhibits most yeasts at < 100 mM at pH 4.0. In the experiments shown here, *Z. kombuchaensis* was seen to be resistant to acetic acid, 300 mM at pH 4.0, to a degree slightly exceeding most strains of *Z. lentus* (Table 3). Kurtzman et al. [15] showed similar data: while *Z. kombuchaensis* grew on 1.0% acetic acid agar, some strains of *Z. lentus* grew only at 0.9% acetic acid. Other yeast species also reported in kombucha, e.g. *Z. bailii*, *Z. bisporus*, or *Zygosaccharomyces microellipsoides*, may find their intrinsic acetic acid resistance to be a selective advantage in this habitat.

4.3. *Z. kombuchaensis* and sorbic acid resistance

Strains of a new yeast species, *Z. kombuchaensis*, are shown to be resistant to acetic acid but very sensitive to sorbic acid (Table 4). The physiology of this species is highly unusual in that *Zygosaccharomyces* spp. spoilage yeasts, *Z. bailii*, *Z. bisporus* and *Z. lentus*, are significantly resistant to several weak-acid preservatives, including acetic acid, benzoic acid and sorbic acid [17,23]. This co-resistance to weak-acid preservatives has contributed to the widespread assumption that all weak acids have similar mechanisms of action and that mechanisms of resistance will protect yeast cells equally against all weak-acid preservatives.

In aqueous solution, weak acids form dynamic equilibria between the uncharged acid molecules and their respective charged anions, e.g. acetic acid molecules and acetate anions. The classic 'weak-acid preservative theory' proposes that the uncharged acid molecules penetrate the cell by diffusion though the plasma membrane, charged anions being lipid insoluble. Acid molecules dissociate at the near-neutral pH of the cytoplasm, releasing protons and causing the cytoplasm to become acidic, thus inhibiting the cell. The weak-acid theory of cellular acidification has been independently proposed to account for inhibition by acetic acid [24], sulfite [25] and benzoic acid [26]. Measurement of cytoplasmic pH has confirmed cytoplasmic acidification in cells treated with acetic acid [24] and sulfite [27].

However, the role of sorbic acid as a cause of cytoplasmic acidification has been questioned. Other six carbon acids, alcohols and aldehydes also inhibited yeast at broadly similar concentrations, despite the inability of alcohols or aldehydes to dissociate and release protons [28].

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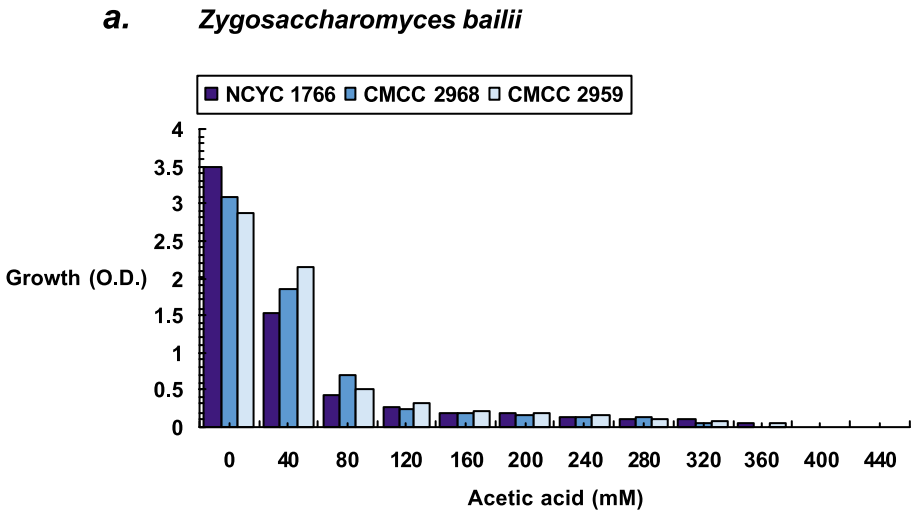
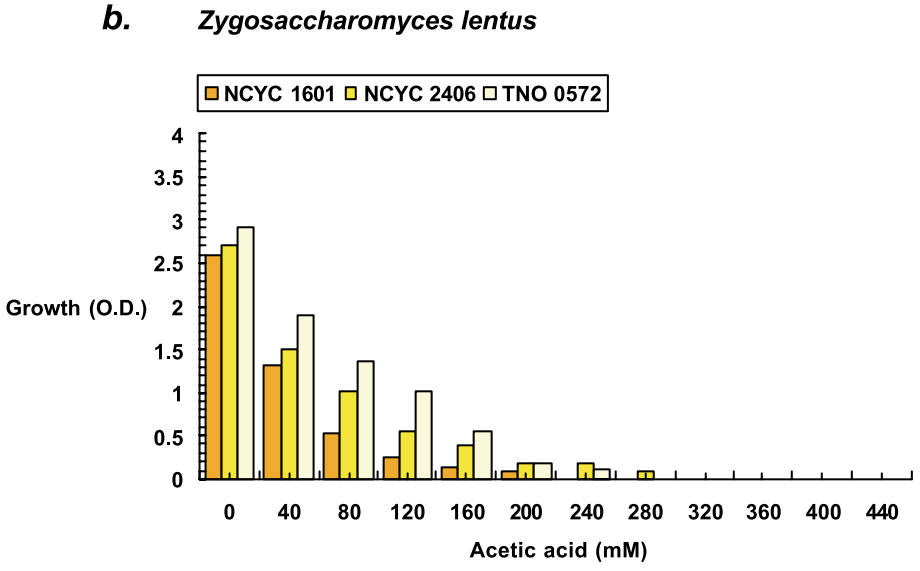
Fig. 3. Growth and inhibition by sorbic acid of *Z. bailii* NCYC 1766, CMCC 2959, CMCC 2968, *Z. lentus* TNO 0572, NCYC 1601, CC 380 and *Z. kombuchaensis* NRRL YB4811, NRRL Y27162, NRRL Y27163, in YEPD broth, pH 4.0 measured after 5 days incubation at 25°C.





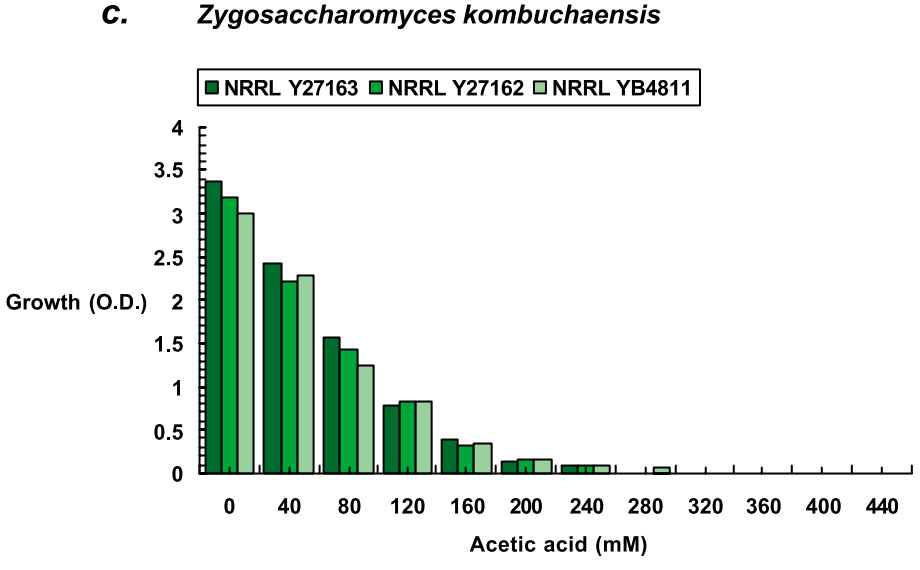


Fig. 4. Growth and inhibition by acetic acid of *Z. bailii* NCYC 1766, CMCC 2959, CMCC 2968, *Z. lentus* TNO 0572, NCYC 1601, CC 380 and *Z. kombuchaensis* NRRL YB4811, NRRL Y27162, NRRL Y27163, in YEPD broth, pH 4.0 measured after 5 days incubation at 25°C.
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The calculated proton release caused by sorbic acid was found to be far smaller than that of acetic acid, and direct tests showed that sorbic acid did not affect cytoplasmic acidification when used at the inhibitory concentration (M. Stratford and J. Ueckert, manuscript in preparation). The close relationship between inhibitory concentration of medium-chain acids, such as sorbic acid, and the partition coefficient suggested that sorbic acid was primarily acting on cell membranes [28].

The co-existence of sorbic acid sensitivity and acetic acid resistance in *Z. kombuchaensis* is strong evidence that these acids are resisted by different mechanisms, *Z. kombuchaensis* expressing resistance to acetic acid but lacking the resistance mechanism to sorbic acid used by *Z. lentus* and *Z. bailii*. Furthermore, the existence of distinct resistance mechanisms to different acids may infer that, in all probability, these acids inhibit yeast cells by different physiological mechanisms. It is hoped that further study and comparison of *Z. kombuchaensis* and *Z. lentus* strains will reveal more of the molecular and physiological mechanisms of resistance to sorbic acid, present in *Z. lentus* but absent in *Z. kombuchaensis*.

Acknowledgements

The authors gratefully acknowledge the technical assistance of Mark Walker and Jo Richens and Chris Capell for critical evaluation of the manuscript.

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