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by Kathleen Boons, Estefanía Noriega, Niels Verherstraeten, Charlotte C. David,
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The Effect of Medium Structure Complexity
on the Growth of *Saccharomyces cerevisiae* in Gelatin-Dextran systems

Kathleen Boons^{a,b}, Estefanía Noriega^{a,b}, Niels Verherstraeten^b,

Charlotte C. David^c, Johan Hofkens^c, Jan F. Van Impe^{a,b}

^aCPMF² - Flemish Cluster Predictive Microbiology in Foods – www.cpmf2.be

^bBioTeC - Chemical and Biochemical Process Technology and Control,
Department of Chemical Engineering, KU Leuven, Leuven, Belgium, [kathleen.boons,
estefania.noriegafernandez, jan.vanimpe][@cit.kuleuven.be](mailto:jan.vanimpe@cit.kuleuven.be),
niels.verherstraeten[@student.kuleuven.be](mailto:niels.verherstraeten@student.kuleuven.be)

^cMolecular Visualization and Photonics, KU Leuven, Leuven, Belgium, [charlotte.david,
johan.hofkens][@chem.kuleuven.be](mailto:johan.hofkens@chem.kuleuven.be)

Correspondence to:

Prof. J. F. Van Impe

Chemical and Biochemical Process Technology and Control (BioTeC),

Department of Chemical Engineering, KU Leuven,

W. de Croylaan 46, B-3001 Leuven (Belgium)

jan.vanimpe@cit.kuleuven.be

Tel: +32-16-32.14.66

Fax: +32-16-32.29.91

Abstract

As most food systems are (semi-) solid, the effect of food structure on bacterial growth has been widely acknowledged. However, studies on the growth dynamics of yeasts have neglected the effect of food structure.

In this paper, the growth dynamics of the spoilage yeast *Saccharomyces cerevisiae* was investigated at 23.5°C in broth, singular, homogeneous biopolymer systems and binary biopolymer systems with a heterogeneous microstructure. The biopolymers gelatin and dextran were used to introduce the different levels of structure. The metabolizing ability of gelatin and dextran by *S. cerevisiae* was examined. To study microbial behavior in the binary systems at the micro level, mixtures were imaged with confocal laser scanning microscopy (CLSM). Growth dynamics and microscopic images of *S. cerevisiae* were compared with those obtained for *E. coli* in the same model system (Boons *et al.*, 2014). Different phase-separated, heterogeneous microstructures were obtained by changing the amount of added gelatin and dextran. Regardless of the microstructure, *S. cerevisiae* was preferentially located in the dextran phase. Metabolizing ability-tests indicated that gelatin could be consumed by *S. cerevisiae* but in the presence of glucose, no change in gelatin concentration was observed. No indication of dextran metabolizing ability was observed. When supplementing broth with gelatin or dextran alone, an enhanced growth rate and maximum cell density were observed. This enhancement was further increased by adding a second biopolymer, introducing a heterogeneous microstructure and hence increasing the medium structure complexity.

The results obtained indicate that food structure complexity plays a significant role in the growth dynamics of *S. cerevisiae*, an important food spoiler.

Keywords: growth kinetics, heterogeneous microstructure, confocal laser scanning microscopy, metabolizing ability, yeast, food biopolymers

1. Introduction

Food spoilage causes major losses of processed food around the world every year (Pitt and Hocking, 2009a), leading to big economic losses (Fleet, 1992). Microbial spoilage is a common type of food spoilage caused by three types of microbial spoilers, i.e., bacteria, molds and yeasts. Yeast spoilage is mostly encountered in food products with low pH, high sugar concentrations and/or high salt concentrations, where competition from bacterial growth is restricted, e.g., dairy products, fresh and processed fruits and sauces. *Saccharomyces cerevisiae* is cataloged as one of the top ten yeast species considered responsible for spoilage of processed and packaged food products as soft drinks, dairy products and fermented foods (Pitt and Hocking, 2009b). Food composition and food processing and storage conditions all have an effect on microbial behavior. Selecting the correct environmental conditions at these different stages, microbial outgrow can be controlled. As such, it is important to characterize the growth behavior of spoilage yeasts, and more specifically *S. cerevisiae*, as a function of different environmental factors in order to prevent food spoilers from growing.

Abundant literature is available on unraveling the effect of environmental factors as temperature, pH and water activity on the growth of *S. cerevisiae* in liquid systems (Arroyo-López *et al.*, 2009; Hobot and Jennings, 1981; Kalathenos *et al.*, 1995; Nielsen and Arneborg, 2007; Praphailong and Fleet, 1997; Thomas *et al.*, 2002; Watson, 1970). However, most food products have a (semi-) solid structure, causing micro-organisms to be immobilized and forced to grow as colonies. The effect of a (semi-)solid environment, mimicked by the addition of a biopolymer with gelling properties, on microbial growth dynamics has been investigated rather extensively for bacteria, reporting a growth limiting effect of the (semi-) solid structure (Brocklehurst *et al.* 1995; Brocklehurst *et al.*, 1997; Wilson *et al.*, 2002). However, studies on the effect of medium structure in food model systems on yeast growth are scarce. Up till now, studies on the effect of immobilization of yeast cells are framed in the domain of fermentation

technology, with focus on the behavior in/on a solid carrier for the use in bio-industry and brewing industry (Bangrak *et al.* 2011; Bezbradica *et al.*, 2007; Burrill *et al.*, 1983; Norton and D'Amore, 1994; Wada *et al.*, 1980; Willaert and Nedovic, 2006). However, nutrients are not incorporated in the solid matrix itself but have to diffuse from the surrounding liquid to the cells in the matrix. It is clear that this does not reflect the situation in real food systems. Mertens *et al.* (2011) studied the growth behavior of *Zygosaccharomyces bailii* in food model systems for acid sauces, based on Carbopol or xanthan gum. Mertens *et al.* (2011) reported a difference in growth probability between experiments in liquid and in semi-solid media. These results lead to the conclusion that studies on the effect of food structure on the growth of spoilage yeasts are needed in order to improve predictions on food spoilage, now based on experimental results obtained in liquid systems. To draw general conclusions on the effect of food structure on yeast growth, the authors believe that experiments should first be performed in reproducible food model systems. Next, studies on real food products can be performed, taking into account the structure and specific formulation and composition of the food product.

In food (model) systems, different structures with increasing level of complexity can be identified. Liquid systems are the simplest medium structures. Supplementing a liquid system with one gelling agent, a homogeneous gel is created, increasing the medium complexity. The most complex forms of medium structure are systems with different phases, i.e., heterogeneous systems. In this category, emulsions, gelled emulsions and heterogeneous gels can be identified. Emulsions and gelled emulsions consist of an oil phase and a water or gel phase. Heterogeneous gels consist out of different phases, each containing different concentrations of the biopolymers present in the mixture. Heterogeneous gels are formed when a biopolymer mixture undergoes phase-separation and (one of) the phases undergo gelation. This results in a kinetically frozen heterogeneous microstructure. In previous work (Boons *et al.*, 2013), a phase-separating biopolymer mixture of gelatin and dextran, both used in food industry, was created based on

the findings of Tromp *et al.* (2001) to investigate the effect of a heterogeneous microstructure on *E. coli* growth behavior. It was found that the presence of a second biopolymer, introducing a heterogeneous microstructure, had an influence on microbial dynamics. As food products are rarely homogeneous in composition and structure, the authors believe that investigating the effect of a heterogeneous medium microstructure on yeast behavior, has an added value.

The aim of the current study was to investigate; the effect of three medium structures with increasing level of complexity on the overall population growth dynamics of *S. cerevisiae*, i.e, growth dynamics in liquid were compared with those obtained in homogeneous media based on gelatin or dextran addition and the heterogeneous systems created in Boons *et al.* (2013). Seven different ratios of gelatin and dextran, leading to seven different microstructures, were used to elucidate the effect of a heterogeneous microstructure on yeast growth. To ensure phase-separation between gelatin and dextran and hence a heterogeneous microstructure, 2.9% (w/v) of NaCl was added, as mentioned by Tromp *et al.* (2001). Since the concentration of added salt used introduced an extra stress factor at a level that is present in many food products, this was a relevant factor to investigate. To study transferability of data between yeast and bacteria, micro- and macro-scale behavior of *S. cerevisiae* was compared with results obtained for *E. coli* in the same heterogeneous gelatin/dextran systems (Boons *et al.*, 2014). The yeast strain used produced the fluorescent protein citrine. Confocal laser scanning microscopy was used to characterize the heterogeneous microstructure and to investigate the distribution of the fluorescent yeast cells in the media. It is reported that some yeast can metabolize gelatin (Abrusci *et al.*, 2007; Mertens *et al.*, 2009). Hence the addition of gelatin would not only alter the structure of the medium but also the nutritional content. For this reason, the ability of *S. cerevisiae* to metabolize one of the added biopolymers was investigated in additional growth experiments.

Unravelling the effect of medium structure complexity on growth dynamics of the spoilage yeast *S. cerevisiae* will help to control food spoilage and reduce economic losses.

2. Material and methods

2.1. Microorganisms and preculture conditions

The experiments were performed with *Saccharomyces cerevisiae* KV1529, expressing the fluorescent protein citrine. The strain was kindly provided by the Centre of Microbial and Plant Genetics (KU Leuven, Belgium). YPD medium was used for the growth experiments, consisting of 1% (w/v) Yeast extract (yeast extract granulated, Merck KGaA, Darmstadt, Germany), 2% (w/v) bacterial Peptone (Oxoid, Basingstoke, United Kingdom) and 2% (w/v) glucose (alpha-D(+)-glucose, 99+%, anhydrous, Acros Organics, Geel, Belgium). Before starting up a growth/metabolizing ability-experiment, yeast was grown on YPD supplemented with 1.4% (w/v) agar (Agar technical No. 3, Oxoid, Basingstoke, UK). The yeast was allowed to grow for 2 days at 30°C (Binder KB-series incubator; Binder Inc., NY, USA). Afterwards, the plate was stored in the fridge at 4°C for maximum 2 months. Inoculum was prepared by transferring a colony from the plate into an Erlenmeyer containing 20 mL YPD. After 9 h at 30°C and 150 rpm (rotary shaker, Heidolph UNIMAX 2010, Led Techno, Belgium), a 20 µL aliquot of the stationary phase culture was inoculated into 20 mL of fresh YPD and incubated for 15 h under the same conditions. The obtained cell suspension is used as the preculture for the growth and metabolizing ability-tests.

2.2. Medium preparation and experimental setup for testing metabolizing ability

In order to determine the metabolizing ability of the different components by *S. cerevisiae*, 2% (w/v) glucose or 2.5% (w/v) dextran (Dextran from *Leuconostoc* spp. Mr ~ 500000, Sigma, Denmark) or 5% (w/v) gelatin (Gelatin from Bovine skin, type B, Sigma, USA) were added to

100 mL of salt solution (2.9% (w/v) NaCl, Analar NORMAPUR, VWR, Belgium) or Yeast extract bacteriological Peptone (YP) supplemented with 2.9% (w/v) NaCl. After 15 min in a water bath at 70°C, media were filter sterilized using a 0.22 µm filter (Filtertop, 150 mL, 0.22 µm, TPP, Switzerland). Samples were inoculated to obtain an initial cell density of about 10⁴ CFU/ mL. After thorough mixing, 8 mL of the different solutions were pipetted into test tubes. Samples were exposed to a sequence of different temperatures to ensure phase-separation (for further details, see Boons *et al.*, 2014). The singular and liquid samples undergo the same temperature cycle to give all cells the same temperature history. At different points in time, one test tube of every solution was removed from the water bath and placed for 5 min at 35°C to melt the samples. 100 µL of the solution was sampled to determine viable plate count. After preparing the appropriate serial decimal dilutions in YPD, samples were plated onto YPD supplemented with 1.4% (w/v) agar. Plates were incubated for at least 36 h at 30°C before viable cell counting. The remaining of the solution was filter sterilized through a 0.2 µm pore size filter (Filtropur S 0.2, SARSTEDT, Germany) with the aid of a syringe. The filtered solution was stored in a freezer at -20°C.

Different protocols were used to determine glucose, dextran and gelatin concentration as a function of time. For the determination of the different components (glucose, dextran or gelatin), another aliquot of the sample was used for each analysis. The amount of glucose in the samples was determined with a glucose kit (Glucose (HK) Assay Kit, Sigma-Aldrich, USA). The appropriate volumes of reagent and sample were mixed. After incubation at room temperature for 15 min, absorbance was measured at 340 nm (DR 5000, Hach Lange, Germany). Gelatin concentration was determined by using the Bradford method (Bradford, 1976) with the Bradford reagent (Sigma-Aldrich, USA). As gelatin is difficult to detect, the micro-assay procedure was applied, i.e., after proper dilution in distilled water, 1 mL of sample was allowed to react with 1 mL of Bradford reagent. After 15 min, absorbance was measured

at 595 nm. The contribution of the proteins in the YP growth medium to the Bradford signal was a factor 20 lower than the contribution of gelatin. As such, the observed trends caused by the variation in gelatin concentration were not altered by the presence of the proteins in the YP growth medium. Dextran concentrations were determined as described in Jacobsson and Hansen (1952). Proteins present in the media were precipitated with trichloroacetic acid (Trichloroacetic acid for analysis, 99.5%, Merck, Germany). Next, the solution was filtered with a 0.45 μm filter (Filtropur S 0.45, SARSTEDT, Germany) and 200 μL of the filtrate was allowed to react with 1 mL of ethanol (Desinfectol, Chem-Lab, Belgium). After 60 min, absorbance was measured at 720 nm. For both gelatin and dextran determinations, the appropriate calibration curves were conducted. For determining glucose concentrations, the calculation described in the Technical Bulletin for the Glucose (HK) Assay Kit (Sigma-Aldrich, 2014) was followed. Analyses were performed in three replicates. In Figure 1 the average and the standard error were plotted.

2.3. Microscopy: sample preparation and image analysis

Preparation of the mixtures for the confocal microscope samples was performed in the same way as described in Boons *et al.* (2014). Before filtration, 30 μL of a 0.01% (w/v) Rhodamine B-solution (R953, Aldrich, Germany) was added to stain the gelatin phase. After inoculation, well chambers (chambered borosilicate coverglass system, Nunc Lab-Tek, USA) were filled with 300 μL of the mixture and incubated at room temperature. As described in Boons *et al.* (2013), images were taken with a commercial laser scanning microscope (FV 1000, Olympus), although a 40X magnification objective was used now.

2.4. Medium preparation and experimental setup for growth experiments

Medium preparation was similar to the procedure mentioned in Boons *et al.* (2014). In summary, different ratios of gelatin and dextran (2.5 (1G), 5.0 (2G), 7.5 (3G) and 10.0% (w/v) gelatin (4G), 2.5 (1D) or 5.0% (w/v) dextran (2D), see Table 2 in Boons *et al.*, 2014) were mixed with 2.9% (w/v) NaCl. After adding the appropriate amounts of YPD, samples were heated, filter sterilized and inoculated to obtain an initial cell density of about 10^4 CFU/mL. Next, sterile glass screw-cap tubes were filled with 1 mL of inoculated medium. Samples were exposed to a sequence of different temperatures to ensure phase-separation (for further details, see Boons *et al.*, 2014). Tubes were placed in a water bath at 23.5°C from which, at regular time steps, one tube of each mixture was removed and placed in another water bath at 35°C, in order to melt the semi-solid medium. After preparing the appropriate serial decimal dilutions in YPD, viable count was determined via plate count. Four independent experiments were conducted for each mixture. In Figure 3, independent replicates performed in the same medium were indicated separately with the same symbol.

2.5. Estimation of growth parameters and statistical analysis

The growth model of Baranyi and Roberts (1994) was fitted to the growth curves and model parameters were estimated. Standard errors of parameters were calculated from the Jacobian matrix. To determine whether there are any significant differences amongst means of parameter estimates analysis of variance (ANOVA) test was performed. Fisher's Least Significant Difference (LSD) test was used to identify which means were significantly different. The letters in Figure 4 indicate the different groups obtained from the LSD-test. For more information on the estimation of the growth parameters and the implementation of the statistical analysis, authors refer to Boons *et al.* (2014). Statistical analyses on the metabolizing analysis-test results for gelatin are performed in Excel (Microsoft 2010) with the ANOVA single factor-method of the Data Analysis-toolbox. Test statistics were regarded as significant when P was ≤ 0.05 .

3. Results

3.1. Metabolizing ability-tests

To investigate the metabolizing ability and hence the nutritional character of the different biopolymers, i.e., gelatin and dextran, metabolizing ability-tests were performed with *S. cerevisiae*. Appropriate tests were conducted to determine the concentration of gelatin, dextran, and, as a control, glucose, as a function of time. Figure 1 displays the viable count and the concentration of unconsumed glucose, dextran and gelatin as a function of time.

As can be seen from Figure 1a, yeast growth was not detected in the mixtures with salt solution. For the mixtures containing glucose or dextran, microbial inactivation was observed. However, the viable counts in salt solution with gelatin stayed constant with time (survival). For all mixtures containing YP, Figure 1b shows that growth was observed. After approximately 50 h, the stationary phase was reached for all experiments performed in YP.

The amount of unconsumed glucose is displayed in Figure 1c. No consumption of glucose was observed when *S. cerevisiae* was grown in salt solution supplemented with glucose. Growth in YP supplemented with glucose resulted in a decrease in glucose as a function of time, where glucose depletion was reached at approximately 110 h. For the YP mixture supplemented with glucose, dextran and gelatin, depletion of glucose was reached, around 70 h.

It is clear from Figure 1d that *S. cerevisiae* did not use dextran as a nutrient source. For the experiments both in salt solution and YP the amount of unconsumed dextran stayed constant as a function of time.

In Figure 1e, a significant decrease of the amount of unconsumed gelatin in YP growth medium was observed. This indicated that *S. cerevisiae* could metabolize gelatin when glucose was not available. In the YP mixture supplemented with glucose, dextran and gelatin, no decrease in gelatin concentration was observed. In the absence of YP, no consumption of gelatin was observed.

3.2. Microscopy

A confocal laser scanning microscope was used to characterize the microstructure of the binary mixtures and to determine the distribution of yeast cells in these microstructures. In Figure 2, confocal images are shown for the different mixtures studied. The gelatin phase stained with rhodamine B is shown in green, the black phase represents the dextran phase. For all binary mixtures studied, phase-separation between gelatin and dextran was observed. Additionally, changing the gelatin-dextran ratio led to the formation of different microstructures. In the 1G/1D system, gelatin domains were dispersed in a dextran matrix. Increasing the gelatin content led to an increase in the size of the gelatin domains, which included dextran spheres. When adding even more gelatin, i.e., for 3G/1D and 4G/1D systems, a gelatin matrix containing dextran spheres of similar sizes was observed (Figure 2 III and IV). Increasing the dextran concentration of the 2G/1D system to 2G/2D, led to smaller, more sphere-like gelatin domains without dextran spheres inside. Supplementing this 2G/2D system with more gelatin, resulting in the 3G/2D and 4G/2D system, led to phase inversion, resulting in dextran spheres of different sizes dispersed in a gelatin matrix (Figure 2 VI and VII). *S. cerevisiae*, made visible by the production of the fluorescent protein citrine, was shown in red. As can be seen from the images, the yeast grew preferably in the dextran phase, regardless the microstructure. Some cells appeared as green in the microstructure, probably caused by the diffusion of rhodamine B into the yeast cells, resulting in a detection of the cells at wavelengths related to rhodamine B. As such, cells were marked with the color of the rhodamine B-channel, i.e., green.

3.3. Growth experiments

Figure 3 displays the growth curves, i.e., population cell densities as a function of time, obtained in the different mixtures. For all the experiments a similar, sigmoid growth curve was observed with the different growth phases present, i.e., lag phase, growth phase and stationary phase.

By fitting the growth model of Baranyi and Roberts (1994) to the experimental data, parameter estimates were obtained. As can be seen from Figure 4a, average lag phases values for the singular dextran systems and the liquid system were respectively approximately 4 and 2 times longer than those for the binary and singular gelatin systems. However, error bars were large, rendering the lag phase-value for the liquid not significant different from the ones observed for the binary systems.

The maximum growth rate (Figure 4b) was similar for all binary systems with an equal amount of dextran. Increasing the amount of dextran in the binary systems led to a small ($\pm 14\%$) but significant decrease in maximum growth rate. The lowest value was observed for the experiments performed in liquid and the 1D system. The 2D and 4G system displayed values in the range of the ones observed for the binary systems with the highest amount of dextran.

For the 1D binary systems, maximum cell densities (Figure 4c) were similar for all the systems, except the 3G/1D system, which displayed a slightly higher ($\pm 1\%$) value. Increasing the dextran concentration in the binary systems from 1D to 2D led to a decrease (± 0.4 [ln(CFU/ml)]) in the maximum cell density. Shifting from binary to singular systems caused an additional downshift in the maximum cell density. The liquid system exhibited the lowest values of maximum cell density.

4. Discussion

4.1. Phase-separation in gelatin-dextran systems

In Boons *et al.* (2014), phase-separation was observed in gelatin-dextran mixtures supplemented with BHI. In the present study, BHI was replaced by YPD in order to support

yeast growth. The gelatin-dextran mixtures supplemented with YPD also showed phase-separation. This is in accordance with what was described by Grinberg and Tolstoguzov (1997), i.e., a high ionic strength (> 0.5 M, ensured by the addition of NaCl) and a pH value (5.2-5.6) that exceeds the pI of gelatin (4.7-5.2, Sigma, Product Information Sheet Gelatin), will lead to phase-separation.

4.2. Preferential phase behavior of *S. cerevisiae* in gelatin-dextran systems

In Boons *et al.* (2014), *E. coli* growth was observed to preferentially happen in the dextran phase of the phase-separated gelatin-dextran system. A combination of two reasons was hypothesized to explain this behavior. At first, it was suggested that the negatively-charged *E. coli* was repelled from the negatively-charged gelatin molecules. Secondly, it was believed that the hydrophilic *E. coli* cells favored the hydrophilic dextran phase over the gelatin phase, which is consisting of molecules with both hydrophilic and hydrophobic groups. As observed for *E. coli*, *S. cerevisiae* grew in the dextran phase. The same explanation for the preferential growth phase as given for *E. coli*, is believed to be applicable for *S. cerevisiae* growth. *S. cerevisiae* is known to be a negatively-charged strain (Kang and Choi, 2005; Nekari-Setälä *et al.*, 2002; White *et al.*, 2011). In addition, White *et al.* (2011) and Kang and Choi (2005) reported on the hydrophilic nature of *S. cerevisiae*, which was here confirmed for this specific strain (MATH-test as described in Van den Broeck *et al.* (2011), results not shown). As such, the combination of electronegativity and hydrophilicity is believed to be the cause for the preferential phase behavior of *S. cerevisiae*.

4.3. Effect of cell immobilization

The present study reports on an increase in growth rate and maximum cell density of *S. cerevisiae* when biopolymers were added to broth. A small increase in maximum growth rate and maximum cell density was observed when broth is supplemented with one biopolymer, i.e.,

gelatin or dextran. When increasing medium structure complexity by introducing a second biopolymer and as such a heterogeneous microstructure, an even bigger increase of both growth parameters was observed in comparison to liquid systems. These observations indicate the importance of medium structure complexity on the growth behavior of *S. cerevisiae*. Also for *E. coli*, Boons et al., (2014) observed an increase in maximum growth rate when biopolymers were added to broth. However, no clear trend was observed for the maximum cell density. It must be noted that the use of YPD for yeast growth instead of BHI for bacterial growth, causes an alteration in microstructure, possibly causing a change in macro-scale growth behavior.

Studies on the effect of food structure on yeasts growth, as presented in this article, are scarce. Most studies were performed in relation to fermentation technology; they focus on the effect of yeast immobilization in/on a solid carrier on growth dynamics and metabolism. It is generally accepted that immobilization of yeast cells in solid carriers causes changes in growth behavior. In contrast to the presented results, most studies reported on a decreased growth rate when comparing immobilized growth in/on a solid carrier with freely dispersed cells (Bezbradica *et al.*, 2007; Doran and Bailey, 1986; Junter *et al.*, 2002; Parascandola and de Alteriis, 1996; van Iersel *et al.*, 2000). However, Junter *et al.* (2002) also referred to studies where no changes in maximum growth rate were observed and, as observed in the present paper, studies where immobilization caused an enhancement in maximum growth rate. As observed for the addition of gelatin and/or dextran to liquid systems, literature generally reported on a higher maximum cell density when yeast cells were immobilized (Bezbradica *et al.*, 2007; Nedovic *et al.*, 2001; Wada *et al.*, 1980; Wang and Hettwer, 1982). It must be stressed that in fermentation processes the solid structure is used as carrier and does not contain any nutrients to start with (Bangrak *et al.* 2011; Bezbradica *et al.* 2007; Burrill *et al.* 1983; Parascandola and de Alteriis, 1996; Wada *et al.* 1980). As such, mass transfer limitations will be different, affecting microbial growth dynamics.

To the knowledge of the authors, Mertens *et al.* (2011) were the first to study the effect of medium structure on the growth of yeast in food model systems. They reported on a general increased growth probability when a biopolymer structuring agent, i.e., Carbopol or xanthan gum, was added to the system. As observed in the presented results, Mertens and coworkers also noticed that the growth rate appeared to be faster in broth supplemented with biopolymers compared to growth in broth.

4.4. Effect of gelatin concentration

In contrast to what was observed for *E. coli* in Boons *et al.* (2014), the maximum growth rate and maximum cell density of *S. cerevisiae* were not affected by changing the gelatin concentration in the binary systems between 2.5 (1G) and 10% (4G).

As was already reported in literature (Abrusci *et al.*, 2007; Mertens *et al.*, 2009), the results in Figure 1e indicated that *S. cerevisiae* can metabolize gelatin. Changing the gelatin concentration hence led to a change in the concentration of metabolizable compounds, which could alter microbial growth. However, in the presence of glucose, gelatin did not seem to be consumed. It was hypothesized that in the mixtures in which growth parameters were studied, i.e., in the presence of 2% glucose, gelatin was not metabolized by *S. cerevisiae*.

Keeping the dextran concentration constant, changing the gelatin concentration led to a change in microstructure. In Boons *et al.* (2014), it was reported that for *E. coli* growth, there was a relation between the microscopic determined percentage of dextran phase and the population maximum cell density, i.e., the maximum cell density increased with increasing available dextran phase. However, this relation was only valid for microstructures exhibiting a disperse dextran phase in a gelatin matrix (2G/1D, 3G/1D and 4G/1D for systems supplemented with BHI). Different G/D ratios, resulting in different microstructures led to different maximum cell densities but the relation between the maximum cell density and the percentage of dextran phase did no longer hold. In the present study, changing the gelatin concentration in the mixtures led

to different microstructures, possibly affecting the microbial growth. However, different microstructures gave rise to similar growth parameters, making it difficult to define the microstructure by a certain (set of) parameter(s), e.g., percentage of dextran phase, and link these to the macroscopic behavior.

4.5. Effect of dextran concentration

Although no clear trend was observed when performing experiments with *E. coli*, increasing the dextran concentration in the binary system had a negative effect on the growth rate as well as on the maximum cell density of *S. cerevisiae*.

Dextran is not toxic to microorganisms (Ashwood and Warby, 1971), which is confirmed by the fact that increasing the dextran concentration in the singular systems, did not lead to a negative effect on the growth behavior. Adding dextran to the mixture may influence a_w and pH values and hence, affect microbial growth. The global water activity and pH values were within the same range for all binary mixtures (results not shown), excluding an influence of those global parameters. However, these global parameters do not reflect properties at the micro-scale, i.e., the scale at which microbial growth takes place. The local water activity or pH values sensed by the cells might reasonably change when increasing the dextran concentration and therefore, have an influence on microbial behavior.

No consumption of dextran was observed in Figure 1, ruling out the possible effect of nutrient concentration on growth dynamics.

Changing the dextran concentration in the mixtures led to different microstructures, possibly affecting the microbial growth. However, keeping the gelatin concentration the same, only two different dextran concentrations and hence microstructures were tested. This makes it difficult to draw general conclusions on the effect of certain microstructural properties on growth dynamics.

In conclusion, predictions based on experiments in liquid or media supplemented with a single biopolymer will give a fail dangerous prediction for systems containing phase-separating biopolymer mixtures. Medium composition in terms of biopolymers, i.e., gelling agents and the resulting medium structure complexity cannot be ignored in predicting growth behavior of food spoilers in food systems. Similar micro-scale behavior for *E. coli* and *S. cerevisiae* in the heterogeneous gelatin/dextran systems was observed. Although the addition of biopolymers to the growth media causes an increase in maximum growth for both *E. coli* and *S. cerevisiae*, the response of the bacteria and yeast to the different ratios of gelatin and dextran is different. Hence, in contrast to the similar micro-scale behavior and the comparable response to cell immobilization, the effect of the concentration of added biopolymers on the growth parameters are not transferrable from bacteria to yeast.

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Figures

Figure 1: Average values and standard errors of metabolizing ability-tests: a) Ln-transformed viable count of *Saccharomyces cerevisiae* in salt solution (2.9% NaCl) supplemented with 2% glucose (o), 2.5% dextran (□) and 5% gelatin (◇); b) Ln-transformed viable count of *Saccharomyces cerevisiae* and in YP supplemented with glucose (◁), dextran (△), gelatin (▷)

and glucose, dextran and gelatin (*); c) Percentage of unconsumed glucose in salt solution (o) and YP (◁) supplemented with glucose and YP supplemented with glucose, dextran and gelatin (*); d) Percentage of unconsumed dextran in salt solution (□) and YP (△) supplemented with dextran and YP supplemented with glucose, dextran and gelatin (*); e) Percentage of unconsumed gelatin in salt solution (◇) and YP (▷) supplemented with gelatin and YP supplemented with glucose, dextran and gelatin (*).

Figure 2: Confocal microscope images of *S. cerevisiae* (red) in gelatin (G, green)-dextran (D, black) mixtures: I) 1G/1D, II) 2G/1D, III) 3G/1D, IV) 4G/1D, V) 2G/2D, VI) 3G/2D and VII) 4G/2D.

Figure 3: Growth curves of *S. cerevisiae* at 23.5°C in different mixtures of gelatin (G) and dextran (D): 1G/1D ($\triangleright$), 2G/1D ($\circ$), 3G/1D ($+$), 4G/1D ($\square$), 2G/2D ($\diamond$), 3G/2D ($\triangleleft$), 4G/2D ($*$), 1D ($\blacktriangle$), 2D ($\blacktriangleright$), 4G ($\blacktriangleleft$) and YPG broth ($\bullet$).

Figure 4: Growth parameters and standard errors for *S. cerevisiae* at 23.5°C in G/D binary and singular systems and YPD broth: a) the duration of the lag phase, b) the maximum specific growth rate and c) the maximum cell density.