RESEARCH ARTICLE

Alcohol-based quorum sensing plays a role in adhesion and sliding motility of the yeast *Debaryomyces hansenii*

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Abstract

The yeast *Debaryomyces hansenii* was investigated for its production of alcohol-based quorum sensing (QS) molecules including the aromatic alcohols phenylethanol, tyrosol, tryptophol and the aliphatic alcohol farnesol. *Debaryomyces hansenii* produced phenylethanol and tyrosol, which were primarily detected from the end of exponential phase indicating that they are potential QS molecules in *D. hansenii* as previously shown for other yeast species. Yields of phenylethanol and tyrosol produced by *D. hansenii* were, however, lower than those produced by *Candida albicans* and *Saccharomyces cerevisiae* and varied with growth conditions such as the availability of aromatic amino acids, ammonium sulphate, NaCl, pH and temperature. Tryptophol was only produced in the presence of tryptophane, whereas farnesol in general was not detectable. Especially, the type strain of *D. hansenii* (CBS767) had good adhesion and sliding motility abilities, which seemed to be related to a higher hydrophobicity of the cell surface of *D. hansenii* (CBS767) rather than the ability to form pseudomycelium. Addition of phenylethanol, tyrosol, tryptophol and farnesol was found to influence both adhesion and sliding motility of *D. hansenii*.

Introduction

The hemiascomycetes yeast *Debaryomyces hansenii* is a heterogeneous species characterized by its ability to grow under extreme conditions including low pH, low temperatures and high NaCl concentrations (Norkrans, 1966; Petersen & Jespersen, 2004; Gori *et al.*, 2005; Gunde-Cimerman *et al.*, 2009). *Debaryomyces hansenii* has been isolated from sea water and other salty environments correlating neatly with its high NaCl tolerance. *Debaryomyces hansenii* is of importance for the food industry, where it is used as a starter culture for the production of cheeses and meat products (Eliskases-Lechner & Ginzinger, 1995; Jessen, 1995). Additionally, it has been suggested for the production of alditols and lyctic enzymes (Breuer & Harms, 2006).

Many yeasts including *Candida albicans* and *Saccharomyces cerevisiae* are dimorphic, which means that they have the capacity to undergo transition from a unicellular to a filamentous form and vice versa (O'shea & Walsh, 1996; San-Blas *et al.*, 1997). However, to the best of our knowledge, only a single study has reported dimorphism of *D. hansenii* during continuous fermentation of acid hydrolysed barley bran (Cruz *et al.*, 2000). The transition from yeast-to-mycelium may be induced by environmental factors such as suboptimal temperatures, limited availability of oxygen, high osmolality, pH or substrate limitation including nitrogen starvation (Johnson & Mcdonald, 1983; Mcneil *et al.*, 1989; Sipiczki *et al.*, 1998).

Quorum sensing (QS) molecules are typically produced in a cell density-dependent manner; i.e., at high cell densities at the shift between exponential and stationary phase, and thus coordinating different behaviours at this stage of growth (Waters & Bassler, 2005). QS has been shown control the transition from yeast-to-mycelium. In *C. albicans*, the aromatic alcohol tyrosol has been found to be a QS molecule simulating pseudomycelium formation and thus to shorten lag phase time (Chen & Fink, 2006), whereas the aliphatic alcohol farnesol has been

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found to be a QS molecule inhibiting pseudomycelium formation (Hornby *et al.*, 2001). More recently, the aromatic alcohols phenylethanol and tryptophol were identified as QS molecules stimulating pseudohyphal growth in *S. cerevisiae* (Chen & Fink, 2006). In addition, phenylethanol was found to stimulate invasive growth, which was further promoted by addition of tryptophol. However, tryptophol alone did not have an effect on invasive growth. Alcohol-based QS is unknown in *D. hansenii*; however, this species has shown QS properties with ammonia as QS molecule involved in coordination of growth on agar plates (Gori *et al.*, 2007).

Adhesion and biofilm formation of microorganisms are well recognized in medical systems including dental plaque on tooth surfaces and implant-related infections in which adherent microbial populations are found on device surfaces (Potera, 1999; Donlan, 2001). Furthermore, adhesion and biofilm formation of yeasts to solid food matrices, for example, cheese surfaces, are a crucial prerequisite for their establishment and growth on the surface and contribution to the final product quality (Mortensen *et al.*, 2005). Adhesion properties of *C. albicans* and
S. cerevisiae have mainly been explained by cell wall glucoproteins (Verstrepen & Klis, 2006; Goossens & Willaert, 2010), whereas mechanisms involved in adhesion of *D. hansenii* are only partly known (Mortensen *et al.*, 2005). Biofilm formation has been shown to be tightly connected to sliding motility, i.e., mat formation defined as the ability to grow on low-solid surfaces has previously been related to biofilm formation (Reynolds & Fink, 2001).

The aim of the present study was to investigate whether *D. hansenii* produced any alcohol-based QS molecules and further in that case to investigate any QS effects on adhesion and sliding motility.

Materials and methods

Yeast strains, culture conditions, growth and preparation of supernatants

Three strains of *Debaryomyces hansenii* var. *hansenii* were used: CBS767 (Centraalbureau voor Schimmelcultures, CBS, Baarn and Delft, The Netherlands), D18335 (Petersen *et al.*, 2001, 2002) and MD02 (Arla Innovation, Brabrand, Denmark). As references, the type strains of *C. albicans* (CBS5878) and *S. cerevisiae* (CBS1171) were included (both from CBS). The cultures were maintained at −80 °C in yeast peptone dextrose (YPD) broth [per litre: 5 g yeast extract (Difco Laboratories, Detroit, MI), 10 g glucose (Merck, Darmstadt, Germany), 10 g Bacto peptone (Difco Laboratories), pH 5.6] containing 20% (v/v) glycerol (Merck). Yeast cultures were grown in yeast nitrogen base (YNB) without amino acids and ammonium sulphate (233510; BD, Franklin Lakes, NJ) [supplemented with 2% glucose (Merck) and 5 mM L-Proline (P5607; Sigma-Aldrich, St. Louis, MO), pH 4.3]. Yeast cultures were propagated in two steps. YNB (25 mL) was inoculated with freeze culture and incubated for 48 h at 25 °C with shaking at 120 r.p.m. Cells were counted, and 100 mL of YNB was inoculated with 1×10^6 cells mL^{-1} and incubated at the same conditions as in the first propagation step. At mid exponential phase, cultures were used to inoculate 100 mL of YNB (1×10^6 cells mL^{-1}), and growth was continued under the same conditions. For investigation of the influence of different environmental conditions, YNB and growth conditions were changed as specified. At appropriate time intervals, optical density was measured at 600 nm using a Helios Alpha UV-Vis spectrophotometer (Thermo Spectronic, Cambridge, UK) to establish growth phase. Concomitantly, samples were harvested and supernatants were prepared by centrifugation at 5000 ***g*** for 10 min and filtration through a 0.22 μm filter (DGS02025SO; Millipore, Billerica, MA). Supernatants were stored at −20 °C, before they were analysed for their content of phenylethanol, tyrosol, tryptophol and farnesol.

Preparation of phenylethanol, tyrosol, tryptophol and farnesol

Stock solutions (100 mM) of phenylethanol, tyrosol, tryptophol and farnesol were prepared in ethanol in the range 10–1000 μM.

High-performance liquid chromatography-coupled to MS/MS

All solvents were high-performance liquid chromatography (HPLC)-grade where other chemicals were of analytical-grade and from Sigma-Aldrich. Water was purified on a Milli-Q system (Millipore). Calibration standards of tyrosol, phenylethanol, tryptophol, farnesol and the two internal standards D_5-phenylethanol (hydrogens in the aromatic ring substituted with deuterium) and farnesal were because of the variations in the sample matrix, prepared in acetonitrile. The calibration standards were prepared to the final concentrations of 12.5, 6.25, 3.125, 0.781, 0.391 and 0.195 μg mL^{-1}. The internal standards D_5-phenylethanol and farnesal were added to all samples to a final concentration of 10 ng mL^{-1}.

Detection and quantification of the alcohols were performed using a HPLC-tandem mass spectrometer (HPLC-MS/MS) composed of a HP1100 liquid chromatograph system (Aligent Technologies, Palo Alto, CA) interfaced with a Micromass (Manchester, UK) Quattro Ultima mass

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spectrometer operated in the electrospray positive ionization mode. Subsamples of 3 μL were injected on a Kinetex PFP, 2.1 mm i.d. × 5 mm, 2.6 μm (Phenomenex, Torrance, CA) column held at 80 °C. Samples were eluted using a linear gradient going from 10% acetonitrile in water to 43% in 4 min at a flow rate of 350 μL min^{-1} and thereafter increased to 100% at 7 min at a flow rate of 600 μL min^{-1} and kept at that level until 9 min before reversion to original conditions in 12 min. Tandem mass spectrometry was operated in multiple reaction mode at the following transitions listed in Table 1.

Adhesion to polystyrene

Yeast cells were washed twice with YNB with 0.1% glucose. Yeast cells (100 μL) with a final OD_{600nm} between 0.03–1.0 in YNB with 0.1% glucose were inoculated into a 96-well polystyrene plate (flat bottom) (269787; Nunc, Roskilde, Denmark). Phenylethanol, tyrosol, tryptophol and farnesol were added in the range from 10 to 1000 μM, whereas supernatants taken out at 24, 48, 72, 96 and 120 h were added in a concentration of 80% (w/v). The treated cell cultures were incubated for 24 h at 25 °C. After incubation, nonadherent cells were removed by washing three times with 0.85% (w/v) NaCl. To measure adherence, 200 μL of 1% (w/v) crystal violet was added and left for 15 min. The wells were washed five times with 0.85% (w/v) NaCl, and bound crystal violet was eluted by the addition of 200 μL 96% (w/v) ethanol and determined by measurement at 590 nm.

Sliding motility

Mid exponential yeast cells were washed twice in 0.85% (w/v) NaCl, and 5 μL containing 10^6 cells mL^{-1} was inoculated in the centre of the YPD plates containing 0.3% and 2% agar. Phenylethanol, tyrosol, tryptophol and farnesol were added in the concentrations of 100, 500 and 1000 μM, whereas 120 h supernatants were added in a concentration of 80% (w/v). Plates were photographed after 14 days of incubation at 25 °C.

Pseudohyphal and invasive growth

To induce pseudohyphal growth, mid exponential yeast cells were restreaked on synthetic low-ammonium dextrose (SLAD) agar (1× YNB without amino acids and ammonium sulphate, 2% glucose and 50 μM ammonium sulphate). Phenylethanol, tyrosol, tryptophol and farnesol were added in the concentrations of 100, 500 and 1000 μM, whereas 120 h supernatants were added in a concentration of 80% (w/v). Plates were incubated at 25 °C for 7 days. Colony morphology was observed and photographed through a microscope. Invasive growth assay was performed on YPD and SD plates (1× YNB without amino acids, 2% glucose and 2% agar). Five microlitres containing 10^6 cells mL^{-1} was inoculated on plates and incubated for 5–7 days at 25 °C. Colony mass was carefully wash off with 0.85% (w/v) NaCl, whereafter plates were visually examined for invasive.

Aqueous-hydrocarbon biphasic hydrophobicity assay

A modification of the method of Rosenberg *et al.* (1980) was used. Briefly, mid exponential yeast cells were washed twice in 0.85% (w/v) NaCl and suspended in PUM buffer [L^{-1}: 22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ (pH 7.1)] to yield an OD_{400nm} of 0.40. Onto 2.4 mL of cell suspension, 0.60 mL of octane was overlaid. The phases were vigorously mixed for 3 min using a vortexer (VF2; IKA, Staufen, Germany) and then allowed to separate. The per cent change in OD_{400nm} was considered the hydrophobicity value of the yeast population.

Results

Detection of alcohol-based QS molecules by HPLC-coupled to MS/MS

A HPLC-MS/MS method was developed for the quantification of the aromatic alcohols phenylethanol, tyrosol, tryptophol and the aliphatic alcohol farnesol. Calibration

Table 1. Mass spectrometer operation conditions and transitions for the metabolites of choice

Name	Formula	Retention time (min)	Quantifying ion	Collision energy (eV)	Qualifying ion	Collision energy (eV)	Cone voltage (V)
Tyrosol	$C_8H_{10}O_2$	1.54	77	25	51	45	29
Phenylethanol	$C_8H_{10}O$	4.30	77	60	51	30	29
Tryptophol	$C_{10}H_{11}NO$	4.20	115	45	91	40	35
Farnesol	$C_{15}H_{26}O$	4.54	121	30	93	20	29
Farnesal (IS)	$C_{15}H_{24}O$	7.00	147	20	133	20	25
Phenylethanol (ring-D5) (IS)	C_8H_{10}	7.07	80	30	79	30	25

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curves were linear for the concentrations: phenylethanol 6.38–51.2 μM, tyrosol 2.75–90.5 μM, tryptophol 2.36–77.5 μM and farnesol 3.51–56.2 μM (results not shown). For all values above, concentrations have been extrapolated from the standard curves. The lower limit of quantification (LOQ) (average relative standard deviation better than 20%) was determined to be at least 6.39, 2.75 and 4.84 for phenylethanol, tyrosol and tryptophol, respectively. Limit of detection was at least 3.3, 0.72 and 1.2 μM, respectively. Owing to a slight carryover of farnesol in the autosampler or precolumn, we quantified it only in samples, where the peak area exceeded 3× the maximum areas observed in the blank samples.

Production of alcohol-based QS molecules

Debaryomyces hansenii (CBS767, D18335 and MD02) produced phenylethanol and tyrosol at standard conditions (Fig. 1). Phenylethanol and tyrosol production were observed at the shift from exponential to stationary phase. Phenylethanol concentrations at 120 h of fermentation were between 65–78 μg g^{-1} dry wt (13–21 μM), whereas tyrosol concentrations at 120 h of fermentation were between LOQ-24 μg g^{-1} dry wt (LOQ-6.1 μM). For comparison, the concentrations of phenylethanol and tyrosol at 120 h of fermentation for *C. albicans* (CBS8758) were 3342 μg g^{-1} dry wt (475 μM) and 3342 μg g^{-1} dry wt (120 μM), respectively, and for *S. cerevisiae* (CBS1171), 394 μg g^{-1} dry wt (46 μM) and 200 μg g^{-1} dry wt (21 μM), respectively (Fig. 1).

Contrary to *D. hansenii* (CBS767, D18335 and MD02) and *S. cerevisiae* (CBS1171), *C. albicans* (CBS8758) produced tryptophol at standard conditions resulting in 964 μg g^{-1} dry wt (11 μM) tryptophol at 120 h of fermentation (results not shown). However, addition of tryptophane (the precursor for tryptophol) resulted in tryptophol production by *D. hansenii* (CBS767, D18335 and MD02) (Fig. 2). The tryptophol concentration increased to a maximum [6025–14 294 μg g^{-1} dry wt (11–27 μM)] during exponential phase (24–48 h) and subsequently decreased during stationary phase to 432–3167 μg g^{-1} dry wt (2–11 μM) at 120 h of fermentation.

To test whether cell density controls the production of aromatic alcohols in *D. hansenii*, production of a freshly inoculated low-density culture (5×10^5 cells mL^{-1}) and high-density culture (5×10^7 cells mL^{-1}) in YNB with the amino acids phenylalanine, tyrosine and tryptophan was analysed for its content of aromatic alcohol-based QS molecules. Supernatants from the high-density cultures contained a higher concentration of aromatic alcohol-based QS molecules than supernatants from the low-density cultures, although the aromatic alcohol concentrations of low-density cultures were below LOQ (results not shown). This indicates that cells at high

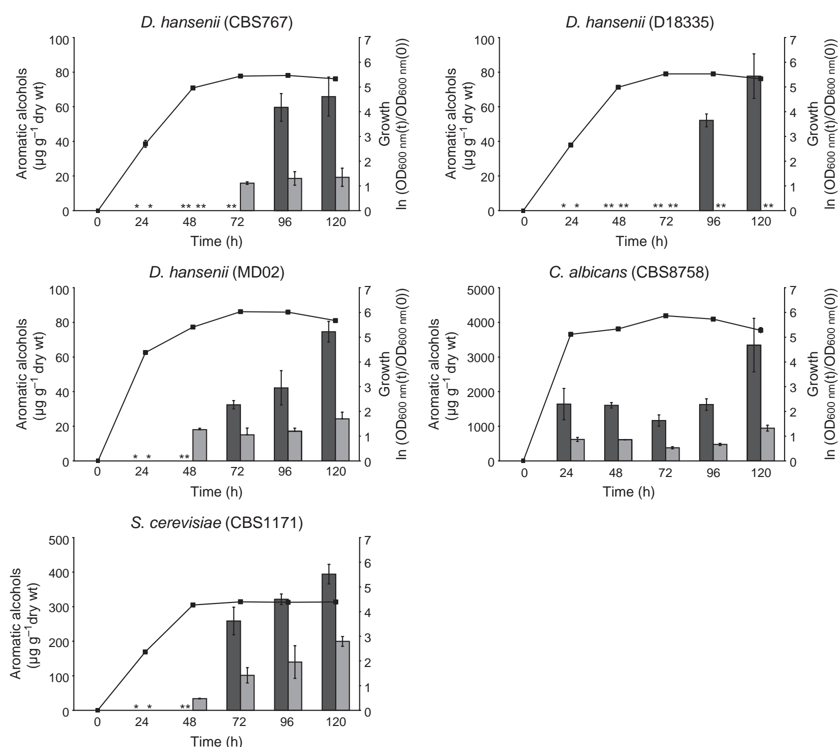

Fig. 1. Phenylethanol and tyrosol production in μg g^{-1} dry wt for *Debaryomyces hansenii* (CBS767, D18335 and MD02), *Saccharomyces cerevisiae* (CBS1171) and *Candida albicans* (CBS8758) grown in YNB without amino acids and ammonium sulphate supplemented with 2% glucose and 5 mM L-proline, pH 4.3. (■) Phenylethanol, (□) Tyrosol. Growth curves are included to illustrate that phenylethanol and tyrosol were produced in a growth-dependent manner. (■) Growth [ln ($OD_{600nm}(t)$/$OD_{600nm}(0)$)], *t* refers to the time. Results are the average of three independent determinations (the error bars indicate standard deviations). *Not detected, **below LOQ.

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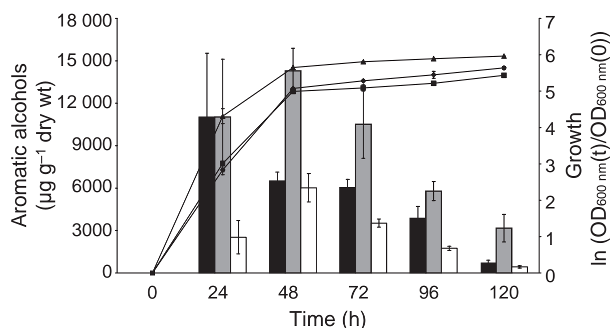

Fig. 2. Tryptophol production in μg g^{-1} dry wt for *Debaryomyces hansenii* (CBS767, D18335 and MD02) grown in YNB without amino acids and ammonium sulphate supplemented with 2% glucose and 50 mg mL^{-1} tryptophan, pH 4.3. (■) CBS767, (▒) D18335 and (□) MD02. Growth curves are included to illustrate that tryptophol was produced in a growth-dependent manner. Growth [ln ($OD_{600nm}(t)$/$OD_{600nm}(0)$)], *t* refers to the time. (■) CBS767, (♦) D18335 and (▲) MD02. Results are the average of two independent determinations (the error bars indicate standard deviations).

density produce more of the aromatic alcohols per cell than cells at low density.

Phenylethanol and tyrosol production at different environmental conditions

For all three strains of *D. hansenii*, different environmental conditions were found to influence the production of alcohol-based QS molecules phenylethanol and tyrosol (Table 2). Supplementation of the aromatic amino acid phenylalanine (the precursor for phenylethanol) increased phenylethanol production between 12–26-fold, whereas supplementation of the aromatic amino acid tyrosine (the precursor for tyrosol production) increased tyrosol production between 16–79-fold. Furthermore, supplementation of tryptophan (the precursor of tryptophol production) slightly increased phenylethanol production up to threefold and tyrosol production up to fivefold in *D. hansenii*. Ammonium sulphate (50–37 000 mM) decreased phenylethanol and tyrosol production. All three *D. hansenii* strains (CBS767, D18335 and MD02) seemed to produce the highest yield of the two aromatic alcohols at pH 6.0. Phenylethanol was not detected for pH values higher than 7.0, whereas tyrosol was not detected at pH values higher than 8.0. Increasing NaCl concentrations decreased phenylethanol and tyrosol production in *D. hansenii* (CBS767 and MD02). In contrast, *D. hansenii* (D18335) produced the highest yield of the two aromatic alcohols in the presence of 2% (w/v) NaCl with an increase in phenylethanol and tyrosol production of 1.9- and 6.2-fold, respectively, compared to 0% (w/v) NaCl. Changing the temperature from 25 to 15 °C did not significantly influence the production of phenylethanol and tyrosol. In *D. hansenii* (MD02), tyrosol and tryptophol seemed to have an autostimulatory effect on phenylethanol production as addition of these two aromatic alcohols increased phenylethanol 2.0- and 2.3-fold, respectively. In a similar fashion, phenylethanol and tryptophol seemed to have an autostimulatory effect on tyrosol production as addition of these two aromatic alcohols increased tyrosol production 1.9- and 2.0-fold. No autostimulatory effects were determined for *D. hansenii* (CBS767 and D18335).

Adhesion to polystyrene

As shown in Fig. 3a, *D. hansenii* (CBS767) was found to posses a particular high ability to adhere to polystyrene. Adhesion of *D. hansenii* (D18335) was only observed when inoculated at a high cell titre, whereas *D. hansenii* (MD02) rarely adhered (Fig. 3a). As shown in Fig. 3b, addition of phenylethanol and tyrosol ($\geq$ 100 μM) slightly increased the adhesion of *D. hansenii* (CBS767), whereas addition of tryptophol ($\geq$ 500 μM) decreased the adhesion of *D. hansenii* (CBS767). Farnesol was shown to decrease the adhesion of *D. hansenii* (CBS767) at concentrations as low as 10 μM (Fig. 3b). For concentrations above 500 μM, adhesion was not even observed for *D. hansenii* (CBS767) (Fig. 3b). As shown in Fig. 3c, contrary to supernatants taken out at 24 h of fermentation (exponential phase), those of *D. hansenii* (CBS767) and *S. cerevisiae* (CBS1171) taken out between 48–120 h of fermentation (stationary phase) decreased the adhesion of *D. hansenii* (CBS767). Supernatants taken out between 24–120 h (stationary phase) of *C. albicans* (CBS8758) decreased the adhesion of *D. hansenii* (CBS767).

Mat formation assay

When inoculated in the centre of 0.3% agar plates, the three strains of *D. hansenii* (CBS767, D18335 and MD02) produced round colonies covering a larger surface area than those of the same strains inoculated on 2% agar plates (Fig. 4). Especially, the type strain of *D. hansenii* (CBS767) was found to possess a particular high sliding capacity as colony areas at 0.3% agar compared to 2% agar were 23- and 73-fold larger at day 3 and 5, respectively. At day 6, *D. hansenii* (CBS767) had fully overgrown the 0.3% agar plate. Addition of phenylethanol, tyrosol and tryptophol (1000 μM) changed the growth pattern of *D. hansenii* (CBS767) to a more filamentous form. The filamentous form was also observed for aromatic alcohols concentrations below 1000 μM; however, results were not consistent. Addition of aromatic alcohols to *D. hansenii* (D18335) resulted only in minor filamentation, whereas no change in the growth pattern

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Table 2. Effects of environmental conditions on production of phenylethanol and tyrosol in *Debaryomyces hansenii* (CBS767, D18335 and MD02)

Added compound/changed environmental conditions	Changes in alcohol production					
	Phenylethanol			Tyrosol		
	CBS767	D18335	MD02	CBS767	D18335	MD02
Aromatic amino acid (50 μg mL^{-1})						
Phenylalanine	26 ± 3*	13 ± 0.6	12 ± 1.4	< LOQ	< LOQ	3.0 ± 0.7*
Tyrosine	< LOQ	1.3 ± 0.02	1.8 ± 0.6	79 ± 16†	16 ± 2*	16 ± 2*
Tryptophan	2.9 ± 0.9*	2.3 ± 0.1	3.0 ± 0.3	< LOQ	2.3 ± 0.04*	4.8 ± 0.6*
Ammonium sulphate						
50μM	0.66 ± 0.2	0.54 ± 0.07	0.81 ± 0.05	nd	nd	nd
100μM	0.75 ± 0.5	0.41 ± 0.3	0.56 ± 0.01	nd	nd	nd
500μM	0.77 ± 0.0003	0.47 ± 0.3	0.60 ± 0.01	< LOQ	< LOQ	< LOQ
1000μM	0.87 ± 0.2	0.73 ± 0.03	0.78 ± 0.05	< LOQ	< LOQ	< LOQ
5000μM	0.64 ± 0.2	0.73 ± 0.02	0.77 ± 0.2	< LOQ	< LOQ	< LOQ
37000μM	0.64 ± 0.04	0.64 ± 0.3	0.97 ± 0.2	< LOQ	< LOQ	0.46 ± 0.6
pH						
5.0	< LOQ	0.32 ± 0.06*	1.1 ± 0.05	< LOQ	< LOQ	0.94 ± 0.2
6.0	< LOQ	0.38 ± 0.008*	1.4 ± 0.03	< LOQ	< LOQ	1.8 ± 0.2
7.0	nd	nd	nd	< LOQ	< LOQ	< LOQ
8.0	nd	nd	nd	nd	nd	nd
9.0	nd	nd	nd	nd	nd	nd
NaCl						
2% (w/v)	0.82 ± 0.1	1.9 ± 0.4	1.1 ± 0.09	0.61 ± 0.05	6.4 ± 2	0.82 ± 0.2
4% (w/v)	0.92 ± 0.2	< LOQ	0.71 ± 0.2	0.65 ± 0.2	< LOQ	< LOQ
6% (w/v)	< LOQ	nd	0.55 ± 0.07	< LOQ	nd	< LOQ
8% (w/v)	< LOQ	nd	< LOQ	< LOQ	nd	< LOQ
Temperature						
15°C	1.1 ± 0.1	0.71 ± 0.09	0.66 ± 0.02	< LOQ	< LOQ	< LOQ
Aromatic alcohols (1000 μM)						
Phenylethanol	–	–	–	0.31 ± 0.02*	0.32 ± 0.01*	1.9 ± 0.2
Tyrosol	< LOQ	0.95 ± 0.1	2.0 ± 0.2	–	–	–
Tryptophol	1.2 ± 0.04	< LOQ	2.3 ± 0.3	0.42 ± 0.04	< LOQ	2.0 ± 0.3

Changes in the production of alcohol-based QS molecules phenylethanol and tyrosol are based on concentrations determined after 120 h of fermentation in YNB without amino acids and ammonium sulphate supplemented with 2% glucose and 5 mM L-proline, pH 4.3.
< LOQ, below LOQ.
*Based on half the value of LOQ.
†Based on half the value of limit of detection (LOD).

of *D. hansenii* (MD02) was observed when the aromatic alcohols were added. Addition of farnesol inhibited the sliding motility of all three strains of *D. hansenii*. Furthermore, farnesol induced the production of cable-like structures in *D. hansenii* (CBS767). Addition of 120 h supernatant of *D. hansenii* (CBS767) slightly stimulated the filamentous form in *D. hansenii* (CBS767) (Fig. 5). Addition of 120 h supernatant of *S. cerevisiae* (CBS1171), however, did not stimulate the filamentous form, whereas addition of 120 h supernatant of *C. albicans* (CBS8758) heavily stimulated the filamentous form.

Pseudohyphal and invasive growth

Contrary to *D. hansenii* (CBS767 and D18335), *D. hansenii* (MD02) showed pseudohyphal growth on SLAD agar (Fig. 6). Neither of the investigated aromatic alcohols was found to stimulate pseudohyphal growth (results not shown). None of the investigated *D. hansenii* strains showed significant invasive growth on either YPD or SD (results not shown).

Hydrophobicity

The hydrophobicity assay displayed large differences in cell surface hydrophobicity of the three strains of *D. hansenii*. The type strain of *D. hansenii*, CBS767, and the dairy isolate D18335 were found to be very hydrophobic as they showed affinities of 92 ± 3% and 80 ± 7% to octane. Contrarily, *D. hansenii* MD02 was very hydrophilic displaying an affinity of only 31 ± 7% to octane (results not shown).

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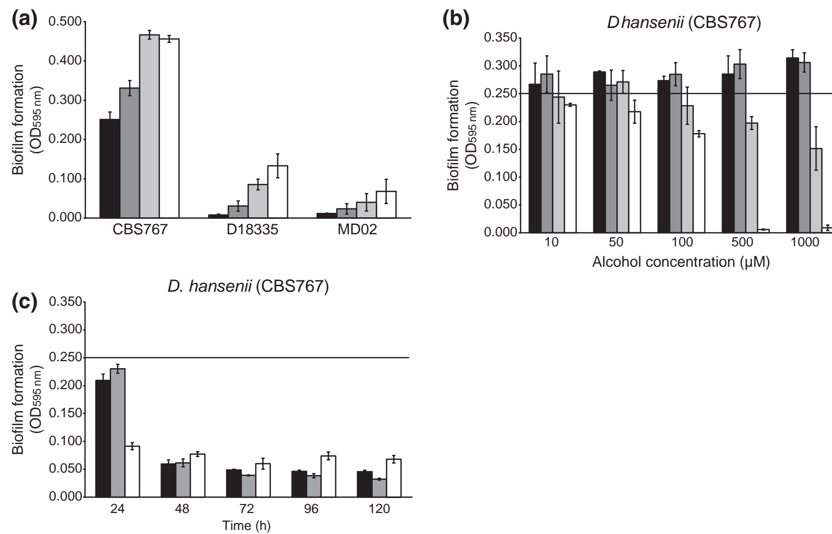

Fig. 3. Adhesion to polystyrene by *Debaryomyces hansenii* (CBS767, D18335 and MD02). (a) Influence of the initial OD_{600nm}. (■) OD_{600nm} 0.03, (■) OD_{600nm} 0.1, (□) OD_{600nm} 0.5, (□) OD_{600nm} 1.0. (b) Influence of pure alcohols on adhesion to polystyrene by *D. hansenii* (CBS767). (■) Phenylethanol, (■) tyrosol, (□) tryptophol, (□) farnesol. Initial OD_{600nm} 0.03. (c) Influence of supernatants on adhesion to polystyrene by *D. hansenii* (CBS767); (■) 80% (w/v) supernatant from *D. hansenii* (CBS767) taken out at 24, 48, 72, 96 and 120 h of fermentation; (□) 80% (w/v) supernatant from *Saccharomyces cerevisiae* (CBS1171) taken out at 24, 48, 72, 96 and 120 h of fermentation and (□) 80% (w/v) supernatant from *Candida albicans* (CBS8758) taken out at 24, 48, 72, 96 and 120 h of fermentation. Initial OD_{600nm} 0.03. The line in (b) and (c) indicates the biofilm formation of *D. hansenii* (CBS767) inoculated with an initial OD_{600nm} 0.03 in YNB without amino acids and ammonium sulphate supplemented with 2% glucose. All results were obtained after 24 h of incubation at 25 °C and given as the average of two independent determinations (the error bars indicate standard deviations).

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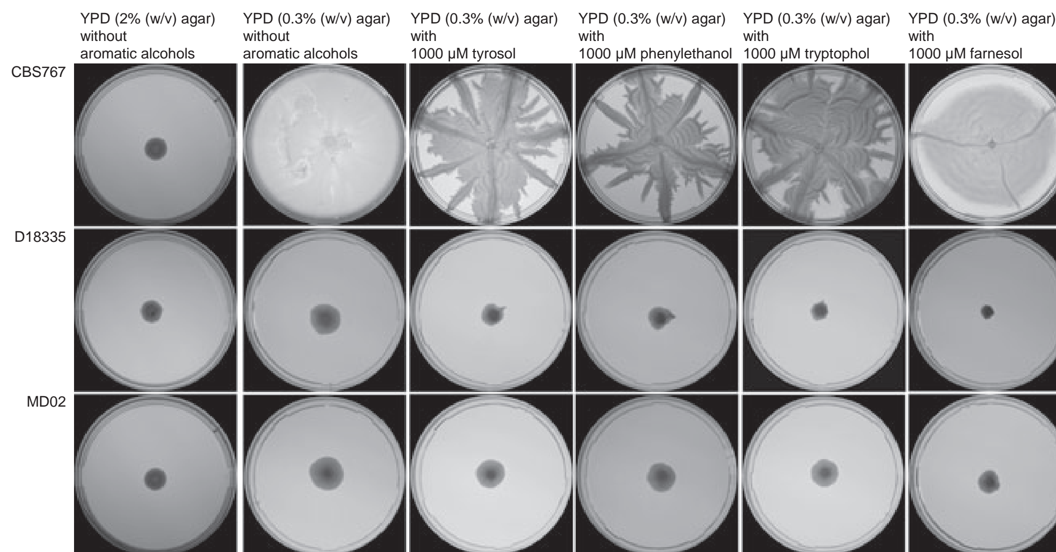

Fig. 4. Mat formation of *Debaryomyces hansenii* (CBS767, D18335 and MD02) on YPD added 2.0% and 0.3% (w/v) agar, respectively. Furthermore, mat formation on low-solid YPD (0.3% (w/v) agar) added phenylethanol, tyrosol, tryptophol and farnesol (1000 μM) are shown. Plates were photographed after 14 days of incubation.

Discussion

Debaryomyces hansenii was shown to produce the aromatic alcohols phenylethanol and tyrosol in similar concentrations as previous studies have found for *C. albicans* and *S. cerevisiae* (Chen *et al.*, 2004; Chen & Fink, 2006). However, in the present study, phenylethanol and tyrosol concentrations detected for *D. hansenii* were lower than

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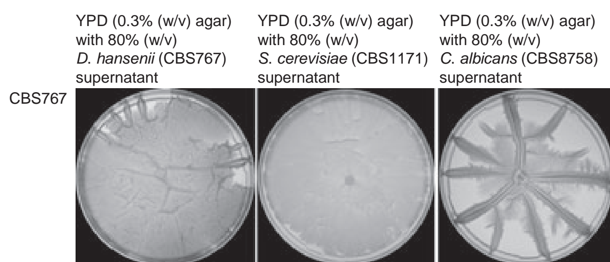

Fig. 5. Mat formation of *Debaryomyces hansenii* (CBS767, D18335 and MD02) on low-solid YPD agar added 80% (v/v) supernatants of *D. hansenii* (CBS767), *Saccharomyces cerevisiae* (CBS1171) and *Candida albicans* (CBS8758). Plates were photographed after 14 days of incubation.

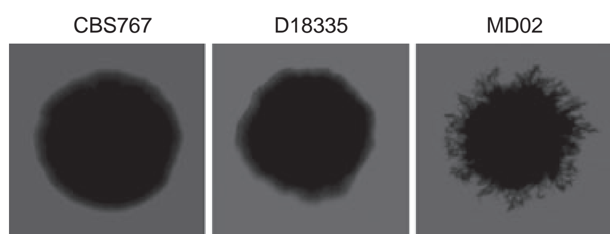

Fig. 6. Filamentation of *Debaryomyces hansenii* (CBS767, D18335 and MD02) on SLAD agar. Colonies were photographed after 14 days of incubation.

those detected for the included reference strains of *C. albicans* and *S. cerevisiae*. Nevertheless, aromatic alcohol production of *D. hansenii* was highly influenced by environmental conditions as previously shown for other yeast species (Chen & Fink, 2006; Ghosh *et al.*, 2008). Consistent with previous studies (Ghosh *et al.*, 2008), addition of the aromatic amino acids phenylalanine, tyrosine and tryptophan, precursors for phenylethanol, tyrosol and tryptophol, respectively, increased aromatic alcohol production most significantly. In addition, ammonium suppression was confirmed. However, contrary to *C. albicans*, which has been shown to produce the highest yields of aromatic QS molecules at alkaline pH values (Ghosh *et al.*, 2008), *D. hansenii* showed no aromatic alcohol production at pH values higher than 6.0.

Production of aromatic alcohols was primarily determined from the end of exponential phase indicating a connection to cell density. Furthermore, high-cell-density cultures seemed to produce higher concentrations of aromatic alcohol-based QS molecules compared to low-cell-density cultures. These results suggest that aromatic alcohols are potential QS molecules in *D. hansenii* as previously found for *C. albicans* and *S. cerevisiae* (Chen *et al.*, 2004; Chen & Fink, 2006).

Strains of *D. hansenii* were found to adhere to polystyrene, which suggests that *D. hansenii* can initiate biofilm formation. However, significant strain variation in adhesion to polystyrene was observed. Biofilm formation has been shown to be controlled by alcohol-based QS in *C. albicans* (Ramage *et al.*, 2002; Alem *et al.*, 2006). In the present study, adhesion to polystyrene of *D. hansenii* (CBS767) and thus its ability to form biofilms was found to be influenced by the reported aromatic alcohols. Phenylethanol and tyrosol slightly stimulated adhesion to polystyrene, whereas tryptophol and farnesol inhibited adhesion. This indicates that potential biofilm formation of *D. hansenii* is controlled by the presence of these molecules. Furthermore, adhesion to polystyrene of *D. hansenii* (CBS767) was inhibited by supernatant from itself as well as by supernatants from *S. cerevisiae* (CBS1171) and *C. albicans* (CBS8758).

Sliding motility, i.e., mat formation defined as the ability to grow on low-solid surfaces has previously been related to biofilm formation (Reynolds & Fink, 2001). The close relationship between biofilm formation and sliding motility was confirmed in the present study as the *D. hansenii* strain with the highest ability to adhere to polystyrene also had the highest ability with respect to sliding motility. Similar to adhesion to polystyrene, sliding motility of *D. hansenii* was also found to be influenced by the previously reported alcohol-based QS molecules. The growth pattern of *D. hansenii* (CBS767) changed to a more filamentous form by the addition of aromatic alcohols, whereas farnesol both inhibited the colony area as well as induced the production of cable-like structures. Furthermore, the filamentous form of *D. hansenii* (CBS767) was slightly observed by the addition of supernatant from itself, whereas supernatant from *C. albicans* (CBS8758) stimulated the filamentous form similarly to the pure alcohols. Supernatant from *S. cerevisiae* (CBS1171) did not stimulate the filamentation of *D. hansenii* (CBS767). Adhesion to polystyrene and sliding motility of *D. hansenii* (CBS767) was influenced by supernatants from itself as well as supernatants from *S. cerevisiae* (CBS1171) and *C. albicans* (CBS8758). However, in general, the necessary concentration of pure alcohols needed for influencing adhesion to polystyrene and sliding motility was generally higher than those found in supernatants. This indicates that other molecules than the suggested might be present in the supernatants and thus influencing these properties.

Saccharomyces cerevisiae biofilms require the cell surface adhesions, e.g., FLO11 as well as FLO8, a yeast gene that encodes a regulatory protein required for *FLO11* expression (Reynolds & Fink, 2001; Zara *et al.*, 2005). With respect to the expression of *FLO* genes in the recently sequenced *D. hansenii*, no orthologues to these genes seem to be present. The *FLO* genes have been reported to be important for both biofilm formation and sliding motility in *S. cerevisiae*, because they increase cell wall

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hydrophobicity (Van Mulders *et al.*, 2009). When we measured the cell wall hydrophobicity of strains by their ability to partition between water and octane, we found that the strain with highest ability for biofilm and mat formation had the most hydrophobic cell wall.

Another ability related to biofilm formation and sliding motility is the ability to form pseudomycelium (Chen & Fink, 2006). Dimorphism defined as the ability to switch between a unicellular yeast form and a filamentous form because of environmental cues is a well-known phenomenon among yeasts. In addition to nitrogen starvation (Gimeno *et al.*, 1992; Biswas & Morschhauser, 2005), pseudomycelium formation of *C. albicans* and *S. cerevisiae* has been shown to be dependent on QS molecules (Hornby *et al.*, 2001; Chen *et al.*, 2004; Chen & Fink, 2006). Only a single study has reported pseudomycelium formation of *D. hansenii* when grown in continuous fermentation with xylose as carbon source and limited O_2 levels (Cruz *et al.*, 2000). In the present study, pseudohyphal development was observed for the dairy isolate *D. hansenii* MD02 when grown on SLAD agar, whereas *D. hansenii* (CBS767 and D18335) did not show pseudohyphal growth. However, addition of alcohol-based QS molecules did not seem to influence pseudohyphal development.

In conclusion, *D. hansenii* was found to produce previously reported alcohol-based QS molecules with phenylethanol and tyrosol. Although *D. hansenii* does not produce the same high levels of these molecules compared to yeast species as *C. albicans* and *S. cerevisiae*, addition of the compounds in pure form was found to regulate several traits involved in adhesion and growth of *D. hansenii*. By understanding the production of QS molecules and their importance for adhesion and biofilm formation, it will be possible to apply and better control the growth yeast starter cultures to foods, where adhesion and biofilm formation are important such as for solid food fermentation. The results are also of importance for preventing the biofilm formation of yeast on medical devices and other polystyrene materials.

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