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Growth of Yeasts Isolated from Cheeses on Organic Acids in the Presence of Sodium Chloride

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Summary

Twenty six species of yeasts isolated from cheeses were examined for their growth on citric, lactic and acetic acids in the presence of sodium chloride up to 15 % (w/v) Debaryomyces hansenii gave the strongest growth reactions on citric and lactic acids in the presence of salt, followed by good responses from Candida famata, Candida catenulata and Candida intermedia. Significant to weaker growth reactions on the acids were found for Candida lipolytica, Candida tropicalis, Cryptococcus albidus and Kluyveromyces marxianus. Only C. catenulata gave growth on acetic acid. Growth rates of D. hansenii were similar on substrates of glucose, citric acid and lactic acid, but maximum cell populations were 5–10 times less for growth on the acids. Growth rates and cell populations on these substrates were decreased in the presence of NaCl. The concentration of organic acid end-products in culture filtrates varied with the growth substrate. Glycerol and arabitol were produced at intracellular and extracellular locations during growth on either glucose, lactic acid or citric acid in the presence of NaCl.

Key words: Debaryomyces hansenii, Candida famata, C. catenulata, C. intermedia, C. lipolytica, C. tropicalis, Cryptococcus albidus and Kluyveromyces marxianus, yeast growth, cheese ripening, polyols

Introduction

Yeasts are part of the microflora associated with the maturation of certain styles of cheeses, such as the Camembert, Brie and Blue-veined varieties. They develop as natural contaminants of the process or they may be deliberately inoculated (*1,2*).

Cheeses contain significant concentrations of lactic acid, and lesser amounts of citric and acetic acids. Metabolism of these acids by yeasts is considered to be a key reaction in the maturation process (*3*). Their utilisation leads to a decrease in product acidity and an increase in pH which now permits the growth of both desirable and undesirable bacteria. Apart from being used as a property in the taxonomic identification of yeast species, (*4*), utilisation of organic acid by yeasts has received limited study. Most cheeses contain salt (NaCl), the concentration of which can be as high as 10–15 % in the aqueous phase, and this could impact on yeast utilisation of organic acids.

This paper reports the ability of 26 yeasts isolated from cheeses (*5*) to utilise lactic, citric and acetic acids in the presence of NaCl at concentrations up to 15 % (w/v). This analysis is followed by a more detailed kinetic study of the growth of *Debaryomyces hansenii* upon lactic and citric acids.

Materials and Methods

Yeast strains and culture

The yeast species examined in this study were isolated from Camembert and Blue-veined cheeses as described in Roostita and Fleet (*5*). Yeasts were grown in a defined basal medium of Yeast Nitrogen Base (Difco) containing either 2 % (w/v) of glucose, lactic acid, citric acid or acetic acid as the carbon substrate. To test the effect of salt concentration on growth, the medium was

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prepared to contain either 0, 5, 10 or 15 (w/v) of NaCl. The pH of the medium was adjusted to 5.5 and was sterilised by autoclaving at 121 °C for 15 minutes. Acetic acid was added to the medium after autoclaving and cooling to avoid loss due to volatility. Yeasts were grown as either agar plate cultures or as liquid cultures (10 mL) in test tubes rotated at 100 r.p.m. on a Roller Drum. Cultures were incubated at either 25 or 10 °C for up to 10 days. Growth was recorded as formation of visible biomass (colony) on the plates or as the development of turbidity in the liquid media.

In one series of experiments, *Debaryomyces hansenii* was grown as liquid cultures in 500 mL of medium contained in one litre conical flasks. The cultures were incubated at 25 °C for up to 10 days with orbital shaking (100 r.p.m.). At regular intervals, samples were removed for microbiological and chemical analyses. Samples (30 mL) were centrifuged at 8000 × g for 10 minutes to give a supernatant which was filtered through a 0.45 μm membrane and stored at –20 °C until chemical analysis.

Organic acids, sugars and ethanol

The concentrations of these components were determined by HPLC as described by Roostita and Fleet (*6*).

Polyols

Glycerol, arabitol and erythritol were determined within yeast cells (intracellular) and as extracellular products of yeast growth. Cultures (30 mL samples) were centrifuged at 8000 × g for 10 minutes at 5 °C to yield a pellet of yeast cells and the supernatant (stored at –20 °C). The pellet of cells was washed twice in 40 mL of 0.02 M phosphate buffer (pH = 6.5) containing 5 mM $MgCl_2$ and the appropriate concentration of NaCl (5, 10 and 15 % w/v) and resuspended in distilled water (about 20 mL) to a constant turbidity. The suspension was cooled to 4 °C, and homogenised with glass beads (diam. 0.45 mm) for 3 × 1 minute intervals in a Braun Homogeniser to disintegrate the cells. The homogenate was centrifuged at 8000 × g for 15 min at 4 °C and the supernatant was retained (frozen) for measuring the concentration of intracellular polyols (*7,8*).

The concentration of polyols was determined by HPLC. The instrumentation consisted of: a pump (Waters, model M-45), injector (Waters, model U6K) and a differential refractive index detector (Waters, model R401) fitted with a silica Radial Pak HP Cartridge 4 μm, (8 mm × 100 mm) in a compressor module (Waters, model RCM-100) or Z-module. The column was eluted at 2 mL/min with a mobile phase of acetonitrile-water (770:210 volume fraction) containing 0.1 % silica modifier, SAM Reagent 1 (Waters, SM1). The samples were filtered through a 0.45 μm membrane just before injection into the column. For samples containing salt, which interfered with the resolution of sugar peaks, a NH_2 Rad Pak cartridge (Waters) was used with a mobile phase of acetonitrile-water (800:200 volume fraction) containing 0.75 % volume fraction of silica modifier (PIC A).

The concentration of glycerol in samples was also determined with the enzymatic kits of Boehringer Mannheim.

All experiments were done as independent duplicates. Analyses within each experiment were done in duplicate and the mean values obtained. The data presented are from a typical experiment.

Results

Growth of yeast species on organic acids in the presence of sodium chloride

Tables 1 and 2 show the ability of various yeast species to grow on either lactic or citric acids as carbon sub-

Table 1. Growth of yeast species on citric acid as a carbon substrate in the presence of different concentrations of sodium chloride

Strain	Yeast	*w*(NaCl) / %							
		0		5		10		15	
		t / °C							
		25	10	25	10	25	10	25	10
001	*D. hansenii*	+++	++	+++	++	++	++	++	+
002	*D. hansenii*	+++	++	++	++	++	++	++	+
005	*D. hansenii*	+++	++	+++	+	++	+	+	+
008	*D. hansenii*	+++	+	+++	+	++	+	++	+
009	*D. hansenii*	+++	+	++	+	+	+	+	+
003	*C. famata*	+/–	–	–	–	–	–	–	–
004	*C. famata*	++	+	++	+	++	+/–	+	–
006	*C. famata*	+	+	+	+	+	+	+	+
007	*C. famata*	++	–	++	–	+	–	+	–
010	*C. famata*	++	+	++	+	++	+	+	+
014	*C. intermedia*	+	+	+/–	+/–	+/–	+/–	+/–	+/–
019	*C. intermedia*	++	+	++	+	+	+	+	+
020	*C. intermedia*	++	+	+	+	+	+	+/–	+
023	*C. intermedia*	+	+	+	+	+	+	+	+
029	*C. intermedia*	++	+	++	+	+	+	+	+
016	*C. tropicalis*	+	+	+/–	+	+/–	+/–	+/–	+/–
017	*C. tropicalis*	+	+	+/–	+	+/–	+	–	–
018	*C. tropicalis*	+	+	–	+	–	+	–	–
025	*C. catenulata*	++	+	+	+	+/–	+	–	–
030	*C. catenulata*	++	+	++	+	+/–	+/–	–	–
033	*C. catenulata*	++	+	+	+/–	–	+	–	–
032	*C. lipolytica*	+	+	+/–	+/–	–	–	–	–
040	*C. lipolytica*	+/–	+/–	–	–	–	–	–	–
011	*Cryp. albidus*	+	–	+/–	–	–	–	–	–
012	*Cryp. albidus*	+	–	+/–	–	–	–	–	–
013	*Cryp. albidus*	+	+/–	+/–	–	–	–	–	–
015	*Cryp. albidus*	+	+	+	+	+/–	+	+/–	+/–
027	*Kluy. marxianus*	+/–	–	–	–	–	–	–	–
028	*Kluy. marxianus*	+/–	–	+/–	–	–	–	–	–
024	*Sacch. cerevisiae*	–	–	–	–	–	–	–	–
026	*Sacch. cerevisiae*	–	–	–	–	–	–	–	–

Growth indicator: +++ Very strong; ++ Strong; + Growth; +/– Weak; – No growth

Data obtained from growth of yeasts on agar plates of the medium. All of the tests were done in duplicate.

strates in the presence of different concentrations of sodium chloride. Of all the species examined, *D. hansenii* exhibited the strongest growth on lactic and citric acids. This growth was best at 0–5 % sodium chloride and at 25 °C but, nevertheless, still very significant at 10 °C and the higher salt concentrations up to 15 %. None of the strains of *D. hansenii* showed growth on acetic acid. After *D. hansenii*, *C. famata* and *C. catenulata* showed the best growth on lactic and citric acids, although this growth was decreased at the higher concentrations (10–15 %) of salt and lower temperature, 10 °C. Of all the species tested, *C. catenulata* gave strong growth on acetic acid at NaCl concentrations up to 10 %. The other species gave very weak or no growth on acetic acid as substrate (data not shown).

The growth of *C. intermedia* was better on citric than on lactic acid. Some strains of this species gave better growth at 10 than at 25 °C on lactic acid as substrate. *Candida tropicalis*, *C. lipolytica*, *Cryp. albidus*, *Kluy. marxianus* exhibited either very weak to clear growth on lactic and citric acids, especially at 25 °C and at 0–5 % salt. *Saccharomyces cerevisiae* gave weak growth on lactic acid in the absence of salt, but did not grow on citric acid.

Similar growth responses were found when the yeasts were cultured in a liquid medium. Particularly noteworthy was confirmation of the growth of strains of *C. catenulata* on acetic acid as carbon substrate, and in the presence of NaCl up to 10 %.

Table 2. Growth of yeast species on lactic acid as a carbon substrate in the presence of different concentrations of sodium chloride

Strain	Yeast	NaCl / %							
		0		5		10		15	
		t / °C							
		25	10	25	10	25	10	25	10
001	*D. hansenii*	+++	+	+++	+	++	+	++	+
002	*D. hansenii*	+++	+	+++	+	++	+	++	–
005	*D. hansenii*	++	+	++	+	+	+	+	+
008	*D. hansenii*	+++	++	+++	++	++	++	++	+
009	*D. hansenii*	++	++	++	++	+	+	+	+
003	*C. famata*	+	+	+	+/–	+/–	+/–	–	–
004	*C. famata*	++	+	++	+/–	++	+/–	+	–
006	*C. famata*	+	+	+	+	+/–	+	–	+
007	*C. famata*	++	+	++	+/–	+	+/–	+	–
010	*C. famata*	++	+	++	+/–	+	+/–	+/–	+/–
014	*C. intermedia*	+/–	+	+/–	+	–	+	–	+/–
019	*C. intermedia*	+/–	+	+/–	+	–	+	–	+/–
020	*C. intermedia*	+	+	+	+	–	+	–	–
023	*C. intermedia*	+	+	+/–	+	+/–	+	–	+
029	*C. intermedia*	+	+	+	–	+	–	–	–
016	*C. tropicalis*	+	+	+	+	+	+/–	+/–	–
017	*C. tropicalis*	+	+	+	+	+	+/–	+/–	–
018	*C. tropicalis*	+	+	+	+	–	+	–	–
025	*C. catenulata*	++	+	+	+/–	+	–	+/–	–
030	*C. catenulata*	++	+	+	+	+	+/–	+/–	–
033	*C. catenulata*	++	+	+	+/–	+	+/–	–	–
032	*C. lipolytica*	+	+	+/–	+/–	–	–	–	–
040	*C. lipolytica*	+	+	+/–	–	+/–	–	+/–	–
011	*Cryp. albidus*	+	+	+	–	–	–	–	–
012	*Cryp. albidus*	+/–	+	+/–	–	–	–	–	–
013	*Cryp. albidus*	+	+	+	+	–	+/–	–	+/–
015	*Cryp. albidus*	+	+	+/–	+	–	+	–	+/–
027	*Kluy. marxianus*	+	+/–	+/–	+/–	+/–	–	+/–	–
028	*Kluy. marxianus*	+/–	+/–	+/–	+/–	+/–	–	–	–
024	*Sacch. cerevisiae*	+/–	–	–	–	–	–	–	–
026	*Sacch. cerevisiae*	+/–	–	–	–	–	–	–	–

Growth indicator: +++ Very strong +/– Weak
++ Strong – No growth
+ Growth

Data obtained from growth of yeasts on agar plates of the medium. All of the tests were done in duplicate.

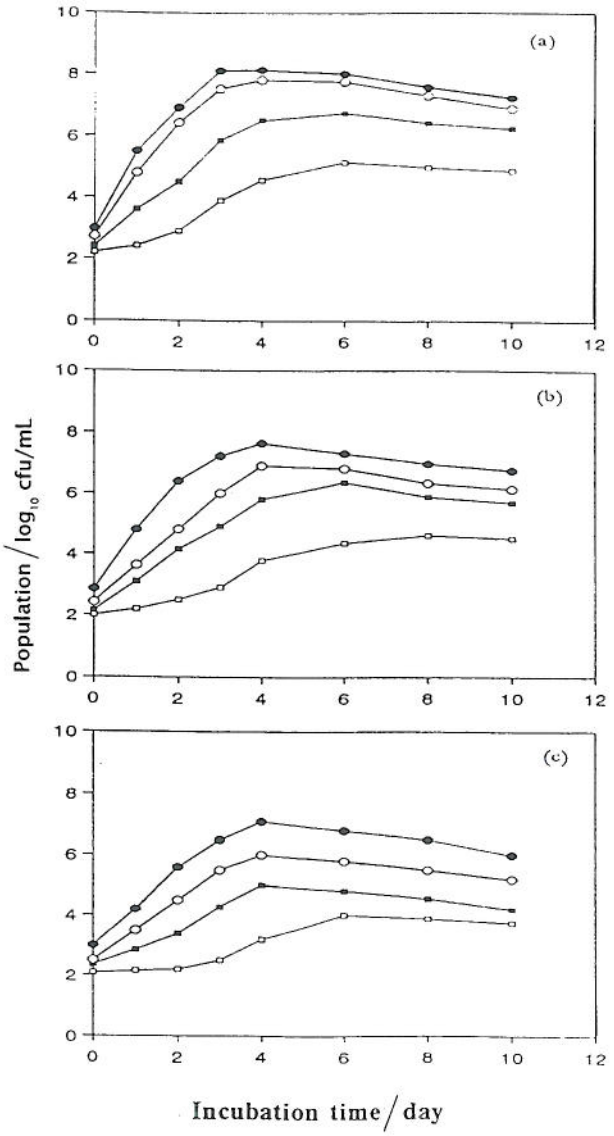

Fig. 1. The growth of *Debaryomyces hansenii* in media of different carbon substrates (a) glucose, (b) citric acid and (c) lactic acid, containing different concentrations of sodium chloride: 0 % ●, 5 % ○, 10 % ■ and 15 % □. Cultures were grown at 25 °C

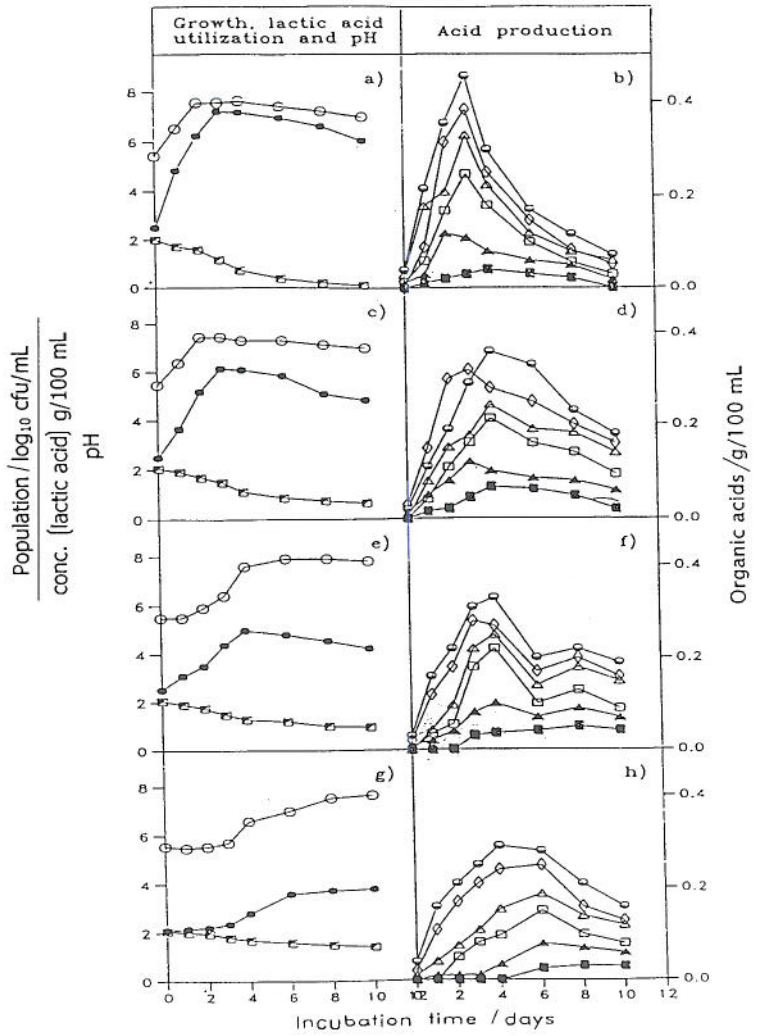

Fig. 2. Changes in the concentrations of organic acids and pH during the growth of *Debaryomyces hansenii* in lactic acid – YNB medium containing 0 % (a-b); 5 % (c-d); 10 % (e-f) and 15 % (g-h) of sodium chloride. Population ●; lactic acid ◪, pH=○. Organic acids: oxaloacetic ■; citric □; pyruvic ▲; succinic △; fumaric ◐; and acetic ◊.

Growth kinetics of Debaryomyces hansenii on organic acids

Debaryomyces hansenii was grown in 500 mL of YNB medium (pH = 5.5) containing either 2 % glucose, lactic acid or citric acid, as well as NaCl at either 0, 5, 10 or 15 %. The cultures were grown at 25 °C with orbital shaking (100 r.p.m.).

Fig. 1 compares the growth of *D. hansenii* on glucose, citric acid and lactic acid in the presence of 0, 5, 10 and 15 % NaCl. At 0 % NaCl, the growth rates were similar on all three substrates, but the maximum cell populations were 5–10 fold less for growth on either citric or lactic acids. Growth rates and maximum cell populations decreased with increasing concentration of NaCl, but the decrease was greater for growth on the organic acids, especially lactic acid.

Organic acid end-products were produced during the exponential phase of growth and, in most cases, re-utilised during the stationary phase. Fig. 2 shows the profile of changes that occurred for growth on lactic acid. Similar profiles were found for growth on glucose and citric acid. However, the concentrations of individual organic acid end-products varied with the growth substrate (Table 3). The predominant acid end-products for growth on glucose were fumaric and citric, but for growth on citric acid they were fumaric, oxaloacetic, lactic and pyruvic acids and for growth on lactic acids they were fumaric, acetic, succinic and citric acids. The concentration of NaCl did not affect these profiles although it decreased the concentrations of individual acids by decreasing growth. Growth on glucose was accompanied by a decrease in pH from 5.5 to 3.0, while growth on citric or lactic acids caused an increase in pH from 5.5 to about 8.0–8.5 by the end of the exponential phase.

Polyol production by Debaryomyces hansenii during growth on organic acids

The polyols, glycerol and arabitol were produced at both intracellular and extracellular locations in response to growth in the presence of NaCl for either glucose, lactic acid or citric acid substrates (Table 4). Generally, pro-

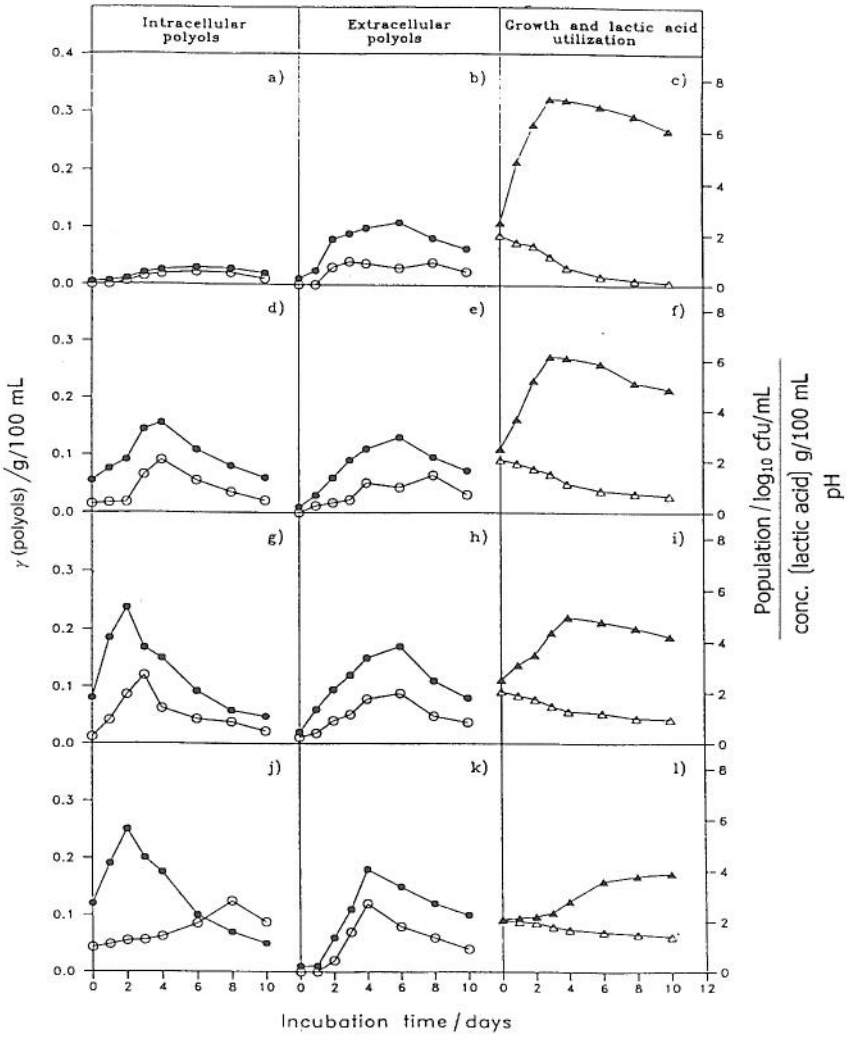

Fig. 3. Changes in the concentrations of intra- and extracellular polyols during the growth of *Debaryomyces hansenii* in lactic acid –YNB medium containing 0 % (a-c); 5 % (d-f); 10 % (g-i) and 15 % (j-l) of sodium chloride. Population ▲; lactic acid Δ, glycerol ●; and arabitol O; at 25 °C.

Table 3. Concentrations of organic acids produced by growth of *Debaryomyces hansenii* on substrates of either glucose, citric acid or lactic acid in the presence and absence of sodium chloride

γ (*acid*) / *g* / 100 mL	Growth on substrate					
	Glucose		Citric acid		Lactic acid	
	w(NaCl) / %					
	0	15	0	15	0	15
Fumaric	0.38	0.22	0.32	0.20	0.55	0.27
Succinic	–	–	–	–	0.35	0.18
Oxaloacetic	0.08	0.04	0.15	0.18	0.03	0.01
Pyruvic	0.02	0.06	0.14	0.10	0.12	0.08
Acetic	–	–	–	–	0.37	0.21
Lactic	–	–	0.25	0.11	–	–
Citric	0.19	0.21	–	–	0.24	0.15

Concentrations were measured in culture filtrates taken at the end of the exponential phase

Table 4. Concentrations of polyols produced by growth of *Debaryomyces hansenii* on substrates of either glucose, citric acid or lactic acid in the presence and absence of sodium chloride

Polyol concentration	Growth on substrate					
	Glucose		Citric acid		Lactic acid	
	w(NaCl) / %					
	0	15	0	15	0	15
Intracellular: mg/mL cell extract						
glycerol	0.05	0.15	0.01	0.14	0.01	0.10
arabitol	0.04	0.16	0.01	0.10	0.01	0.07
erythritol	0.02	0.05	–	–	–	–
Extracellular: mg/mL culture filtrate						
glycerol	0.03	0.25	0.06	0.16	0.1	0.20
arabitol	0.04	0.21	0.10	0.08	0.06	0.12
erythritol	–	0.06	–	–	–	–

Concentrations were measured in cells and culture filtrates taken at the end of the exponential phase

duction of the polyols occurred during exponential growth, and decreases in their concentration during the stationary phase suggested their re-metabolism (Fig. 3). Erythritol was also produced when glucose was the growth substrate.

Discussion

As reported by previous researchers (*3,9,10*), the metabolism of organic acids is a key mechanism by which yeasts contribute to the maturation of certain cheeses. Despite such conclusions, there is limited experimental evidence demonstrating that yeasts found in cheese actually utilise organic acids, especially lactic acid, as growth substrates. Our studies have demonstrated the capability of a range of yeast species isolated from cheese to utilise lactic acid and citric acid. Moreover, they show the potential for many of these species to grow on these acids in the presence of high concentrations of NaCl, and at the low temperature of 10 °C – conditions simulating the environmental stresses imposed by some cheeses during maturation and retailing.

Of the species examined, *D. hansenii* and its asporogenous counterpart, *C. famata*, exhibited the strongest growth on these acid substrates. Undoubtedly, these properties contribute to the dominance of these yeasts in cheeses such as the Camembert and Blue-veined types (*5*). *Candida catenulata* and to a lesser extent *C. lipolytica* also exhibited good ability to grow on lactic and citric acids in the presence of salt and this may account for their frequent isolation from cheeses (*5*). *Candida catenulata* was the only species that gave strong growth on acetic acid. Acetic acid does not occur in milk but would be expected at low concentration in cheeses, where it would arise from bacterial metabolism, either from the lactic acid bacteria associated with the fermentation of the milk, or the bacteria that develop in cheese as secondary flora (*11,12*). Generally, most yeast species are unable to grow on acetic acid, and are inhibited by its presence, especially at the lower pH. A few well known spoilage yeast species such as *Zygosaccharomyces bailii, Z. rouxii, Z. bisporus, C. krusei, Kluy. fragilis, Sacch. cerevisiae, C. tropicalis, C. versatilis.* and *Schizosaccharomyces pombe* are somewhat resistant to acetic acid (*13,14*) and *C. catenulata* can be added to this list. Its tolerance of and growth on acetic acid has not been reported previously.

Because of the significance of *D. hansenii* in the maturation process of cheeses, its kinetics of growth on organic substrates were examined in more detail in a defined liquid medium. These studies confirmed that this species was capable of growth to quantitatively high populations (10^6–10^8 cfu/mL) on either citric or lactic acids as carbon source, but the rate of growth and cell yields were decreased as the concentration of salt was increased. The growth kinetics of *D. hansenii* on glucose in the presence and absence of salt have been reported by Hobot and Jennings (*15*) and Burke and Jennings (*16*) and, essentially, show salt to increase the lag phase, decrease the growth rate and decrease final cell yield. However, the growth response of the yeast to salt concentration was influenced by the pH of the medium, with significant differences being observed for growth at pH = 5.0, 7.2 and 8.3. Medium pH was not controlled in the present study and, in this context, it is important to note that growth on the acids gave substantial increases in medium pH to produce an alkaline condition, whereas growth on glucose resulted in a decrease in pH to give an acidic environment. Such differences are likely to influence the growth response and metabolic activities of cells in the later stages of growth. The growth characteristics of *D. hansenii* on organic acids have not been reported in detail. Hobot and Jennings (*15*) noted the growth of this yeast on citric acid, while Deiana *et al.* (*17,18*) reported its growth on lactic acid with a concomitant increase in medium pH to about 8.0.

Organic acids were produced during the growth of *D. hansenii*, with the profile of acid production showing differences depending on the growth substrate. Fumaric acid was produced in the highest amounts on all substrates (glucose, citric acid and lactic acid) along with lesser quantities of oxaloacetic and pyruvic acids. Succinic and acetic acids were produced from the growth on lactic acid, but not on the other substrates. Deiana *et al.* (*18*) briefly noted the production of acetic acid by *D. hansenii* when grown on a mixed glucose-lactic acid substrate. They also noted its production of the flavour volatiles diacetyl and acetoin under these conditions. Lactic acid was produced from the growth of *D. hansenii* on citric acid but not from the growth on glucose or lactic acid. The production and excretion of individual acids into the medium was most apparent during the exponential stage of growth, whereafter, re-utilisation of the acids became evident, especially for growth on the organic acid substrates. The biochemistry of organic acid metabolism by yeasts has been reviewed by Whiting (*19*) and Radler (*20*). There appears to be no specific study on *D. hansenii* in this respect. Presumably, the acids are metabolised by the TCA cycle under aerobic conditions. Apart from cell biomass and CO_2, the primary and secondary end products of acid metabolism have not been reported. These end products could have sensory significance, which would be important in cheese maturation, given the dominant growth of *D. hansenii* during this process, and its potential to utilise lactic acid. Fundamental studies are needed to understand the biochemistry of organic acid utilisation and production by *D. hansenii* and other yeast species significant in cheese maturation.

The production of intracellular and extracellular polyols as osmoregulatory metabolites in response to growth in the presence of NaCl is well documented for *D. hansenii* (*21–24*). The major polyols produced by this species are glycerol and arabitol. Our findings agree with existing literature, although polyol production for growth on lactic acid and citric acid has not been reported previously. Essentially, there is an accumulation of intracellular glycerol during the exponential phase of growth, followed by a decrease as it is leaked into the extracellular environment. The intracellular and extracellular production of arabitol tends to occur more towards the later stages of growth (*7,8,22,24*), this being linked to altered medium pH and nutrient availability. Glycerol production was more significant than arabitol production for growth on citric and lactic acids. Nobre and Da Costa (*8*) reported higher glycerol production by *D. hansenii* when grown on acetate as a substrate. The

production of erythritol by *D. hansenii* has not been reported before, although its role as an osmoregulator in other species (e.g. *Pichia miso*, *C. polymorpha* and *C. famata*) has been noted (*25*).

Since cheeses present an environment of elevated salt concentration, yeast growth within or on them during maturation and retailing should be accompanied by polyol production. As these polyols have, characteristic flavour notes, their presence should impact on cheese sensory quality. There appears to be no information on the concentration of the different polyols in cheeses. Future studies should aim to obtain this information and correlate the presence and concentration of these components with yeast growth. It is possible that yeasts could have a novel impact on the quality of cheeses through their production of polyols.

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Rast kvasaca izoliranih iz sireva na organskim kiselinama u prisutnosti natrijeva klorida

Sažetak

Ispitana je mogućnost rasta dvadesetšest sojeva kvasaca, izoliranih iz sireva, na limunskoj, mliječnoj i octenoj kiselini u prisutnosti natrijeva klorida koncentracije do 15 %. Debaryomyces hansenii najbolje je rastao na limunskoj i mliječnoj kiselini u prisutnosti soli, a dobro su rasli i Candida famata, Candida catenulata i Candida intermedia. Signifikantno je slabiji rast na kiselinama opažen u bakterija Candida lipolytica, Candida tropicalis, Cryptococcus albidus i Kluyveromyces marxianus. Na octenoj kiselini rasla je samo C. catenulata. D. hansenii imao je slične brzine rasta na supstratima: glukozi, limunskoj i mliječnoj kiselini, ali je maksimalni broj stanica bio 5–10 puta slabiji pri rastu na kiselinama. Brzina rasta i broj stanica na tim supstratima smanjivala se u prisutnosti NaCl. Koncentracija organskih kiselina kao konačnih proizvoda u filtratu podloge mijenjala se s njezinim sastavom. Intracelularno i ekstracelularno dobiveni su glicerol i arabitol tijekom rasta bilo na glukozi, mliječnoj kiselini, bilo octenoj kiselini u prisutnosti NaCl.