Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Assessment of quorum sensing effects of tyrosol on fermentative performance by chief ethnic fermentative yeasts from northeast India

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Keywords
ethanol fermentation, morphogenesis, quorum sensing, tyrosol, yeast.

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2020/1521: received 21 July 2020, revised 28 September 2020 and accepted 21 October 2020

doi:10.1111/jam.14908

Abstract

Aim: Tyrosol, a quorum sensing molecule in yeasts, was reported to reduce lag phase and induces hyphae formation during cell proliferation. However, evidence of any enhancing effect of tyrosol in cellular proliferation within fermentative environment is unclear. In this investigation, selected yeast cells were assessed for their ability to synthesize tyrosol followed by examining the role of the molecule during fermentation.

Methods and Results: Tyrosols were characterized in four fermentative yeasts viz., *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Candida glabrata* and *Candida tropicalis* isolated from traditional fermentative cakes of northeast India. All the isolates synthesized tyrosol while *C. tropicalis* exhibited filamentous growth in response to tyrosols retrieved from other isolates. Purified tyrosols showed protective behaviour in *C. tropicalis* and *S. cerevisiae* under ethanol mediated oxidative stress. During fermentation, tyrosol significantly enhanced growth of *W. anomalus* in starch medium while *C. tropicalis* exhibited growth enhancement in starch and glucose sources. The chief fermentative yeast *S. cerevisiae* showed notable enhancement in fermentative capacity in starch medium under the influence of tyrosol con-commitment of ethanol production.

Conclusion: The study concludes that tyrosol exerts unusual effect in cellular growth and fermentative ability of both *Saccharomyces* and non-*Saccharomyces* yeasts.

Significance and Impact of the Study: This is the first report of expression of tyrosol by non-conventional yeasts, where the molecule was found to exert enhancing effect during fermentation, thereby augmenting the process of metabolite production during traditional fermentation.

Introduction

Quorum sensing (QS) is a population density-dependent phenomenon and a complex process of cell-to-cell communication, where participating individual organisms detect, analyse and respond to the concentration of certain quorum sensing molecules (QSMs), resulting in alteration of phenotypic traits (Wuster and Babu 2008). These autoinducing hormone-like molecules accumulate above a particular threshold level when the density of microbial cell population is at its highest (Sprague and Winans 2006) which, in turn, activates or repress certain genes leading to expression of various complex cellular traits (Fuqua *et al.* 1994). A diverse range of QSMs are reported to be expressed by bacterial population during bioluminescence, horizontal DNA transfer, biofilm formation, expression of pathogenic factors, production of antibiotics and other secondary metabolites (Whitehead *et al.* 2001), while in eukaryotes the mechanism is primarily involved in morphogenic transition (Hornby *et al.* 2001; Chen *et al.* 2004; Chen and Fink 2006; Gori *et al.* 2011). The eukaryotic mechanism of QS is well known in *Candida albicans*,

(Sprague and Winans 2006) and among all the identified QSMs, tyrosol had been observed to stimulate the maximum conversion of yeast to filamentous form (Chen *et al.* 2004). Tyrosol is abundantly found in natural sources like olive and argan oil and is known to harbour antioxidant property. The molecule affects biofilm formation (Alem *et al.* 2006) and induces oxidative stress responses in *C. albicans* (Cremer *et al.* 1999). The evidence of QS activity of molecules like tyrosol in both conventional and non-conventional yeasts during fermentation has not attained much clarity, although the molecule is widely known to be expressed by yeasts.

Avbelj *et al.* (2016) had reviewed the effect of QS molecules in yeasts in relation to cell density and emphasized on the probable role of such molecules during wine fermentation. While intraspecies and interspecies interaction is common between yeasts and other microbes coexisting in wine ecosystem (Fleet 2003), such interactions among large microbial communities and their resulting physiological expression are believed to affect the final quality of wine produced (Ciani *et al.* 2016). The role of ethanol and other metabolites produced by some yeast species acts as potent inhibitors of other yeasts, resulting in promotion of cell death (Pérez-Nevado *et al.* 2006). Previously, death of non-*Saccharomyces* yeasts during early stages of fermentation was reported to be due to direct physical interaction (Nissen *et al.* 2003; Nissen and Arneborg 2003; Renault *et al.* 2013), but recent reports state that such death is triggered only upon attaining a threshold cell density during culture indicating that growing cells perceive and respond to arrest of growth in dense population (Nissen and Arneborg 2003; Pérez-Nevado *et al.* 2006). Although this mechanism of growth arrest is believed to be a QS phenomenon, yet, confirmatory evidence of the involvement of QS molecules in cell-to-cell interaction is still unclear (Avbelj *et al.* 2016). Application of tyrosol in various biotechnological techniques has already been reported which include evaluation of wine quality (González-Marco *et al.* 2010) and enhancement of aroma in foods and drinks (Etschmann *et al.* 2003; Wang *et al.* 2011). *Saccharomyces cerevisiae* has been found to employ defensive mechanisms and secretion of antimicrobial peptides against microbes which impart significant impact on wine profile during alcoholic fermentation (Albergaria and Arneborg 2016). In addition, QS mechanism also controls the growth of food spoiling microbes (Avbelj *et al.* 2016). It is reported that K2 and Klus type yeast killer toxins regulate proliferation of spoilage yeasts (Comitini *et al.* 2004; Rodríguez-Cousiño *et al.* 2011). Furthermore, QS mechanism regulates the expression of food spoiling enzymes such as cellulase, lipases, chitinase, nucleases, pectate lyase and various proteases (Skandamis and Nychas 2012).

The tradition of preparing indigenous fermented food is widespread among native communities dwelling in different parts of the world. Spontaneous starter culture aided solid state fermentation involving yeasts and other micro-organisms play a steering role in the preparation of traditional fermented foods and beverages which constitute an essential part of the diet in various communities around the globe (Bhalla and Savitri 2017). These longstanding practices involve cultivation of microbes from the local environment and has been perpetuated for hundreds of years through generations indoctrinating indigenous knowledge system (Tsuyoshi *et al.* 2005). A wide variety of yeast species in unison or in cooperation are known to involve during fermentation, especially alcoholic beverages. From northeast India, Parasar *et al.* (2017) revealed the coexistence of *Wickerhamomyces anomalus*, *Candida glabrata*, *S. cerevisiae* and *Candida tropicalis* in starter cultures of Rabha, Karbi, Kachari and Ahom communities. The occurrence of these isolates questions the probable role of QS activity and their direct or indirect association during traditional fermentation.

It has been observed that very scanty literatures are available that reflects the effect of tyrosol and tyrosol-like molecules on alcoholic fermentation. Besides, the characterization of yeasts inherent in traditional fermentation systems from India has also not received much attention. With our previous experiences on isolating both conventional and non-conventional yeasts from traditional starter cultures of northeast India, we attempted to assess the ability of selected yeasts pertaining to both *Saccharomyces* and non-*Saccharomyces* groups to synthesize tyrosol as the primary QS molecule in culture, characterized them and screened their effects on morphogenesis of the isolates. Furthermore, we evaluated the effect of retrieved tyrosols from each of the yeasts on one another and thereby elucidated the effect of the molecule on fermentation process. The probable stimulatory effect was also screened to monitor the role of the molecule in enhancement of fermentation *in vitro*.

Materials and methods

Yeast isolates

For the investigation, four yeast isolates that were observed to be dominant in traditional starter cultures maintained by four major indigenous communities from various locations of Assam (Nath *et al.* 2020) were selected. These included *W. anomalus* GUBT1R (GenBank accession no. MH012188.1), *C. tropicalis* GUBT28A (GenBank accession no. MH012189.1), *S. cerevisiae* GUBT42K (GenBank accession no. MH012190.1) and *C. glabrata* GUBT43C (GenBank accession no. MH012191.1).

Extraction of tyrosol from cell-free supernatant

Prior to extraction of tyrosol, tested isolates were grown in SD medium which is a synthetic minimal liquid medium (pH 4·3) as mentioned by Chen *et al.* (2004). The cultures were incubated for 24 ± 2 h and absorbance was noted at 600 nm in a UV-VIS spectrophotometer (Eppendorf, Hamburg, Germany). The OD_{600} of all isolates were adjusted to ~1·0 with fresh SD medium. The cells were then harvested by centrifugation at 12 000 ***g*** for 5 min at 4°C, washed twice with distilled water, resuspended in 50 ml of freshly prepared SD medium and were incubated at 30°C for 24 ± 2 h in a rotary shaker maintained at 120 rev min^{-1}.

For tyrosol extraction, cell-free supernatants of cultured yeasts were obtained through centrifugation at 6500 ***g*** for 15 min at 4°C and was filter sterilized through vacuum filtration using Whatman 0·45-mm PVDF membrane filters. Extraction was done by both solid phase and liquid–liquid extraction processes (Hornby *et al.* 2001; Alem *et al.* 2006).

Liquid–liquid extraction

Liquid–liquid extraction was done according to the method described by Hornby *et al.* (2001) with minute modifications. To extract tyrosols from the spent medium, 25 ml ethyl acetate was added to 100 ml of cell-free supernatant in a 250 ml separating funnel, agitated gently for 5 min and the mixture was allowed to stand for 4 h in the funnel for separating phases of liquids. After phase separation, the upper organic layer was collected and the ethyl acetate was allowed to evaporate under reduced pressure in a rotary evaporator set at 40°C. After evaporation, the residue was dissolved in 1 ml of absolute methanol and was stored at −20°C. The samples were later subjected to gas-chromatography mass-spectrometer (GC-MS) analysis.

Solid phase extraction

Solid phase extraction (SPE) of tyrosol was done according to Alem *et al.* (2006) with slight modifications. Prior to extraction, 100 ml of cell-free supernatant collected as above from each isolate was acidified by adding 0·4 ml of 0·1 mol l^{-1} H_2SO_4 (prepared by adding 0·272 ml of 98% H_2SO_4 in 50 ml of distilled water). For SPE, Sep-Pak C18 Plus short cartridge (360 mg sorbent per cartridge, 55–105 µm particle size, WAT020515; Waters, Milford, CT) was used. The cartridge was first conditioned with 10 ml of methanol (high-performance liquid chromatography (HPLC) grade; Merck, Darmstadt, Germany), followed by another wash with 10 ml of 1 mmol l^{-1} H_2SO_4 (prepared by mixing 27 µl 98% H_2SO_4 in 500 ml of distilled water). The acidified supernatants were then loaded onto the cartridge. The probable tyrosol molecules from the cartridge were eluted with 10 ml of 7·5% acetonitrile in 1 mmol l^{-1} H_2SO_4 and stored at 4°C. The SPE extracts were later subjected to HPLC analysis.

Characterization of tyrosols by gas-chromatography mass-spectrometer

The probable tyrosol molecules in liquid–liquid extracts were detected and characterized by GC-MS following the methodology of Ghosh *et al.* (2008). Chromatography was performed in a Perkin Elmer Clarus 680 GC-MS instrument coupled with autosampler. Separation of samples was done in Perkin Elmer Elite-35ms capillary column (60 m × 0·25 mm) with a film thickness of 0·25 µm. 2 µl of samples was injected by autosampler and the flow rate of helium (carrier gas) was maintained at 1 ml min^{-1} The inlet temperature was set at 250°C while the oven temperature was programmed as follows: initial temperature of 60°C for 2 min followed by first temperature ramp of 5°C min^{-1} until the temperature reached 160°C. The temperature was then held constant at 160°C for 2 min. The second temperature ramp comprised of an increase in 5°C min^{-1} until 300°C was achieved and the temperature was held constant at 300°C for 5 min. Total run time consisted of 60 min. The MS used a 6-min solvent delay. TurboMass NIST 2008 was used for detection and characterization of splitted molecules. The obtained mass spectrum of detected molecules was compared with MS library of NIST (National Institute of Standard and Technology) and the identity of the molecules was confirmed.

High-performance liquid chromatography analysis

The SPE eluents were subjected to both analytical and preparative HPLC to quantify the amount of produced tyrosols and obtain their purified fractions.

Quantification of tyrosol by analytical HPLC

To quantify the amount of tyrosols present in the SPE extracts, analytical HPLC was performed following the methodology of Alem *et al.* (2006) with minor modifications. HPLC was carried out at room temperature using an Agilent Zorbax Eclipse Plus C-18 analytical column (4·6 × 250 mm, 5 µm particles size). The mobile phase consisted of three phase compositions with a total runtime of 30 min. The initial 10 min of the mobile phase comprised of acetonitrile–distilled water in the ratio of 10 : 90% v/v. The mobile phase for washing the column consisted of acetonitrile and distilled water in the ratio 50 : 50% v/v. Finally, mobile phase for reconditioning comprised of acetonitrile

and distilled water in the ratio 10 : 90% v/v. The flow rate of the mobile phase was set at 1·5 ml min^{-1}. 10 µl of the samples were injected onto the machine and photodiode array was set to record data at 215 nm. OpenLab EZChrome software was used to interpret the HPLC chromatograms. Calibration curve of tyrosol was prepared by calibrating the instrument with 10, 20, 30, 50 and 100 µmol l^{-1} of standard tyrosol (Tyr^{st}) following similar procedure.

Purification of tyrosol by preparative HPLC

To purify and retrieve tyrosols from SPE columns, the extracts were subjected to preparative HPLC following the methodology of Chen *et al.* (2004). For the separation, a reverse-phase C18 HPLC column (Agilent ZORBAX SB-C18, semi-preparative 9·4 × 50 mm, 5 µm) was used. The mobile phase comprised of a gradient of 9–15% acetonitrile used for a total runtime of 60 min maintained at a flow rate of 2 ml min^{-1}. 800 µl of each sample was injected individually and purified fractions were collected in vials for lyophilization in a vacuum concentrator (Concentrator Plus; Eppendorf). The resultant samples were then dissolved in 0·1% acetonitrile and 50 µmol l^{-1} of each was stored separately for morphogenetic and ethanol tolerance study.

Effect of tyrosol on morphogenesis

Activity of purified tyrosols on morphogenesis of yeast isolates were tested on 12 well plates. First, each isolate was inoculated onto 250 ml YPD broth and incubated for 24 ± 2 h in a rotary shaking incubator set at 120 rev min^{-1}. After incubation, 1 ml of cell cultures (2×10^6 CFU per ml) was transferred to the designated wells and were incubated at 30°C for 2 h so as to allow the cells to adhere to the surface of the wells (Kuhn *et al.* 2002). The non-adherent cells were removed by rinsing the wells twice with distilled water. This was followed by incubation in SD broth (pH 7·0) (Chen *et al.* 2004) supplemented with purified tyrosol retrieved from isolates as well as commercial tyrosol (Fluka; Sigma-Aldrich, St. Louis, MO, product code 9058-500MG-F) at equal concentrations (50 µmol l^{-1}). For comparison, a blank (without tyrosol) inoculation was also prepared. The plates were incubated for 48 ± 2 h at 30°C. After incubation, morphogenesis was observed under phase contrast microscope (Leica DM750) both in suspension and under adhered conditions.

Assessment on ethanol tolerance of yeasts aided by tyrosol

The protective effect of tyrosol over exogenous ethanol was tested by incubating the isolates in the presence of 12% ethanol supplemented with tyrosol retrieved from each isolate. For this, pre-cultures were prepared by growing the isolates in YPD broth for 24 ± 2 h at 30°C in a rotatory shaker set at a speed of 120 rev min^{-1}. After incubation, cells were pelleted at 12 000 ***g***, washed twice with distilled water and resuspended in fresh YPD medium by adjusting the OD_{600} to ~1·0. 1 ml of adjusted cell culture was centrifuged again at 12 000 ***g*** for 5 min, the supernatant was discarded and the pellet was reconstituted in 12% ethanol supplemented with 50 µmol l^{-1} retrieved tyrosols and allowed to incubate further for 10, 30 and 60 min, respectively. After each time interval, 100 µl of cells was withdrawn from the treatments, diluted 100 times and 20 µl of the diluted cells was placed onto YPD agar plates to determine the viable colony forming units. The plates were incubated for 24 ± 2 h at 30°C for the colonies to attain observable growth.

Assessing the enhancement of growth and ethanol fermentation in the presence of tyrosol

To examine the enhancing effect of tyrosol on cell growth and ethanol fermentation, the cells were grown in dual carbon sources viz starch and glucose, with or without tyrosol.

Growth medium

Prior to the experiment, pre-cultured cells were grown in YPD broth for 24 ± 2 h at 30°C in a rotary shaking incubator. After incubation, 1 ml of cell culture was pelleted at 12 000 ***g*** and reconstituted in liquid SD medium (Chen *et al.* 2004) by adjusting the OD_{600} to ~1·0 for each test isolate. The isolates were then grown in two sets of 10 ml SD medium with one set supplemented with 2% soluble starch while the other set was supplemented with 2% glucose. Isolates were incubated for 72 ± 2 h at 30 ± 2°C in a shaker maintained at 120 rev min^{-1}. After incubation, OD_{600} of each culture was recorded, cells were centrifuged at 6500 ***g*** for 15 min at 4°C and cell-free supernatant was analysed for ethanol and residual carbon contents.

Total cell number, ethanol content, residual carbon and fermentation efficiency

Total cell number was counted in a haemocytometer (Neubaur, Hatfield, PA, UK). The ethanol content (in grams) was measured following the methodology described by Seo *et al.* (2009). Briefly, 1 ml of cell-free supernatant from each culture was added to 1 ml of Tri-n-butyl phosphate (Himedia, Beleghata, Kolkata, India) in a 2 ml Eppendorf tube and vortexed for 15 min. After phase separation, 750 µl of the upper solvent phase was

transferred to a new tube to which 750 µl of dichromate reagent was added (5 g potassium dichromate dissolved in 100 ml of 5 mol l^{-1} sulphuric acid) and vortexed for another 15 min. 200 µl of dichromate reagent from the phase separated lower phase was transferred to a new tube and absorbance was measured at 595 nm. The amount of residual starch was determined in cell-free supernatant following the methodology of Vähäsalo and Holmbom (2004) with minimal adjustments. To achieve this, 5 ml of cell-free supernatant from each culture was diluted with 5 ml of distilled water to which 1 ml of 0·1 mol l^{-1} iodine solution was added (0·645 g iodine added with 2 g of potassium iodide in 50 ml distilled water). The solution was allowed to rest for 1 min at room temperature followed by measuring the absorbance at 580 nm. Residual glucose was measured by dinitrosalicylic (DNS) acid method (Zhang *et al.* 2009). For this, 1 ml of the cell-free supernatant from each culture was made up to 3 ml with distilled water to which 3 ml of DNS reagent (1g of DNS acid, 200 mg of crystalline phenol and 50 mg of sodium sulphite dissolved in 100 ml of 1% NaOH solution) was added and the mixture was heated for 5 min in a boiling water bath. After the development of colour, 1 ml of 40% Rochelle salt solution (40 g sodium potassium tartrate in 100 ml distilled water) was added (in warm contents) and mixed thoroughly. Absorbance was recorded at 540 nm after cooling the constituents. Standard curves were prepared for starch, glucose and ethanol following similar protocols. Ethanol yield, 'Y' was calculated from ratio of grams of ethanol produced per gram of sugar utilized and the percentage fermentation efficiency was calculated on the basis of the relationship between the sugar consumed and alcohol produced following the fermentation stoichiometry, where 1 g of total reducing sugar was estimated to produce 0·461 g ethyl alcohol (Okunowo *et al.* 2005). Finally, ethanol fermentation capacity was calculated from the ratio of grams of ethanol produced per ml divided by total number of cells per ml of culture.

Statistical analysis

Data were analysed in GraphPad Prism ver. 7.04 software and reported as means ± SEM of triplicate measurements for all the experiments. Two-way ANOVA was carried out and Tukey's multiple range test (*post hoc*) was performed to analyse the growth of isolates with or without tyrosol. Utilization of carbon source and corresponding ethanol fermentation in the presence and absence of external tyrosol was also analysed through Tukey's multiple range test. Significance of the data interpreted was determined at three levels ($^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}001$ and $^{***}P < 0{\cdot}0001$).

Results

Detection and characterization of tyrosol by GC-MS

The mass spectral analysis from GC confirmed the presence of tyrosol in all the selected isolates. The peaks were analysed in TurboMASS software and the peaks displaying 100% similarity hit were recorded in TIC (total ion chromatogram). The retention peaks corresponding to tyrosol mass spectrum were found to be at 33·22 min (0·102 % peak area) for *W. anomalus* GUBT1R, 34·28 min (0·843% peak area) for *C. tropicalis* GUBT28A, 34·10 min (0·231% peak area) for *S. cerevisiae* GUBT42K and 34·14 min (0·867% peak area) for *C. glabrata* GUBT43C isolate. All the peaks showed similarity to the peak obtained for commercial tyrosol. Details of the peaks are shown in Fig. 1a–d.

Quantification of tyrosol by HPLC in solid phase extracts of isolates

The amount of tyrosol in µmol l^{-1} per 100 ml of cell-free supernatant was quantified using analytical HPLC. The amount of tyrosol determined are listed in Table 1. The chromatograms obtained are displayed in Fig. 2a–e. The HPLC chromatograms exhibited the presence of peaks corresponding to that of standard tyrosol. Each of the SPE purified extract showed peak at 7·5 min retention time similar to that of the standard. This confirmed the presence of tyrosol in the SPE purified extract.

Assessing morphogenesis under the influence of tyrosol

Assessment of morphogenesis showed that out of the five sets (20 combinations) of tyrosol-treated yeast, six treatments resulted in elongated growth or morphogenesis (Table 2, Fig. 3). For comparison purpose, one set of isolates was inoculated without the addition of external tyrosol and was considered as the standard. The pH of the cultures was maintained at 7 as per earlier reports studied on hyphae formation (Chen *et al.* 2004). *Candida tropicalis* GUBT28A exhibited morphogenic changes in response to its self-induced tyrosol (Tyr^{Ct}) and tyrosols purified from *S. cerevisiae* GUBT42K (Tyr^{Sc}), *W. anomalus* GUBT1R (Tyr^{Wa}) as well as standard tyrosol. However, it did not respond to tyrosol from *C. glabrata* GUBT43C (Tyr^{Cg}). Similarly, *W. anomalus* GUBT1R showed changes in cellular appearance in response to Tyr^{Ct} from *C. tropicalis* and standard tyrosol (Tyr^{St}). On the reverse, *S. cerevisiae* GUBT42K and *C. glabrata* GUBT43C did not undergo any morphogenic transition when treated with the purified tyrosols including the self-expressed ones.

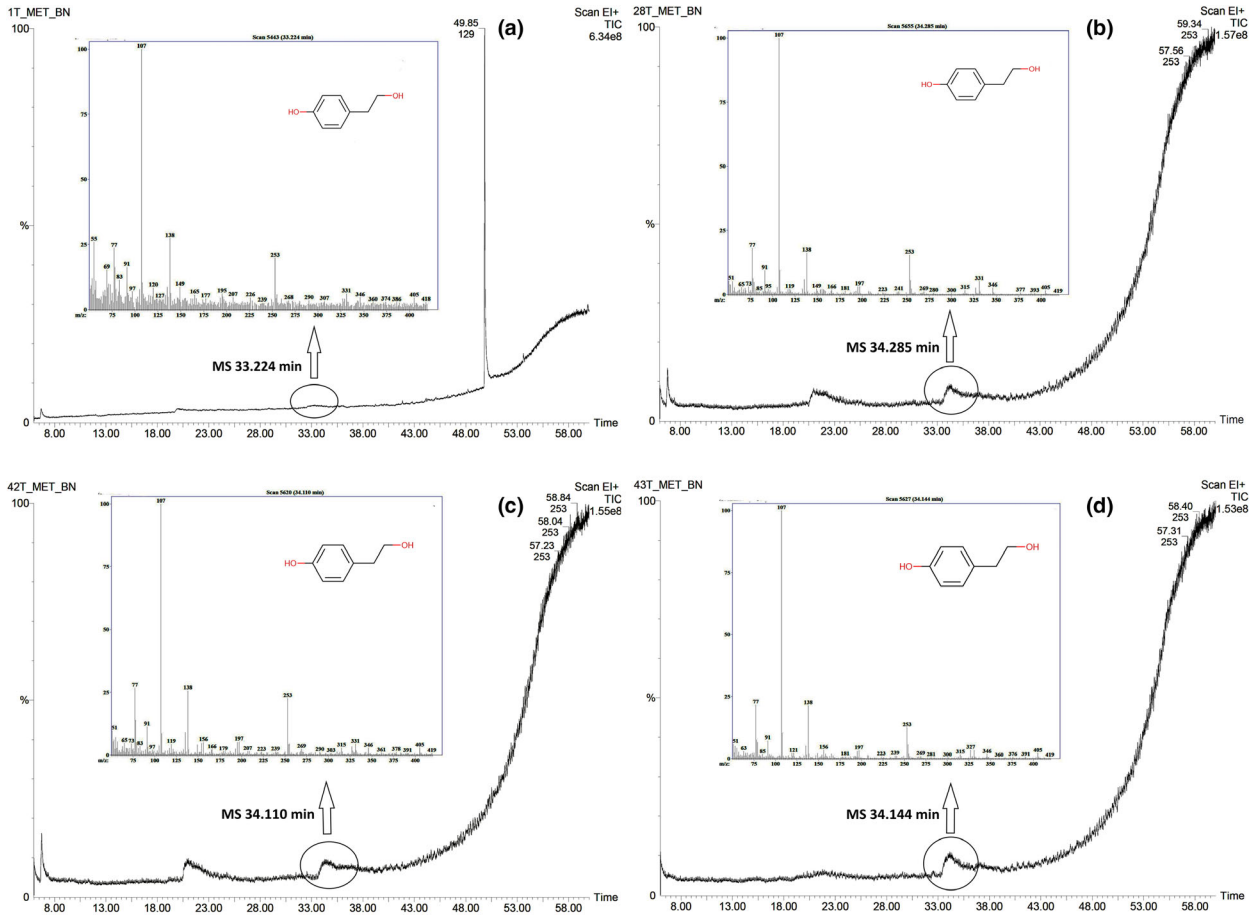

Figure 1 Mass spectrum fingerprint analysis showing *m/z* fragmentation of peaks from GC Total ion chromatogram (TIC) of isolates (a) *Wickerhamomyces anomalus* GUBT1R, (b) *Candida tropicalis* GUBT28A (c) *Saccharomyces cerevisiae* GUBT42K, (d) *Candida glabrata* GUBT43C.

Table 1 Table showing amount of quantified tyrosol in SD medium without amino acids of SPE extracts for each isolate

SPE source isolate	SPE eluent	μmol l^{-1} tyrosol per 100 ml of cell culture	Name of purified tyrosol
Wickerhamomyces anomalus GUBT1R	7·5% acetonitrile	198·51 ± 0·71$^{\Delta}$	Tyr^{Wa}
Candida tropicalis GUBT28A	7·5% acetonitrile	617·23 ± 1·49$^{\Delta}$	Tyr^{Ct}
Saccharomyces cerevisiae GUBT42K	7·5% acetonitrile	614·88 ± 1·37$^{\Delta}$	Tyr^{Sc}
Candida glabrata GUBT43C	7·5% acetonitrile	264·25 ± 0·84$^{\Delta}$	Tyr^{Cg}

Δ = standard error calculated from triplicate readings.

Examining the probable protective role of tyrosols during ethanol stress

The probable role of extracted tyrosols as protective agents of yeasts during 12% ethanol stress and their subsequent effect on basic fermentation parameters were examined. The drop count in YPD agar plates is depicted in Fig. 4. It was observed that isolates *C. tropicalis* GUBT28A and *S. cerevisiae* GUBT42K demonstrated appreciable growth under the influence of all tested tyrosols including self-expressed tyrosol. Although the growth was less compared to control condition (without any ethanol stress), yet these two isolates survived 12% ethanol concentration under tyrosol influence for approximately 60 min (Fig. 4), which is an unusual behaviour compared to our earlier experiment where these isolates could not survive even 10% ethanol stress without any

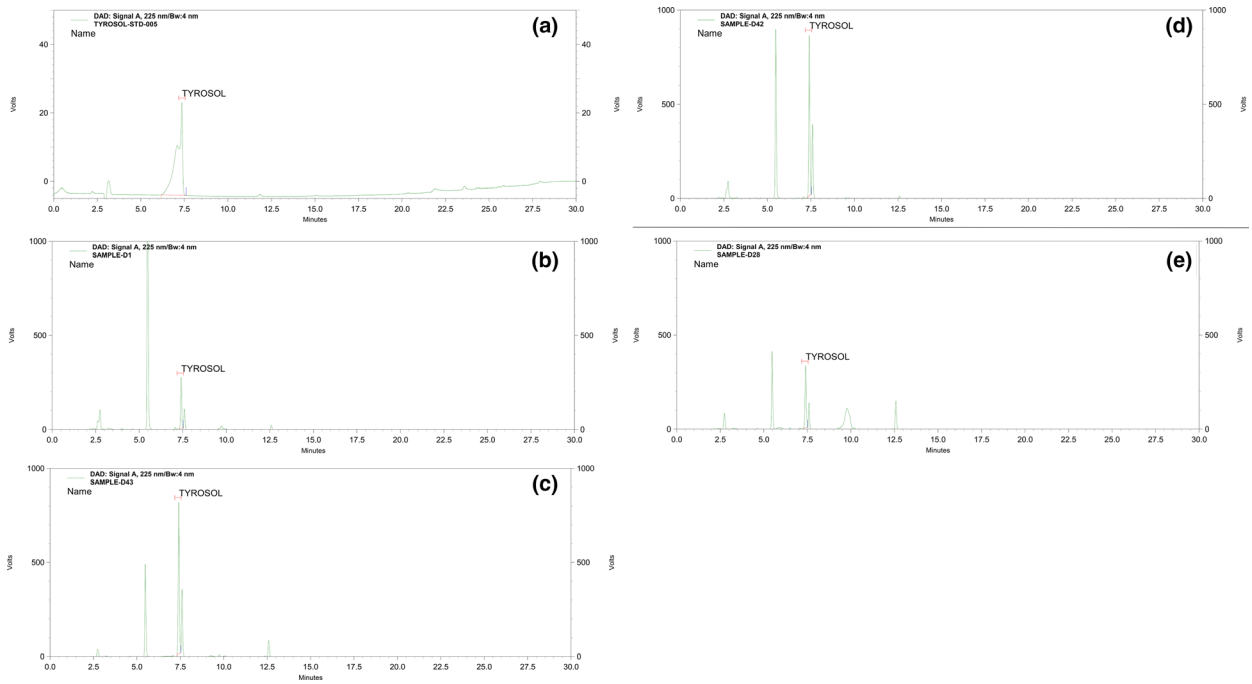

Figure 2 HPLC chromatograms of 7·5% acetonitrile-treated SPE eluent of (a) 100 μmol l^{-1} tyrosol standard, (b) *Wickerhamomyces anomalus* GUBT1R, (c) *Candida tropicalis* GUBT28A. (d) *Saccharomyces cerevisiae* GUBT42K and (e) *Candida glabrata* GUBT43C.

Table 2 Table showing morphogenic transition test of the experimented yeasts under the influence of tyrosols Tyr^{Ct}, Tyr^{Cg}, Tyr^{Sc}, Tyr^{Wa} and standard tyrosol Tyr^{St}. Presence of pseudo-hyphae on treated cells is denoted by (+) while the absence of any morphogenic change is denoted by (−)

	Tyrosol source					
Isolates	*Candida tropicalis* Tyr^{Ct}	*Candida glabrata* Tyr^{Cg}	*Saccharomyces cerevisiae* Tyr^{Sc}	*W. anomalus* Tyr^{Wa}	Standard Tyr^{St}	Blank
Wickerhamomyces anomalus GUBT1R	+	−	−	−	+	−
Candida tropicalis GUBT28A	+	−	+	+	+	−
Saccharomyces cerevisiae GUBT42K	−	−	−	−	−	−
Candida glabrata GUBT43C	−	−	−	−	−	−

tyrosol addition (data not shown). Notably, *W. anomalus* GUBT1R sustained growth for initial 10 min of ethanol exposure, but ceased to show any growth after 30 and 60 min exposure of Tyr^{Wa}, Tyr^{Ct},Tyr^{Sc} and Tyr^{Cg} treatments.. Contrarily, although *C. glabrata* GUBT43C did not show growth under the influence of Tyr^{Wa}, Tyr^{Ct} and Tyr^{Sc}, but it was observed to resist 12% ethanol even after 30 and 60 min of exposure to self-expressed tyrosol Tyr^{Cg}, implying the protective behaviour of Tyr^{Cg} during oxidative stress (Fig. 4e). Except for *W anomalus*, the ability of the isolates to endure ethanol stress under the influence of tyrosol indicates the possible protective role of the molecule towards the cell during fermentation.

Total cell number and fermentation efficiency

The probable role of tyrosol on enhancing fermentation ability by yeasts was determined by calculating total cell number for each isolate after incubation. Fermentation capacity was determined by dividing produced ethanol by total number of cells per ml. Comparative analysis of cell number and fermentation capacity is shown in Fig. 5a,b, respectively.

The growth of isolates in starch medium was lower compared to the glucose medium (Fig. 5a). However, application of 50 μmol l^{-1} tyrosol exhibited a significant increase in cell growth in *W. anomalus* (GUBT1R), *C. tropicalis* (GUBT28A) and *S. cerevisiae* (GUBT42K). *W. anomalus* exhibited

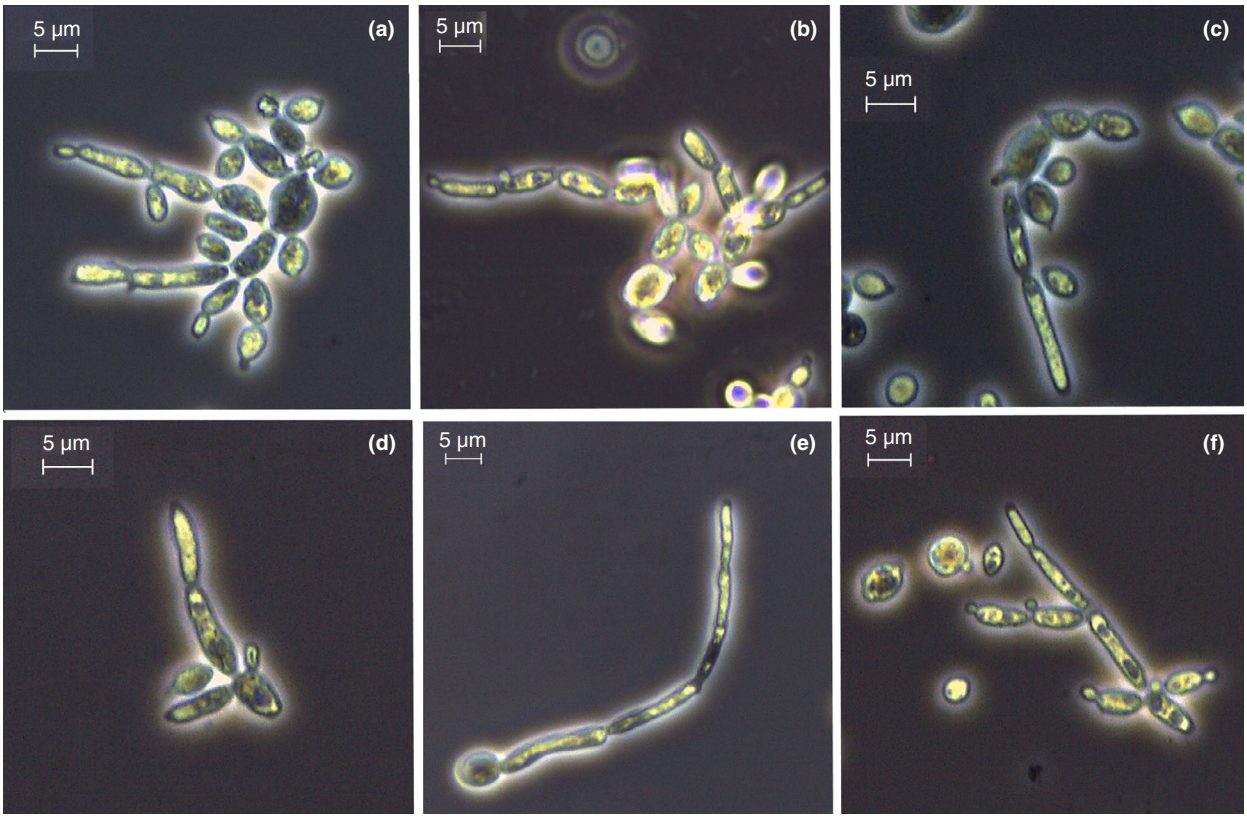

Figure 3 Figure showing pseudo-hypha formation in (a) *Candida tropicalis* under the influence of Tyr^{Wa}, (b) *Candida tropicalis* under the influence of self-expressed Tyr^{Ct}, (c) *C. tropicalis* under the influence of Tyr^{Sc}, (d) *C. tropicalis* under the influence of standard tyrosol Tyr^{St}, (e) *Wickerhamomyces anomalus* under the influence of Tyr^{Ct} and (f) *W. anomalus* under the influence of Tyr^{St}.

~1·3-fold increase ($^{*}P < 0{\cdot}05$) in starch medium, whereas *S. cerevisiae* displayed ~1·2 fold increase ($^{**}P < 0{\cdot}001$) in glucose medium. On the other hand, *C. tropicalis* showed ~1·3-fold ($^{**}P < 0{\cdot}001$) increase in both starch and ~1·2-fold ($^{***}P < 0{\cdot}0001$) increase in glucose medium. However, no growth stimulatory effect of tyrosol could be observed in *C. glabrata* and thus the cell growth was found to be unaffected either in the presence or absence of tyrosol on the isolate.

In addition, tyrosol in growth medium had no positive effect on fermentation capacity of the isolates except *S. cerevisiae* (GUBT42K). *Saccharomyces cerevisiae* is known to be a fermentative yeast and the tested isolate was found to possess the highest fermentative efficiency among the selected isolates (Nath *et al.* 2020). Under the influence of tyrosol in starchy medium, the isolate exhibited ~1·2-fold increase in fermentative capacity substantiating the positive effect of tyrosol on ethanol fermentation. However, in glucose medium, the fermentative capacity was observed to be reduced (Fig. 5b). The basic fermentation parameters and the ANOVA analysis of interaction of isolates with and without tyrosol are presented in Tables 3 and 4 respectively.

Discussion

This is the first report on the assessment of quorum response activity of tyrosol on yeasts isolated from traditional fermentative starter materials which are chiefly involved in indigenous fermentation. Besides, an attempt to decipher the interrelationship between QS mechanism and fermentation process has provided new insight into the possible enhancement of fermentative product directly or indirectly.

QSMs and quorum response in the yeast isolates

Characterization of QSMs in the spent medium revealed the presence of tyrosol in all four isolates viz *W. anomalus*, *C. tropicalis*, *S. cerevisiae* and *C. glabrata*. The presence of tyrosol in *C. tropicalis*, *S. cerevisiae* and *C. glabrata* was found to be in accordance with previous reports. Tyrosol was first reported to be secreted by *Candida albicans* (Chen *et al.* 2004) where the molecule regulated yeast morphogenesis. Chen *et al.* (2004) reported the continuous accumulation of tyrosol into the external environment at a very

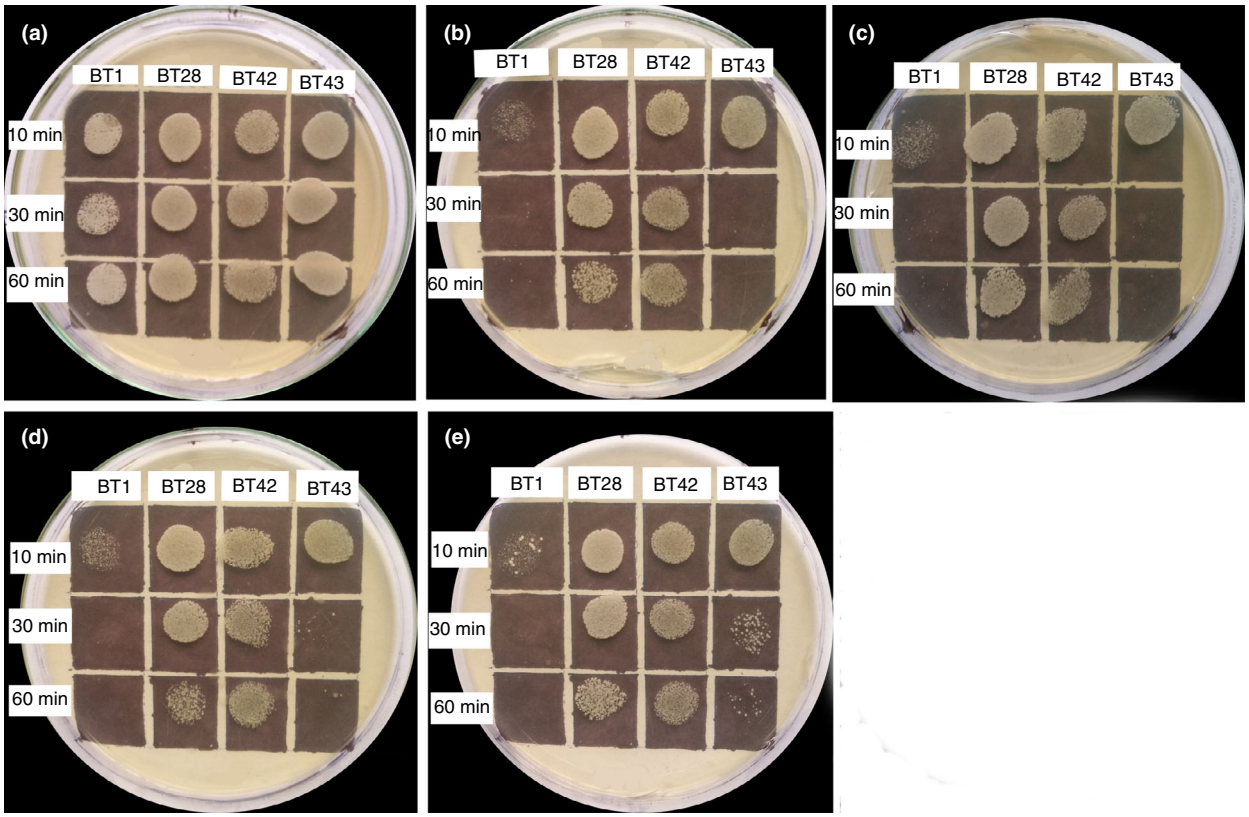

Figure 4 Figure showing colony forming units of isolates (BT1: *Wickerhamomyces anomalus*, BT28: *Candida tropicalis*, BT42: *Saccharomyces cerevisiae* and BT43: *C. glabrata*) after 12% ethanol exposure for 10, 30 and 60 min. Pictures shows CFU of isolates in (a) Blank (no ethanol exposure), (b) under influence of tyrosol from *W. anomalus* (Tyr^{Wa}), (c) under influence of tyrosol from *C. tropicalis* (Tyr^{Ct}), (d) under influence of *S. cerevisiae* tyrosol (Tyr^{Sc}) and (e) under influence of *C. glabrata* tyrosol (Tyr^{Cg}).

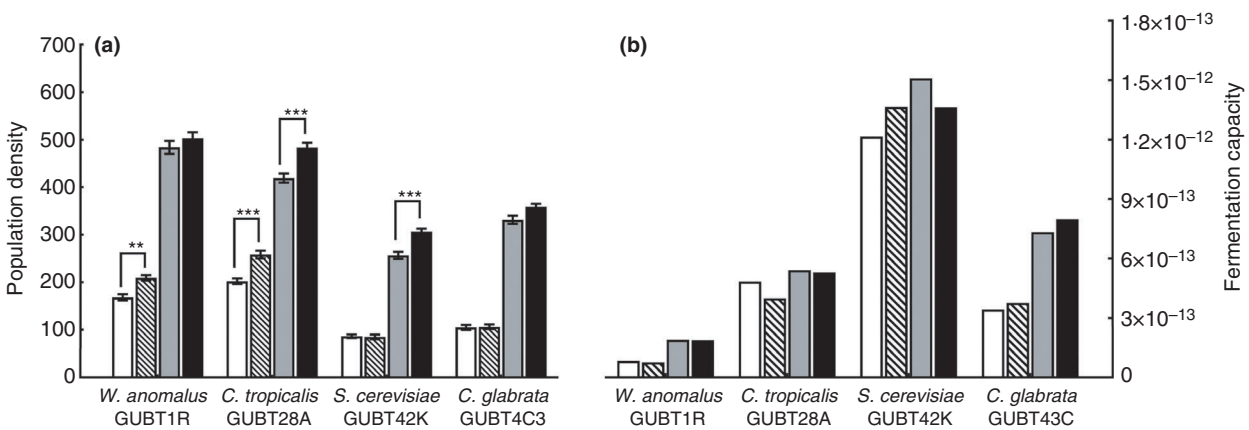

Figure 5 (a) Comparative growth of isolates in the presence and absence of 50 μmol l^{-1} tyrosol in starch and glucose medium. (Population density = Total cell number × 10^7), (b) Fermentation capacity of isolates in the presence and absence of 50 μmol l^{-1} tyrosol in starch and glucose medium. Fermentation capacity was calculated by dividing grams of ethanol produced per ml by total number of cells per ml of growth medium. Growth medium: () starch without tyrosol, () starch with tyrosol, () glucose without tyrosol and () glucose with tyrosol.

low concentration, that is, ~3 μmol l^{-1} at ≥10^8 cells per ml cellular density. Incidentally, Cremer *et al.* (1999) had reported the expression of tyrosol by the genus *Candida* where the highest amount of tyrosols were found to be expressed by *C. tropicalis* strain 54 (48·63 ± 3·83 μmol l^{-1}) and *C. glabrata* strain 497

Table 3 Fermentation parameters in *Wickerhamomyces anomalus* (BT1R), *Candida tropicalis* (BT28A), *Saccharomyces cerevisiae* (BT42K) and *Candida glabrata* (BT43C) subjected to starch and glucose as sole carbon sources with and without 50 μmol l^{-1} tyrosol

Parameters	BT1R	BT28A	BT42K	BT43C
Starch (Tyrosol−)				
Initial starch % (w/v)	2	2	2	2
Utilized starch % (w/v)	0·22 ± 0·02	0·57 ± 0·04	0·29 ± 0·03	0·22 ± 0·04
Ethanol produced % (w/v)	0·014 ± 0·001	0·098 ± 0·008	0·105 ± 0·010	0·036 ± 0·007
Ethanol yield	0·063	0·17	0·36	0·16
Fermentation efficiency	13·7	36·9	78·1	34·7
Starch (Tyrosol+)				
Initial starch % (w/v)	2	2	2	2
Utilized starch % (w/v)	0·26 ± 0·02	0·63 ± 0·04	0·28 ± 0·02	0·19 ± 0·03
Ethanol produced % (w/v)	0·016 ± 0·001	0·103 ± 0·006	0·116 ± 0·005	0·040 ± 0·005
Ethanol yield	0·061	0·16	0·41	0·21
Fermentation efficiency	13·2	34·7	88·9	45·6
Glucose (Tyrosol−)				
Initial starch % (w/v)	2	2	2	2
Utilized glucose % (w/v)	1·26 ± 0·04	1·85 ± 0·05	1·27 ± 0·05	1·30 ± 0·05
Ethanol produced % (w/v)	0·092 ± 0·003	0·227 ± 0·016	0·387 ± 0·015	0·243 ± 0·009
Ethanol yield	0·073	0·12	0·3	0·19
Fermentation efficiency	15·8	26	65·1	41·2
Glucose (Tyrosol+)				
Initial glucose % (w/v)	2	2	2	2
Utilized glucose % (w/v)	1·32 ± 0·07	1·96 ± 0·01	1·34 ± 0·07	1·42 ± 0·10
Ethanol produced % (w/v)	0·095 ± 0·005	0·256 ± 0·013	0·418 ± 0·021	0·286 ± 0·021
Ethanol yield	0·072	0·13	0·31	0·2
Fermentation efficiency	15·6	28·1	67·2	43·4

(1·3 ± 0·17 μmol l^{-1}), respectively. A high tyrosol producing *S. cerevisiae* strain 901 (72·9 ± 1·1 μmol l^{-1}) had also been reported by Soejima *et al.* (2012) which was isolated from Japanese traditional Sake fermentation. Whatsoever, till date, no clear evidence of expression of tyrosol by *Wickerhamomyces* isolates are available.

The amount of tyrosol produced by the tested isolates in this study was found to be highest in *C. tropicalis* (GUBT28A), followed by *S. cerevisiae* (GUBT42K) and *C. glabrata* (GUBT43C). Similar reports of high tyrosol producing *C. tropicalis* (Cremer *et al.* 1999) and *S. cerevisiae* (Soejima *et al.* 2012) yeasts support the finding of this study. Although induction of morphogenesis due to QS activity of tyrosol is well documented in *C. albicans* (Chen *et al.* 2004), yet there is no concrete evidence of such activity in *C tropicalis* and *S. cerevisiae* till date.

Test for morphogenic transition revealed that *C. tropicalis* GUBT28A showed morphogenic shift in response to its self-expressed tyrosol Tyr^{Ct} as well as Tyr^{Sc}, Tyr^{Wa}, Tyr^{St} (tyrosol standard). Development of pseudo-hyphal growth in *C. tropicalis* was reviewed earlier by Thompson *et al.* (2011) and therefore the resultant morphogenesis observed was found to have similar effect. Moreover, *C. tropicalis* was found to form pseudo-hyphae when grown in the presence of foetal bovine serum (Lackey *et al.* 2013), although evidence of the involvement of tyrosol on such morphogenetic shift is not very convincing. The closer genetic similarity of *C. tropicalis* towards *C. albicans* can presumably be considered for this change (Butler *et al.* 2009). Interestingly, *W. anomalus* GUBT1R exhibited morphogenic change in response to Tyr^{St} and Tyr^{Ct}, which was not reported earlier, while *S. cerevisiae* GUBT42K did not undergo any change in morphology in response to any of the tested tyrosol which was in congruence with the reports of Chen and Fink (2006) who stated the induction of pseudo-hyphal growth in *S. cerevisiae* in the presence of tryptophol and phenyl ethanol only. Similarly, *C. glabrata* GUBT43C also did not show any change in morphology on treatment with tyrosol which was probably because the species was reported to be devoid of any polymorphic trait and lack pseudo-hyphal growth (Silva *et al.* 2012) which implies that unlike *C. albicans*, *C. glabrata* do not respond to the morphogenic regulation on tyrosol treatment.

Effect of tyrosol in ethanol oxidative stress and enhanced cell growth during fermentation

The current investigation revealed the protective effect of tyrosol on yeasts subjected to oxidative stress induced by

Table 4 Data of two-way analysis of variance (ANOVA) analysed for screening total interactions between the individual isolates with and without tyrosol. The result shows significance at ***$P < 0{\cdot}001$ for interactions between glucose and starch fermentation and their corresponding ethanol yield in the presence and absence of tyrosol. The multiple comparisons for each of the treated groups with one another were analysed by Tukeys multiple range (*post hoc*) test as tabulated below

Two-way ANOVA	SS	df	MS	*F* (DFn, DFd)	*P* value	Significance
A						
Total interaction (Row *vs* Column)	0·2295	9	0·0255	*F* (9, 32) = 18·3	$P < 0{\cdot}0001$	***
Interaction of isolates grown in starch medium (Row factor)	0·4059	3	0·1353	*F* (3, 32) = 97·06	$P < 0{\cdot}0001$	***
Starch fermentation and ethanol produced with and without tyrosol (column factor)	0·8505	3	0·2835	*F* (3, 32) = 203·4	$P < 0{\cdot}0001$	***
Residual	0·0446	32	0·001394			
B						
Total interaction (Row vs Column)	0·9423	9	0·1047	*F* (9, 32) = 17·99	$P < 0{\cdot}0001$	***
Interaction of isolates grown in glucose medium (Row factor)	0·9073	3	0·3024	*F* (3, 32) = 51·97	$P < 0{\cdot}0001$	***
Glucose fermentation and ethanol produced with and without tyrosol (column factor)	17·77	3	5·924	*F* (3, 32) = 1018	$P < 0{\cdot}0001$	***
Residual	0·1862	32	0·00582			

ethanol. It was observed that isolates *C. tropicalis* GUBT28A and *S. cerevisiae* GUBT42K could survive 12% ethanol stress in the presence of 50 μmol l^{-1} tyrosol concentrations. Notably, *W. anomalus* GUBT1R failed to survive ethanol stress in spite of the presence of treated tyrosols, whereas isolate *C. glabrata* GUBT43C showed stunted growth under the influence of self-expressed tyrosol (Tyr^{Cg}) which was reasonably motivating. Protective behaviour of tyrosol in *C. albicans* was reported earlier by Cremer *et al*. (1999) where the molecule was found to protect the cells from reactive oxygen metabolites released by phagocytizing neutrophils. Despite a difference in approaches of the experiments performed, similar type of findings by Cremer *et al*. (1999) and this investigation suggest a probable protective role of tyrosol from oxidative stress during fermentation.

The direct role of tyrosol during the ethanol fermentation is still unclear, although tyrosol was found to be secreted during fermentation along with other aromatic alcohols (Zupan *et al*. 2013). The primary ethanol fermenting yeast *S. cerevisiae* was found to secret tyrosol into external environment in a density-dependent manner similar to *C. albicans* (Avbelj *et al*. 2015). In addition, tyrosol was reported to be secreted by *Debaryomyces hansenii* (Gori *et al*. 2011) in external media as well. In the present study, a significant increase in cell density was observed in *C. tropicalis*, *S. cerevisiae* and *W. anomalus* in the presence of tyrosol. In an earlier report, the upregulation of genes associated with DNA replication and cell-cycle maintenance were considered to be the reasons for accelerated germ tube formation under the influence of tyrosol (Chen *et al*. 2004). Such upregulation presumably exerts the growth-enhancing effect in cell density and since production of ethanol is dependent on cellular density; therefore, the indirect effect of tyrosol on fermentation capacity can also be justified. However, the inhibitory effect of ethanol can play an important role during the fermentation process. It was observed that addition of external ethanol played an inhibitory role in the production of tyrosol by *S. cerevisiae* and delayed the synthesis of the QSM (Avbelj *et al*. 2015).

In spite of the encouraging results obtained in the present study, the absolute involvement of tyrosol in the process demands more investigations. In this study, the shortening of lag phase during fermentation in the presence of tyrosol may be considered as a beneficial trait which is thought to evoke cell proliferating effect as was detected by Chen *et al*. (2004) who had observed that the addition of external tyrosol or conditioned media (CM) containing tyrosol resulted in shortening of lag phase in diluted cell culture of *C. albicans*. Moreover, the amount of pure tyrosol required for eliciting the shortening effect was found to be much higher (≥10 μmol l^{-1}) than the tyrosol present in the CM (~3 μmol l^{-1}) exerting same effect, which led Chen *et al*. (2004) to guess the presence of other growth stimulatory compounds in the CM, which nullifies the role of tyrosol as sole compound exhibiting such effect. Gene expression analysis performed in the same study (Chen *et al*. 2004) revealed the regulation of certain genes involved in DNA replication machinery and cell-cycle maintenance in the presence of tyrosol. The authors reckoned that tyrosol provided a

stabilizing aid to the transcripts that encoded cell division functions which were otherwise destabilized and degraded in diluted culture, resulting in delayed growth. In the discussion, the authors considered the probable transcriptional regulation of these genes by tyrosol. This provides an evidence of probable involvement of tyrosol in increasing the cell mass during fermentation as was also observed in this present investigation.

Conversely, an indirect role of tyrosol can be anticipated during glycolytic influx (Schwartz and Larsh 1982). As reported in *C. albicans*, the activities of hexokinase enzymes were observed to be almost double in hyphal form as compared to the conventional yeast form (Schwartz and Larsh 1982), indicating higher glycolytic activity during morphogenic shift. Additional published reports supported the evidence of regulation of genes associated with the glycolytic pathway resulting in the regulation of enzymes viz hexokinase II (Doedt *et al.* 2004; García-Sánchez *et al.* 2005; Sexton *et al.* 2007), phosphofructokinase (Sexton *et al.* 2007), glucose-6-phosphote isomerase (Doedt *et al.* 2004), fructose–bisphosphate aldolase (Doedt *et al.* 2004; Yin *et al.* 2004; Enjalbert *et al.* 2006), phosphoglycerate kinase (Yin *et al.* 2004; Enjalbert *et al.* 2006) and pyruvate kinase (Yin *et al.* 2004; Enjalbert *et al.* 2006). All these regulations were studied in *C. albicans* and since tyrosol had already been demonstrated as a responsible QS molecule for hyphal growth (Chen *et al.* 2004), it was therefore pertinent that the molecule was involved during the glycolytic flux. This can further be substantiated by the evidence cited by Shirtliff *et al.* (2009) where the downregulation of several glycolytic enzymes was reported in the presence of the QSM farnesol, which is reported to exert negative effect during filamentous growth of yeasts. Han *et al.* (2011) stated that QSMs are products of central carbon metabolism of yeast, whose production is assumed to reflect flux in the metabolic pathways providing feedback to these pathways and leading to optimized growth under conditions of different environmental set up. These result in the change in metabolic pathway that leads to hyphal formation, which may be due to the filamentation accelerating activity of QSM such as tyrosol. All known yeast QSMs till date are reported to be products of glycolytic pathway whereby aromatic QSMs such as tyrosol, tryptophol and phenylethyl alcohol are derived from phosphoenolpyruvate (Han *et al.* 2011). It had also been speculated that once these QSMs aggregate in the external environment, the passive diffusion of these molecules executes expression of specific genes or enhance activity of certain enzymes leading to various metabolic changes (Han *et al.* 2011). In spite of such obvious connection, the direct role of QSMs in the metabolic regulation resulting in morphogenesis is yet to be ascertained and therefore lack of solid evidence prevents a confirmatory conclusion for such enhancing activity.

From the present investigation, it is vivid that tyrosol can exert certain stimulatory effects on traditionally perpetuated fermentative yeasts. Although lack of available reports derecognizes tyrosol to be declared as a ubiquitous QSM in yeasts other than *C. albicans*, yet the present investigation strongly supports pseudo-hyphae formation capability on *C. tropicalis* and *W. anomalus* isolates subjected to interspecies tyrosols that interact and exert morphogenic effect through molecular cross-talk. In addition, the probable cell-cycle accelerating effect of tyrosol was observed in *W. anomalus*, *C. tropicalis* and *S. cerevisiae* under the influence of tyrosol resulting in increased cell number. Noticeably, the isolate *C. tropicalis* exhibited growth in both starch and glucose medium and the reason for this dual character has been discussed in preceding pages. The effect of retrieved tyrosols from tested isolates on morphogenic shift in *C. tropicalis* was observed to be indifferent and therefore tyrosol produced by *S. cerevisiae* in consortium is presumed to play similar accelerating effect on *C. tropicalis* growth. Since tyrosol do not act as carbon source, hence its utilization by *S. cerevisiae* in energy generation is unlikely, although a rise in fermentative capacity was observed in the isolate under the influence of tyrosol (Fig. 5b), suggesting an indirect effect of the molecule on fermentation, which may be due to the glycolytic influx by the QSM as discussed herein. On the other hand, elevation in cell number was also observed in *W. anomalus* isolate when grown in the presence of tyrosol (Fig. 5a), suggesting a similar growth enhancing effect of the molecule on a non-*Saccharomyces* yeast. This enhancement is vital and beneficial since *W. anomalus* is known to produce several aromatic compounds during fermentation process. However, the exact mechanism of such stimulatory effect of tyrosol is yet to be deciphered at the molecular and genetic levels. One possible approach could be the employment of fluorescent molecule tracking technique to track the involvement of the QSMs during studies on metabolic pathways, leading to changes in morphological, cellular proliferation or enhancement in fermentation products, which could not be accomplished in the present investigation due to the limitations in time and resources.

Acknowledgements

This work was supported by DBT Twinning project (BT/303/NE/TBP/2012 dated 04/01/2013) sanctioned to H.K.S. and A.K.M. by the Department of Biotechnology, Govt. of India. B.J.N. received DBT fellowship (2013–2016) and was a recipient of NFOBC-UGC fellowship (F./2017-18/NFO-2017-18-OBC-ASS-61070/SA-III/Website) for PhD under H.K.S.. Authors are thankful to the indigenous communities for providing raw materials and

Guwahati Biotech Park, IIT Guwahati for providing HPLC and GC-MS facilities.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' contributions

H.K.S. and A.K.M. conceived and designed the experiments. H.K.S. supervised the work. B.J.N. performed all the experiments. B.J.N. and H.K.S. performed the statistical analysis and wrote the paper. A.K.M. is a collaborator of the work.

References

Albergaria, H. and Arneborg, N. (2016) Dominance of *Saccharomyces cerevisiae* in alcoholic fermentation processes: role of physiological fitness and microbial interactions. *Appl Microbiol Biotechnol* **100**, 2035–2046. https://doi.org/10.1007/s00253-015-7255-0.

Alem, M.A.S., Oteef, M.D.Y., Flowers, T.H. and Douglas, L.J. (2006) Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. *Eukaryot Cell* **5**, 1770–1779. https://doi.org/10.1128/EC.00219-06.

Avbelj, M., Zupan, J., Kranjc, L. and Raspor, P. (2015) Quorum-sensing kinetics in *Saccharomyces cerevisiae*: a symphony of ARO genes and aromatic alcohols. *J Agric Food Chem* **63**, 8544–8550. https://doi.org/10.1021/acs.jafc.5b03400.

Avbelj, M., Zupan, J. and Raspor, P. (2016) Quorum-sensing in yeast and its potential in wine making. *Appl Microbiol Biotechnol* **100**, 7841–7852. https://doi.org/10.1007/s00253-016-7758-3.

Bhalla, T.C. and Savitri, P. (2017) Yeasts and traditional fermented foods and beverages. In *Yeast Diversity in Human Welfare* ed. Satyanarayana, T. and Kunze, G. pp. 53–82. Singapore: Springer. https://doi.org/10.1007/978-981-10-2621-8_3.

Butler, G., Rasmussen, D., Lin, M., Santos, M., Sakthikumar, S., Munro, C., Rheinbay, E., Grabherr, M. *et al.* (2009) Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459**, 657–662. https://doi.org/10.1038/nature08064.

Chen, H. and Fink, G.R. (2006) Feedback control of morphogenesis in fungi by aromatic alcohols. *Genes Dev* **20**, 1150–1161. https://doi.org/10.1101/gad.1411806.

Chen, H., Fujita, M., Feng, Q., Clardy, J. and Fink, G.R. (2004) Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc Natl Acad Sci* **101**, 5048–5052. https://doi.org/10.1073/pnas.0401416101.

Ciani, M., Capece, A., Comitini, F., Canonico, L., Siesto, G. and Romano, P. (2016) Yeast interactions in inoculated wine fermentation. *Front Microbiol* **7**, https://doi.org/10.3389/fmicb.2016.00555.

Comitini, F., Di Pietro, N., Zacchi, L., Mannazzu, I. and Ciani, M. (2004) *Kluyveromyces phaffii* killer toxin active against wine spoilage yeasts: purification and characterization. *Microbiology* **150**, 2535–2541. https://doi.org/10.1099/mic.0.27145-0.

Cremer, J., Vatou, V. and Braveny, I. (1999) 2,4-(hydroxyphenyl)-ethanol, an antioxidative agent produced by *Candida* spp., impairs neutrophilic yeast killing in vitro. *FEMS Microbiol Lett* **170**, 319–325. https://doi.org/10.1111/j.1574-6968.1999.tb13390.x.

Doedt, T., Krishnamurthy, S., Bockmühl, D.P., Tebarth, B., Stempel, C., Russell, C.L., Brown, A.J. and Ernst, J.F. (2004) APSES proteins regulate morphogenesis and metabolism in *Candida albicans*. *Mol Biol Cell* **15**, 3167–3180. https://doi.org/10.1091/mbc.e03-11-0782.

Enjalbert, B., Smith, D.A., Cornell, M.J., Alam, I., Nicholls, S., Brown, A.J. and Quinn, J. (2006) Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. *Mol Biol Cell* **17**, 1018–1032. https://doi.org/10.1091/mbc.e05-06-0501.

Etschmann, M.M.W., Sell, D. and Schrader, J. (2003) Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. *Biotechnol Lett* **25**, 531–536. https://doi.org/10.1023/A:1022890119847.

Fleet, G.H. (2003) Yeast interactions and wine flavour. *Int J Food Microbiol* **86**, 11–22. https://doi.org/10.1016/S0168-1605(03)00245-9.

Fuqua, W.C., Winans, S.C. and Greenberg, E.P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**, 269–275. https://doi.org/10.1128/jb.176.2.269-275.1994.

García-Sánchez, S., Mavor, A.L., Russell, C.L., Argimon, S., Dennison, P., Enjalbert, B. and Brown, A.J. (2005) Global roles of Ssn6 in Tup1- and Nrg1-dependent gene regulation in the fungal pathogen, *Candida albicans*. *Mol Biol Cell* **16**, 2913–2925. https://doi.org/10.1091/mbc.e05-01-0071

Ghosh, S., Kebaara, B.W., Atkin, A.L. and Nickerson, K.W. (2008) Regulation of aromatic alcohol production in *Candida albicans*. *Appl Environ Microbiol* **74**, 7211–7218. https://doi.org/10.1128/AEM.01614-08.

González-Marco, A., Jiménez-Moreno, N. and Ancín-Azpilicueta, C. (2010) Influence of nutrients addition to nonlimited-in-nitrogen must on wine volatile composition. *J Food Sci* **75**, S206–S211. https://doi.org/10.1111/j.1750-3841.2010.01578.x.

Gori, K., Knudsen, P.B., Nielsen, K.F., Arneborg, N. and Jespersen, L. (2011) Alcohol-based quorum sensing plays a

role in adhesion and sliding motility of the yeast *Debaryomyces hansenii*. *FEMS Yeast Res* **11**, 643–652. https://doi.org/10.1111/j.1567-1364.2011.00755.x.

Han, T.L., Cannon, R.D. and Villas-Bôas, S.G. (2011) The metabolic basis of *Candida albicans* morphogenesis and quorum sensing. *Fungal Genet Biol* **48**, 747–763. https://doi.org/10.1016/j.fgb.2011.04.002.

Hornby, J.M., Jensen, E.C., Lisec, A.D., Tasto, J., Jahnke, B., Shoemaker, R., Nickerson, K.W., Tasto, J.J. *et al.* (2001) Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* **67**, 2982–2992. https://doi.org/10.1128/AEM.67.7.2982-2992.2001.

Kuhn, D.M., Chandra, J., Mukherjee, P.K. and Ghannoum, M.A. (2002) Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immun* **70**, 878–888. https://doi.org/10.1128/IAI.70.2.878-888.2002.

Lackey, E., Vipulanandan, G., Childers, D.S. and Kadosh, D. (2013) Comparative evolution of morphological regulatory functions in *Candida* species. *Eukaryot Cell* **12**, 1356–1368. https://doi.org/10.1128/EC.00164-13.

Nath, B.J., Verma, E., Sarma, H.K., Mishra, A.K., Tanti, B. and Jha, D.K. (2020) Evaluation of basic fermentation parameters and effective combinations of predominant yeasts from traditional starter materials of indigenous communities from Northeast India. *J Am Soc Brew Chem* **78**, 219–230. https://doi.org/10.1080/03610470.2020.1739601.

Nissen, P. and Arneborg, N. (2003) Characterization of early deaths of non-*Saccharomyces* yeasts in mixed cultures with *Saccharomyces cerevisiae*. *Arch Microbiol* **180**, 257–263. https://doi.org/10.1007/s00203-003-0585-9.

Nissen, P., Nielsen, D. and Arneborg, N. (2003) Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell - cell contact-mediated mechanism. *Yeast* **20**, 331–341. https://doi.org/10.1002/yea.965.

Okunowo, W., Okotore, R. and Osuntoki, A. (2005) The alcoholic fermentative efficiency of indigenous yeast strains of different origin on orange juice. *African J Biotechnol* **4**, 1290–1296.

Parasar, D.P., Sarma, H.K. and Kotoky, J. (2017) Exploring the genealogy and phenomic divergences of indigenous domesticated yeasts cultivated by six ethnic communities of Assam, India. *J BiolSci* **17**, 91–105. https://doi.org/10.3923/jbs.2017.91.105.

Pérez-Nevado, F., Albergaria, H., Hogg, T. and Girio, F. (2006) Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentations with *Saccharomyces cerevisiae*. *Int J Food Microbiol* **108**, 336–345. https://doi.org/10.1016/j.ijfoodmicro.2005.12.012.

Renault, P.E., Albertin, W. and Bely, M. (2013) An innovative tool reveals interaction mechanisms among yeast populations under oenological conditions. *Appl Microbiol Biotechnol* **97**, 4105–4119. https://doi.org/10.1007/s00253-012-4660-5.

Rodríguez-Cousiño, N., Maqueda, M., Ambrona, J., Zamora, E., Esteban, R. and Ramírez, M. (2011) A new wine *Saccharomyces cerevisiae* killer toxin (Klus), encoded by a double-stranded RNA virus, with broad antifungal activity is evolutionarily related to a chromosomal host gene. *Appl Environ Microbiol* **77**, 1822–1832. https://doi.org/10.1128/AEM.02501-10.

Schwartz, D.S. and Larsh, H.W. (1982) Comparative activities of glycolytic enzymes in yeast and mycelial forms of *Candida albicans*. *Mycopathologia* **78**, 93–98. https://doi.org/10.1007/BF00442632.

Seo, H.B., Kim, H.J., Lee, O.K., Ha, J.H., Lee, H.Y. and Jung, K.H. (2009) Measurement of ethanol concentration using solvent extraction and dichromate oxidation and its application to bioethanol production process. *J Ind Microbiol Biotechnol* **36**, 285–292. https://doi.org/10.1007/s10295-008-0497-4.

Sexton, J.A., Brown, V. and Johnston, M. (2007) Regulation of sugar transport and metabolism by the *Candida albicans* Rgt1 transcriptional repressor. *Yeast* **24**, 847–860. https://doi.org/10.1002/yea.1514.

Shirtliff, M.E., Krom, B.P., Meijering, R.A.M., Peters, B.M., Zhu, J., Scheper, M.A., Harris, M.L. and Jabra-Rizk, M.A. (2009) Farnesol-induced apoptosis in *Candida albicans*. *Antimicrob Agents Chemother* **53**, 2392–2401. https://doi.org/10.1128/AAC.01551-08.

Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D.W. and Azeredo, J. (2012) *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: Biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol Rev* **36**, 288–305. https://doi.org/10.1111/j.1574-6976.2011.00278.x.

Skandamis, P.N. and Nychas, G.J.E. (2012) Quorum sensing in the context of food microbiology. *Appl Environ Microbiol* **78**, 5473–5482. https://doi.org/10.1128/AEM.00468-12.

Soejima, H., Tsuge, K., Yoshimura, T., Sawada, K. and Kitagaki, H. (2012) Breeding of a high tyrosol-producing sake yeast by isolation of an ethanol-resistant mutant from a trp3 mutant. *J Inst Brew* **118**, 264–268. https://doi.org/10.1002/jib.46.

Sprague, G.F. Jr and Winans, S.C. (2006) Eukaryotes learn how to count: quorum sensing by yeast. *Genes Dev* **20**, 1045–1049. https://doi.org/10.1101/gad.1432906.

Thompson, D.S., Carlisle, P.L. and Kadosh, D. (2011) Coevolution of morphology and virulence in *Candida* species. *Eukaryot Cell* **10**, 1173–1182. https://doi.org/10.1128/EC.05085-11.

Tsuyoshi, N., Fudou, R., Yamanaka, S., Kozaki, M., Tamang, N., Thapa, S. and Tamang, J.P. (2005) Identification of yeast strains isolated from marcha in Sikkim, a microbial starter for amylolytic fermentation. *Int J Food Microbiol* **99**, 135–146. https://doi.org/10.1016/j.ijfoodmicro.2004.08.011.

Vähäsalo, L. and Holmbom, B. (2004) Reliable spectrophotometric determination of starch concentration in papermaking process waters. *Nord Pulp Pap Res J* **19**, 75–77. https://doi.org/10.3183/npprj-2004-19-01-p075-077.

Wang, H., Dong, Q., Guan, A., Meng, C., Shi, X. and Guo, Y. (2011) Synergistic inhibition effect of 2-phenylethanol and ethanol on bioproduction of natural 2-phenylethanol by *Saccharomyces cerevisiae* and process enhancement. *J Biosci Bioeng* **112**, 26–31. https://doi.org/10.1016/j.jbiosc.2011.03.006.

Whitehead, N.A., Barnard, A.M., Slater, H., Simpson, N.J. and Salmond, G.P. (2001) Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* **25**, 365–404. https://doi.org/10.1111/j.1574-6976.2001.tb00583.x.

Wuster, A. and Babu, M.M. (2008) Chemical molecules that regulate transcription and facilitate cell-to-cell communication. In *Wiley Encyclopedia of Chemical Biology* ed. Begley, T.P. John Wiley and Sons. https://doi.org/10.1002/9780470048672.wecb501.

Yin, Z., Stead, D., Selway, L., Walker, J., Riba-Garcia, I., McInerney, T., Gaskell, S., Oliver, S.G. *et al.* (2004) Proteomic response to amino acid starvation in *Candida albicans* and *Saccharomyces cerevisiae*. *Proteomics* **4**, 2425–2436. https://doi.org/10.1002/pmic.200300760.

Zhang, Y.H.P., Hong, J. and Ye, X. (2009) Cellulase assays. In *Biofuels. Methods in Molecular Biology (Methods and Protocols)* ed. Mielenz, J. pp. 213–231. Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-60761-214-8_14.

Zupan, J., Avbelj, M., Butinar, B., Kosel, J., Šergan, M. and Raspor, P. (2013) Monitoring of quorum-sensing molecules during minifermentation studies in wine yeast. *J Agric Food Chem* **61**, 2496–2505. https://doi.org/10.1021/jf3051363.