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Culture medium optimization for osmotolerant yeasts by use of a parallel fermenter system and rapid microbiological testing

Jens Pfannebecker [a,*], Claudia Schiffer-Hetz [a], Jürgen Fröhlich [b], Barbara Becker [a]

[a] Ostwestfalen-Lippe University of Applied Sciences, Dept. Life Science Technologies, Microbiology, Liebigstraße 87, D-32657 Lemgo, Germany
[b] Erbslöh Geisenheim AG, Erbslöhstraße 1, D-65366 Geisenheim, Germany

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ABSTRACT

In the present study, a culture medium for qualitative detection of osmotolerant yeasts, named OM, was developed. For the development, culture media with different concentrations of glucose, fructose, potassium chloride and glycerin were analyzed in a Biolumix™ test incubator. Selectivity for osmotolerant yeasts was guaranteed by a water activity (a_w)-value of 0.91. The best results regarding fast growth of *Zygosaccharomyces rouxii* (WH 1002) were achieved in a culture medium consisting of 45% glucose, 5% fructose and 0.5% yeast extract and in a medium with 30% glucose, 10% glycerin, 5% potassium chloride and 0.5% yeast extract. Substances to stimulate yeast fermentation rates were analyzed in a RAMOS® parallel fermenter system, enabling online measurement of the carbon dioxide transfer rate (CTR) in shaking flasks. Significant increases of the CTR was achieved by adding especially 0.1–0.2% ammonium salts ($(NH_4)_2HPO_4$, $(NH_4)_2SO_4$ or NH_4NO_3), 0.5% meat peptone and 1% malt extract. Detection times and the CTR of 23 food-borne yeast strains of the genera *Zygosaccharomyces*, *Torulaspora*, *Schizosaccharomyces*, *Candida* and *Wickerhamomyces* were analyzed in OM bouillon in comparison to the selective culture media YEG50, MYG50 and DG18 in the parallel fermenter system. The OM culture medium enabled the detection of 10^2 CFU/g within a time period of 2–3 days, depending on the analyzed yeast species. Compared with YEG50 and MYG50 the detection times could be reduced. As an example, *W. anomalus* (WH 1021) was detected after 124 h in YEG50, 95.5 h in MYG50 and 55 h in OM bouillon. Compared to YEG50 the maximum CO_2 transfer rates for *Z. rouxii* (WH 1001), *T. delbrueckii* (DSM 70526), *S. pombe* (DSM 70576) and *W. anomalus* (WH 1016) increased by a factor ≥2.6. Furthermore, enrichment cultures of inoculated high-sugar products in OM culture medium were analyzed in the Biolumix™ system. The results proved that detection times of 3 days for *Z. rouxii* and *T. delbrueckii* can be realized by using OM in combination with the automated test system even if low initial counts (10^1 CFU/g) are present in the products. In conclusion, the presented data suggest that the OM culture medium is appropriate for the enrichment of osmotolerant yeasts from high-sugar food products.

1. Introduction

Osmotolerant yeasts are able to grow in sugar concentrations up to 40–70% (Lodder, 1970) and frequently cause spoilage in sugar syrup and sugar-rich matrices like chocolate fillings, fruit juice concentrates, molasses, dried fruit, marzipan and honey (Deák, 2008; Fleet, 1992; Tokuoka, 1993; Marvig et al., 2014; Wang et al., 2015b). Products such as dressings, ketchup, mayonnaise, soy sauce and condensed milk are also considered susceptible to a contamination with osmotolerant yeasts (Deák, 2008; Xu et al., 2014). One main problem of a contamination with osmotolerant yeasts is due to the slow, significant CO_2 formation over long storage times. Gas formation leads to product spoilage, for instance, cracking of marzipan or chocolate pralines and packaging swellings (Jermini et al., 1987; Tokuoka et al., 1985; Martorell et al., 2007). Temperature changes may result in condensation of water vapor on product surfaces which accelerates the growth of osmotolerant yeasts (Tokuoka, 1993; Rojo et al., 2014). Moreover, the formation of metabolic compounds such as alcohol, acetic acid or acetaldehyde may cause off-flavors (Fleet, 1992; Wang et al., 2015a). Gas formation and sensory modifications of food entail complaints and product recalls which implicate considerable economic and image losses for the enterprises concerned. As lots of foods are being processed, preserved and stored or transported over long distances nowadays, the importance of osmotolerant spoilage yeasts is increasing (Martorell et al., 2007).

Yeasts of the genus *Zygosaccharomyces* are most frequently isolated from sugar-rich raw materials and products and are often associated with food spoilage (James and Stratford, 2003; Jermini et al., 1987; Martorell et al., 2005; Vermeulen et al., 2012). Furthermore, species of the genera *Schizosaccharomyces*, *Torulaspora*, *Candida*, *Pichia*,

* Corresponding author at: Ostwestfalen-Lippe University of Applied Sciences, Dept. Life Science Technologies, Microbiology, Liebigstr, 87, D-32657 Lemgo, Germany.
E-mail address: jens.pfannebecker@hs-owl.de (J. Pfannebecker).

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Wickerhamomyces, *Kluyveromyces*, *Hanseniaspora* and *Debaryomyces* can also be detected in these matrices (Fleet, 1992; Marvig et al., 2014; Combina et al., 2007; Deák and Beuchat, 1992). Many strains of these species tolerate high amounts of ethanol, acetic acid and preserving agents such as benzoic acid, sorbic acid or sulfur dioxide (Dittrich and Grossmann, 2005; El Halouat and Debevere, 1996; Stratford et al., 2013; Rojo et al., 2015).

Culture media such as YGC, YPD, TGYC (Samson et al., 1992), DG18 (Hocking and Pitt, 1980) or DRBC (King et al., 1979) are frequently used for isolation, cultivation and enumeration of osmotolerant yeasts. DG18 medium applied in ISO/FDIS 21527-2 (2008), is characterized by an a_w-value of 0.95 which is realized by addition of glycerin. Selectivity towards fast growing fungi and bacteria is achieved by adding dichloran and chloramphenicol. Comparative studies illustrated that DG18 agar cannot always be recommended for analyzing yeasts from dry matrices with low a_w-values (Beuchat et al., 2001; Deák et al., 2001). Many company and service laboratories carry out their tests in broth cultures on a qualitative basis (presence-absence tests) by proving gas formation. Apart from the above mentioned culture media standard culture media such as glucose broth or wort broth are often applied for this purpose. All these culture media have in common that the a_w-values are not significantly reduced, i.e., when used in quality control osmotolerant yeasts are not specifically detected.

Another strategy is the use of culture media which attain their selectivity for osmotolerant yeasts by low a_w-values. Jermini et al. (1987) and Pitt and Hocking (1985) published culture media with a_w-values between 0.89 and 0.91 by high glucose concentrations. Detection times in these culture media vary between 5 and 10 days (Jermini et al., 1987; Beuchat, 1993). Although a high selectivity for osmotolerant yeasts is achieved, detection in these culture media may be impeded due to long growth periods and low fermentation rates depending on the yeast species present in the analyzed foods. In general, differences in performance among media are attributed to the diversity of yeasts likely to be present in test foods and differences in nutrients, pH, and water activity requirements for resuscitation of stressed cells and colony development (Beuchat and Mann, 2016). From our perspective, there is a need for a standardized method for qualitative testing of osmotolerant yeasts in high-sugar foods and raw materials which produces comparable results in microbiological quality control laboratories.

The objective of this study was the development of a culture medium for qualitative detection of osmotolerant yeasts in high-sugar foods with special attention to rapid growth and high fermentation rates. The medium was supposed to detect microbial counts from 10^1–10^2 colony forming units (CFU)/mL as well as enabling a selective and rapid detection in <5 days. An automated test incubator was applied for initial experiments concerning fundamental issues of culture medium composition in regard of sugars and osmotically active substances. The influence of individual substances and concentrations on the metabolic activity of osmotolerant yeasts was observed in a RAMOS® parallel fermenter system (HiTec Zang, Herzogenrath, Germany). The focus was particularly on the analysis of the carbon dioxide transfer rate (CTR) during yeast growth. Respiratory rates such as the CTR and the oxygen transfer rate (OTR) are universal parameters to represent the physiological state of a biologic culture (Anderlei et al., 2004). Characteristic measuring curves can be used to depict substrate limitation, diauxic growth or other biologic phenomena (Anderlei and Büchs, 2001).

Based on the data generated through these experiments a culture medium for osmotolerant yeasts, designated OM, was compiled. Yeast growth and fermentation rates in OM culture medium were directly compared to those in YEG50, MYG50 and DG18 under defined conditions. Until now, no data have been available concerning the comparative analysis of yeast fermentation rates in selective culture media with reduced a_w-values. Experimental inoculation tests for the detection of osmotolerant yeasts in high-sugar foods were carried out with simultaneous CO_2 measurements by use of the parallel fermenter system and the automated test incubator.

2. Materials and methods

2.1. Osmotolerant yeast strains

Strains from culture collections and yeast isolates which were originally isolated from high-sugar raw materials and products were applied (Table 1). Strains from culture collections were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands) and the culture collection of the Institute of Microbiology and Wine Research, University of Mainz, Germany. Additional osmotolerant yeasts were isolated from different high-sugar foods and raw materials by enrichment of 10 g product in 90 mL YEG50 bouillon (Jermini et al., 1987). The enrichment samples were incubated for 7 days at 30 °C and streaked out on YEG50 agar for several times to ensure the isolation of pure cultures. The yeast isolates were identified by amplification of the internal transcribed spacer (ITS) region using Primer ITS 1 and ITS 4 (White et al., 1990) and subsequent sequencing (Eurofins Genomics, Ebersberg, Germany). To verify the identification results, the yeast strains were additionally examined by DNA fingerprinting via SAPD-PCR using primer A-Not (Pfannebecker and Fröhlich, 2008). For strain maintenance aliquots were prepared in Cryoinstant (VWR, Darmstadt, Germany) and stored at −152 °C. The yeast strains were cultivated by adding one cryo pearl to 10 mL of the culture medium to be tested with subsequent incubation for 3–5 days at 30 °C. For testing in the Biolumix™-system (I&L Biosystems) and the RAMOS® parallel fermenter system (HiTec Zang, Herzogenrath, Germany), precultures were produced by transferring cultures twice in 10 mL of the culture medium to be tested and incubated under the above-mentioned conditions.

2.2. Use of an automated test incubator for culture medium optimization

The Biolumix system comprises an incubator with analyzer, control software and special test vials filled with culture medium. The test vials consist of an incubation and detection area with an embedded optical carbon dioxide (CO_2) sensor at the bottom of the test vial. CO_2 which occurs due to metabolic processes diffuses into the sensor, reacts

Table 1
Osmotolerant yeast strains used in this study.

Strain	Species	Source
CBS 4512	*Zygosaccharomyces rouxii*	Marzipan
WH 1001	*Zygosaccharomyces rouxii*	Fructose syrup
WH 1002	*Zygosaccharomyces rouxii*	Marzipan
WH 1003	*Zygosaccharomyces rouxii*	Treacle
WH 1004	*Zygosaccharomyces rouxii*	Invert sugar syrup
WH 1005	*Zygosaccharomyces rouxii*	Date
WH 1018	*Zygosaccharomyces rouxii*	Treacle
DSMZ 70492	*Zygosaccharomyces bailii*	Apple juice
CBS 1091	*Zygosaccharomyces mellis*	Honey
DSMZ 70526	*Torulaspora delbrueckii*	Marzipan
WH 1013	*Torulaspora delbrueckii*	Chocolate truffle
DSMZ 70576	*Schizosaccharomyces pombe*	Grape must
CBS 1042	*Schizosaccharomyces pombe*	Grape must
IMW 81	*Schizosaccharomyces pombe*	Wine
IMW 330	*Schizosaccharomyces pombe*	Wine
IMW 339	*Schizosaccharomyces pombe*	Wine
WH 1016	*Wickerhamomyces anomalus*	Chocolate marshmallow
WH 1021	*Wickerhamomyces anomalus*	Praline
WH 1026	*Wickerhamomyces anomalus*	Invert sugar syrup
WH 1010	*Candida parapsilosis*	Chocolate truffle
WH 1015	*Candida lusitaniae*	Nougat filling
WH 1023	*Candida magnoliae*	Honey
WH 1025	*Candida* sp.	Invert sugar syrup

DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. IMW = culture collection of the Institute of Microbiology and Wine Research, University of Mainz, Germany.