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Utilization of the Dicarbonyl Compounds 3-Deoxyglucosone and 3-Deoxymaltosone during Beer Fermentation by *Saccharomyces* Yeasts

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Abstract: In beer production, 1,2-dicarbonyl compounds such as 3-deoxyglucosone (3-DG) and 3-deoxymaltosone (3-DM) are formed via Maillard reaction or caramelization especially during malt kilning or wort boiling, resulting in substantial concentrations in wort. Consequences of dicarbonyl compounds for yeast metabolism are widely unknown. In the present study, the handling of 3-DG and 3-DM by *Saccharomyces* strains from different habitats in wort and during beer fermentation was investigated. We show that beer yeast strains induced a faster 3-DG degradation in Pilsner wort and were additionally more stress-resistant to 3-DG compared to yeasts isolated from natural habitats. In fermentation experiments comparing a light wort and a dark wort prepared from malt extracts, it could be shown that high levels of 3-DM in dark wort influence the utilization of 3-DG by yeasts, and thus higher levels of 3-DG remain in the wort. Beer yeast strains showed an increased formation of 3-deoxyfructose (3-DF) with up to 220 µM, which is possibly due to a preferred metabolization of 3-DM, as indicated by the low degradation rate of 3-DG. In contrast, yeasts isolated from natural habitats produced significantly lower amounts of 3-DF. This suggests an adaptation of technologically used yeasts to metabolization of dicarbonyl compounds, possibly as a result of beer yeast domestication.

Keywords: Maillard reaction; 3-deoxyglucosone; 3-deoxymaltosone; beer wort; *Saccharomyces* yeasts; metabolization; toxicity

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1. Introduction

The Maillard reaction between reducing sugars and amino compounds is of outstanding importance for the color, taste and aroma of heated food [1]. In the advanced stage of the complex reaction cascade, 1,2-dicarbonyl compounds such as 3-deoxyglucosone (3-DG) or 3-deoxymaltosone (3-DM) are formed (see Figure 1), which are highly reactive and represent precursors for many subsequent reactions [2–4].

In beer production, the Maillard reaction is of high importance due to several thermal process steps and is widely researched in this context. Already during malt roasting, a large variety of Maillard reaction products (MRPs) are formed which are transferred into the wort during mashing [5]. In addition, these compounds are formed during wort boiling and ultimately determine the color and the aroma of the final beer [6]. Another important process step is the fermentation by *Saccharomyces* yeasts, which not only converts usable sugars to ethanol and carbon dioxide, but also plays an important role in aroma production by utilizing available substrates such as MRPs in the wort [7]. During beer production and in the final product, a monitoring of MRPs is of particular relevance for

the product quality, as the dicarbonyl compounds such as 3-DG are precursors for the Strecker degradation, a reaction which is of particular importance for flavor formation [8]. 3-DG is the major α-dicarbonyl compound in beer. 3-DG is formed in high amounts during the malting process (21.6–40.7 µmol/100 g of dry weight malt), is partially released into the mash (80 µmol/L) and is formed during wort boiling (179 µmol/L) [6,8,9]. In beer, 3-DG contents of 49–333 µmol/L could be measured [6,8,10–12]. In addition to 3-DG, the formation of the dicarbonyl compound 3-deoxymaltosone (3-DM) from maltose is also of particular relevance in beer, since maltose is the main sugar in beer wort [13]. 3-DM is already formed during the kilning process, especially by higher roasting temperatures, with contents from 99 µmol/kg of dry weight malt (light malt) to 2016 µmol/kg of dry weight malt (dark malt/caramel malt), and subsequently transferred into the wort [14]. Levels of 3–12 µmol/L of 3-DM were detected in Pilsner beer [11,12]. During fermentation, the *Saccharomyces* yeasts come into contact with these highly reactive carbonyl compounds, which may impact the viability of the yeast cells by exerting increased carbonyl stress [15]. Dealing with MRPs such as 3-DG or 3-DM is therefore essential for the yeast cells to work efficiently during fermentation and thus also for the flavor profile of the final product [16].

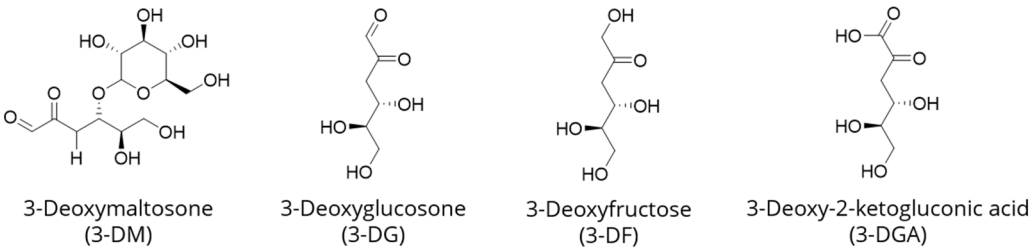

Figure 1. Structures of 3-DM, 3-DG and the yeast metabolites 3-DF and 3-DGA analyzed in this study.

It is known that yeasts have detoxification mechanisms to metabolize cytotoxic substrates into less toxic secondary products [15,17–19]. 3-DG, for example, is reduced to 3-deoxyfructose (3-DF; see Figure 1), which has already been found in beer at levels of 2.4 to 61.5 µmol/L [18]. In preliminary studies, it could be further shown that technologically used *Saccharomyces* beer yeast strains and yeast strains isolated from natural habitats differ in their handling of 3-DG [20]. The beer yeast strains studied showed a different utilization of the 1,2-dicarbonyl compound in terms of the degradation rate, the measured intracellular 3-DG and 3-DF as well as a higher stress resistance to the MRP, which could be shown by determining the dead cell count after incubation experiments. The results indicated that technologically used yeasts may have adapted to higher concentrations of 3-DG, possibly in the course of domestication. However, these experiments were model experiments in a defined minimal medium to investigate the utilization of 3-DG without competing reactions and interfering substances [20]. Furthermore, it is still unknown whether yeasts can utilize the disaccharide 3-DM and if this may have an influence on the metabolization of other compounds such as 3-DG.

Therefore, the aim of this study was to investigate the handling of 3-DG and 3-DM by *Saccharomyces* yeasts under technologically relevant conditions. For this purpose, incubation experiments with 3-DG in Pilsner wort were carried out in order to assess the dicarbonyl utilization in a complex wort matrix. Dead cell count was determined, and growth curves with selected yeast strains were recorded following the addition of different 3-DG concentrations in Pilsner wort, in order to be able to assess the toxicity of the MRP in this habitat. Furthermore, fermentation experiments under conditions relevant to practice using different worts with varying contents of 3-DG and 3-DM were performed. The stability and metabolization of 3-DG and 3-DM in the presence of different yeasts was investigated by HPLC-UV (high-performance liquid chromatography with ultraviolet detection) and GC-MS (gas chromatography with mass-spectrometric detection) in order to obtain information about a possible adaption of beer yeast strains to an efficient use of dicarbonyl compounds in beer wort.

2. Materials and Methods

2.1. Chemicals

The following substances were purchased from commercial suppliers: Yeast extract for microbiology, peptone from soy bean meal (enzymatically digested), malt extract for microbiology and ortho-phenylenediamine (OPD) from Sigma Aldrich, Steinheim, Germany; acetonitrile HPLC grade (Fisher Chemical, Loughborough, UK); agar for microbiology (Marcor, Carlstadt, NJ, USA); pyridine (Acros Organics, Morris Plains, NJ, USA); di-sodium hydrogen phosphate dihydrate, hydroxylamine hydrochloride and sodium dihydrogen phosphate monohydrate from Grüssing GmbH, Filsum, Germany; d-(+)-glucose and acetic acid from Carl Roth GmbH & Co KG, Karlsruhe, Germany; methanol HPLC grade, sodium chloride, n-hexane as well as hydrochloric acid from VWR Chemicals, Fontenay-sous-Bois, France; methylene blue (Merck, Darmstadt, Germany); N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (Supelco, Bellefonte, PA, USA); ultrapure water for the preparation of solutions and HPLC eluents was produced in-house with the Destamat Bi 18E apparatus, QCS GmbH, Maintal, Germany; tris-(hydroxymethyl)-aminomethane (AppliChem GmbH, Darmstadt, Germany); Pilsner malt extract and Dunkel malt extract from Weyermann, Bamberg, Germany. The Polaris hop pellets were purchased by www.brauen.de, accessed on 28 April 2024. The substrates 3-deoxyglucosone (3-DG) [21], 3-deoxyfructose (3-DF) [22], [^{13}C]3-deoxyglucosone ([^{13}C]3-DG) and [^{13}C]3-deoxy-2-ketogluconic acid ([^{13}C]3-DGA) [23] and 3-deoxymaltosone (3-DM) [14] were synthesized according to mentioned literature methods and were used for the incubation experiments and measurements.

2.2. Yeast Strains and Cultivation Conditions

The yeasts studied were *Saccharomyces* species of different origins (isolates from natural habitats such as forest soil or fruits and technologically used top/bottom-fermented brewery yeast strains) and are the same strains as already described in previous studies (see Supplements Table S1) [20,24]. In addition, *S. cerevisiae* BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; deletion strain from *S. cerevisiae* S288C) was included as reference yeast purchased from EUROSCARF (Institute of Microbiology, University of Frankfurt, Germany). This strain was supplied as a cultured agar plate and could be used directly for further culturing. Yeast propagation and cultivation were performed according to Kertsch et al. [20].

2.3. Incubation Experiments in Pilsner Wort—Stability of 3-DG in the Presence of Saccharomyces Strains

At the beginning of the incubation experiments, stock cultures (in the following referred to as main cultures) of the yeasts studied were prepared as described in Kertsch et al. [20]. These were used for all experiments to prepare yeast suspensions with an adjusted optical density (OD) of 5 (incubation and fermentation experiments) or 0.2 (growth curves) in Pilsner wort (prepared from Pilsner malt extract from Weyermann, Bamberg, Germany; for exact production and specifications see fermentation experiments). The incubation experiments were carried out according to Kertsch et al. [22]. Samples were stored at −80 °C until HPLC-UV and GC-MS analysis. Incubations were performed in triplicate. For determination of a potential adsorption of 3-DG to the yeast cells, the experiment was repeated exactly as described above with previously autoclaved main cultures (121 °C, 90 min).

2.4. Cell Disruption for Analysis of Internalized 3-DG and Yeast Metabolites

To completely elucidate the utilization of 3-DG by yeast cells, the 24 h incubation samples and the fermentation samples after day 14 of *Saccharomyces* yeast strains were lysed using glass beads (diameter: 0.5 mm, FR0603, Retsch, Haan, Germany). Cell disruption was performed exactly as described in Kertsch et al. [20]. The supernatants of the lysed

yeast samples were subsequently prepared for HPLC-UV (3-DG and 3-DM concentrations) or GC-MS (concentrations of the metabolites 3-DF and 3-DGA) measurements.

2.5. Dead/Live-Cell Experiments

To evaluate the toxicity of 3-DG on *Saccharomyces* yeasts, the percentages of dead cells in the sample solutions were determined after 24 h of incubation. For this purpose, the yeast samples were diluted 1:25 with 0.9% NaCl solution in a new tube (60 µL of sample solution and 1440 µL of NaCl) and 300 µL of a methylene blue solution (100 mg of methylene blue in 100 mL of ultrapure water) was added and incubated for 10 min. Samples were then shaken again and counted using the Thoma counting chamber on the microscope at 40× magnification. In each case, the total cell number and the number of dead cells in the solutions (blue-colored cells) were determined. For a quantitative evaluation of the toxicity of the substrates, the determined proportion of dead cells in the YBs (incubation without the addition of 3-DG) was subtracted from the respective proportion of dead cells in the samples.

2.6. Growth Curves of Saccharomyces Yeasts with 3-DG in Pilsner Wort

In order to assess the influence of 3-DG on the growth of various *Saccharomyces* yeasts in more detail, growth curves of the selected yeast strains were recorded following addition of different 3-DG concentrations [16,25]. For this purpose, the main cultures of the yeast strains were adjusted to an optical density (OD) of 0.2, slurried in Pilsner wort, in a 2 mL tube. Furthermore, 3-DG standards of different concentrations (0.5 mM, 2 mM, 10 mM, 20 mM) were prepared in ultrapure water and sterile-filtered (0.2 µm) before use. For the assay, 75 µL of each of the 3-DG standards was pipetted into the cavities of a 96-well plate (sterile packed, Sarstedt AG & Co. KG, Nürnbrecht, Germany). An aliquot of 75 µL of each of the adjusted yeast suspensions was added, such that the resulting OD was 0.1. The yeasts were thus exposed to 3-DG concentrations of 0.25 mM, 1 mM, 5 mM and 10 mM, respectively. In addition to the growth experiments, a yeast blank (YB) without the addition of 3-DG was also included (75 µL of yeast suspension + 75 µL of ultrapure water) to detect changes in growth, as well as a medium blank (MB; 75 µL of Pilsner wort + 75 µL of ultrapure water) to demonstrate the sterility of the experimental batch. The plate was incubated for 72 h at 30 °C with continuous orbital shaking to obtain the growth curves. The OD at 600 nm was measured every 20 min using a Biotek EPOCH 2 microplate reader (Agilent, Santa Clara, CA, USA). The end of the lag phase, and thus a growth of the yeasts, was defined as the OD being 2 times higher than the initial OD, meaning that, on average, each yeast cell had divided once (as measured in OD-cell count correlations). To calculate possible growth delay or arrest in the presence of 3-DG, the time of the lag phase measured for yeast growth in the growth samples was divided by the lag time obtained for the yeast growth in the YB on the respective 96-well plate. The calculated growth delay gives a statement about the toxicity of 3-DG on yeast cells. If the OD could not reach the defined factor of 2 during the incubation period of 72 h, the yeasts did not grow, and this effect was defined to be antimycotic. All growth curves were recorded as duplicates.

2.7. Fermentation Experiments in Different Beer Worts—Degradation and Metabolization of 3-DG and 3-DM by Saccharomyces Strains

In order to transfer the incubation experiments to technologically relevant conditions, fermentation studies were carried out in beer wort, which was produced using a brewing system "Braumeister" (Speidel Tank- und Behälterbau GmbH, Ofterdingen, Germany). A quantity of 1.5 kg of malt extract (Pilsner or dark beer malt extract, respectively, both from Weyermann, Bamberg, Germany) was dissolved in 10 L of water to obtain an original gravity of 13 °P (based on the dilution table from Weyermann, Bamberg, Germany), transferred to the Braumeister and heated. At the set temperature of 100 °C, 4.5 g of Polaris hop pellets were added, and the wort was boiled for 1 h. Following boiling, 9.5 L of wort with a final gravity of 13 °P (as measured with a density meter from Anton Paar,

Ostfildern-Scharnhausen, Germany) for both worts were obtained. Aliquots of 1 L of each wort were hot-filtered after boiling (around 90 °C) into sterile 2 L flasks and cooled to room temperature (RT; 21 °C). Then, the yeast pellets were transferred to the worts at a set OD of 20 (prepared from the yeast main cultures [20]), resulting in 1.5×10^7 cells/mL of wort), and the flasks were capped with fermentation lids to ensure continuous pressure equalization. The fermentation flasks were stored at room temperature (21 °C) for 7 days and at 6 °C for another 7 days. Samples of 1 mL each were taken at days 0, 1, 2, 3, 5, 7 and 14 and were transferred to a 2 mL tube and immediately placed on ice. The samples were then stored at −80 °C until analysis. Furthermore, on the sampling days, the extract contents were additionally determined using a density meter (Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) to observe the fermentation process. In addition to the fermentation samples, wort prepared as above but without the addition of yeast was incubated as a reference. Furthermore, the experiment was repeated exactly as described above with previously autoclaved yeast main cultures (121 °C, 90 min; Addition of 1.5×10^7 cells/mL to the worts) to be able to consider a potential adsorption of 3-DG to the yeast cells. The fermentation samples were analyzed by HPLC-UV (degradation of 3-DG and 3-DM) and GC-MS (metabolite formation of 3-DF and 3-DGA) analogous to the incubation samples. The fermentations were performed in duplicate.

2.8. Analysis of 3-DG and 3-DM

The measurement was carried out according to the method published by Degen et al. [10] with some modifications, using a low-pressure gradient system consisting of a mixing chamber (K-1500; Knauer, Berlin, Germany), an autosampler (Basic Marathon, Spark Holland, Emmen, The Netherlands), a pump (Smartline 1000), an online degasser and a diode array detector DAD 2.1L (all from Knauer, Berlin, Germany). Data analysis was performed using ClarityChrom version 6.1.0.130 software (Chrom Tech Inc., Apple Valley, MN, USA). A stainless steel column (250 mm × 4.6 mm, 5 µm) filled with ProntoSIL 60 Phenyl material with an integrated precolumn (5 mm × 4 mm) with the same material (Knauer, Berlin, Germany) was used at room temperature (RT). Solvent A was 0.075% acetic acid in ultrapure water, and solvent B was a mixture of solvent A and methanol (20/80, *v*/*v*). A gradient was applied (0 min, 10% B; 25 min, 50% B; 30 min, 50% B; 34 min, 70% B; 49 min, 70% B; 51 min, 10% B; 58 min, 10% B) at a flow rate of 0.7 mL/min, and the injection volume was 20 µL. Absorbance was measured at 312 nm, and UV spectra were recorded between 190 and 400 nm throughout the run. Prior to analysis, 50 µL of each sample was mixed with 50 µL of methanol/acetonitrile (*v*/*v*, 30/70) and stored at 4 °C for 1 h for protein precipitation. Subsequently, the samples were centrifuged (10,000× *g*, 10 min, 4 °C), diluted with ultrapure water (50 µL each) and mixed with a 0.5 M sodium phosphate buffer (pH = 6.5; 50 µL) and a 0.2% solution of o-phenylenediamine (OPD; 50 µL) for derivatization. The mixtures were stored overnight in the dark, and 180 µL were finally transferred to HPLC vials (1.5 mL, glass, VWR, Darmstadt, Germany) with inserts (5 mm × 31 mm, Macherey-Nagel, Düren, Germany). Concentration determinations were carried out by external calibrations in ultrapure water of the quinoxalines of 3-DG and 3-DM from 5 calibration points each, covering the concentration range from 1.0 µM to 300 µM. Quinoxalines of 3-DG and 3-DM were synthesized according to Hellwig et al., 2010 [26]. Individual concentrations of the Maillard reaction products in the 24 h incubation samples were determined in triplicate; the fermentation samples were analyzed in duplicate.

2.9. Gas Chromatography with Mass-Spectrometric Detection (GC-MS)

GC-MS analysis of yeast metabolites resulting from 3-DG (incubation and fermentation experiments) or 3-DM (fermentation experiments) was performed as described in Kertsch et al. [20]. The parameters of the SIM (selected ion monitoring) method used for 3-DG, the yeast metabolites 3-DF and 3-DGA as well as the internal standards (IS) used ([^{13}C]3-DG and [^{13}C]3-DGA) are listed in Table 1. Mass spectra were obtained in analytical runs using the same gradient in the *m/z* range between 70 and 700.

Table 1. SIM parameters for quantitation of 3-DG and the yeast metabolites 3-DF and 3-DGA in model incubation and fermentation experiments in Pilsner wort.

Compound	Time Frame	Quantifier Ion	Dwell Time	Qualifier Ion	Dwell Time
	min	*m*/*z* *	ms	*m*/*z* *	ms
3-DF [1]	20.60–22.50	214	40	524	70
3-DGA		348	40	538	40
[^{13}C]-3-DGA		352	25	544	40
3-DG	22.85–31.00	347	60	537	60
[^{13}C]-3-DG		351	45	543	45

* Mass/number of charge. [1] 3-DF was quantitatively evaluated by peak area ratio to IS [^{13}C]-3-DG.

Sample preparation of the incubation and fermentation experiments was carried out as described in Kertsch et al. [22] with [^{13}C]3-DGA as an additional internal standard. The supernatants of the incubation samples after cell disruption were directly used without a prior dilution of 1:10. For quantification, an external calibration spiked with the IS [11 µM each] was carried along. This was processed analogously to the samples from the precipitation step with the acetonitrile/methanol mixture and covered a calibration range of 1.0 µM to 20 µM (3-DG) and 0.33 µM to 2 µM (3-DF and 3-DGA) consisting of 7 calibration points. Oximation leads to the formation of syn- and anti-isomers. In the evaluation, the areas of both isomers of 3-DF were considered for quantification.

3. Results and Discussion

3.1. Utilization of 3-DG by Saccharomyces Strains

In our previous work, it was shown for the first time that *Saccharomyces* yeasts differ in their handling of 3-DG in a minimal medium, with technologically relevant yeasts showing a faster 3-DG utilization as well as being more stress-resistant to the dicarbonyl compound compared to strains isolated from natural habitats [20]. The aim of this study was to transfer these preliminary experiments to conditions relevant for beer brewing. In an initial screening experiment, yeasts were incubated in Pilsner wort at 30 °C for 24 h to investigate the influence of the medium on the utilization of 3-DG by yeasts. In order to provide comparable starting conditions, the yeast suspensions were adjusted to an optical density (OD) of 5. By counting the cells of the strains at this OD before incubation experiments, a comparable cell number of 6×10^7 cells/mL on average could be determined, thus ensuring equal incubation conditions for the yeasts. The exact cell counts as well as the calculated cell densities after the 24 h incubation can be found in Supplements Table S2. In parallel with the incubations of 3-DG in the presence of the yeast strains, the stability of the dicarbonyl compound in Pilsner wort without the addition of yeast was assessed (stability blank, SB). 3-DG remained stable (98 ± 3%, n = 3) under these conditions. Therefore, an observed degradation of 3-DG in the incubation samples could be completely attributed to the metabolism of the yeast cells. With the aim of reporting potential growth-modulating effects of 3-DG, yeast viability was monitored by measuring the OD at 600 nm after 24 h of incubation (see Supplements Table S2). Here, it was already noticeable that the beer yeast strains with the addition of 3-DG showed, on average, a growth increase in comparison to the yeast blank (YB) without 3-DG. In contrast, the growth of the yeasts isolated from natural habitats was consistently inhibited in the presence of the 1,2-dicarbonyl compound. This already indicates different sensitivities of the investigated yeasts to 3-DG. Nevertheless, the influences are proportionally low, and therefore, comparable metabolic performances of the yeasts might still be assumed.

Following incubation of *Saccharomyces* yeasts for 24 h at 30 °C in the presence of 450 µM of 3-DG in Pilsner wort, it was found that all strains studied were able to significantly degrade the dicarbonyl compound, with the beer yeast strains being more effective when compared to the strains isolated from natural habitats (see Supplements Figure S1A). For beer yeast strains, a decrease in the amount of 3-DG ranging between 30% and 40% was observed (Figure 2, grey bars), whereas for the strains isolated from natural habitats, only

between 10% and 20% of the dicarbonyl was degraded (Figure 2, black bars). An exception was the bottom-fermenting beer yeast strain w34/70, for which a lower decrease in the content 3-DG was found, which could be due to the higher difference from the strain's usual fermentation temperature of 8 °C to 14 °C [27]. The highest degradation rate of 60% was documented for the wine yeast "Steinberg" (Figure 2, white bar). For the reference yeast BY4741, the lowest degradation of 3-DG with only 8% was observed, thus ranking this yeast among the results obtained for the yeasts isolated from natural habitats (Figure 2, striped bar).

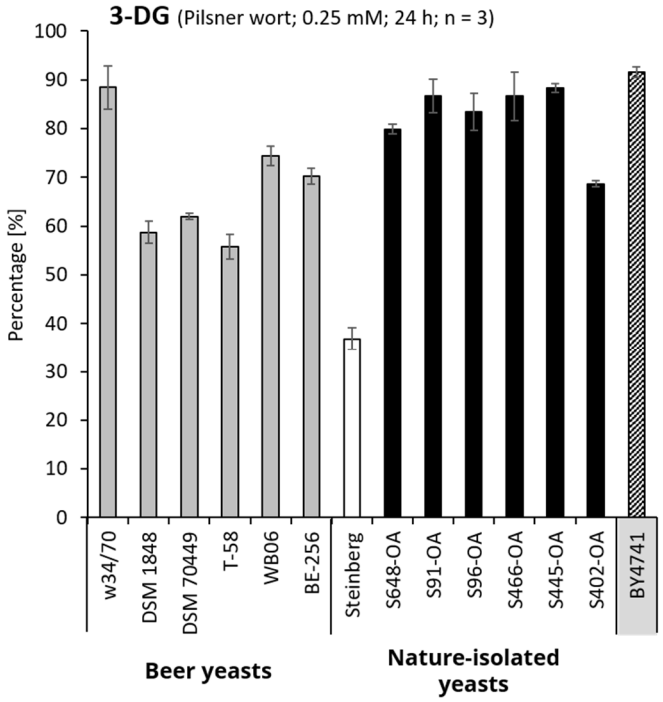

Figure 2. Accounting of the degradation of 3-DG (initial concentration 0.25 mM) following incubation of yeast strains separated into beer yeasts, yeasts isolated from natural habitats and reference (BY4741) for 24 h in Pilsner wort at 30 °C. Data are given as a percentage of the applied concentration of 3-DG, respectively, relative to the non-inoculated sample (stability blank) as mean value (n = 3).

To determine whether the utilization of 3-DG is exclusively due to metabolic processes and to exclude a possible binding of 3-DG to the cells due to its high reactivity [28,29], the incubation was repeated with autoclaved yeast cells. It was found that for some of the yeasts, a slight decrease (from 6% for S445-OA up to 25% for the beer yeast strain BE-256) in the concentration of 3-DG could be quantified after 24 h (see Supplements Table S3). However, this adsorption rate cannot fully explain the total decrease in the amounts of 3-DG in presence of the living yeast strains.

Following cell disruption, the extracellular and intracellular amounts of 3-DF and 3-DGA were quantified via GC-MS (see Supplements Figure S1B and Table S3). Small amounts of 3-DGA were detected for the first time. The oxidation seems to be a minor metabolic pathway. For Pilsner wort, the phenomenon of the previous study, in which only in beer yeast strains 3-DG with around 25 µM and 3-DF with up to 5 µM could be detected intracellularly [20], could not be confirmed. Here, in all yeast cells both 3-DG (up to 25 µM) and the metabolites 3-DF (around 10 µM) and 3-DGA (2 µM) were present, with a yeast-specific distribution. No differences between beer yeasts and strains isolated from nature were observed. This suggests that all yeasts studied metabolize 3-DG via a similar or even the same enzyme system but with a different activity.

3.2. Influence of 3-DG on the Activity and Growth of Saccharomyces Yeasts

3-DG can have a cytotoxic effect on yeasts due to its high reactivity, as the MRP irreversibly modifies enzymes, proteins or lipids [30,31]. After measuring the OD of the 24 h incubation samples, an influence of 3-DG on the cell density of the yeast suspension as well as differences between beer yeast strains and yeasts isolated from nature could already be determined. In previous studies, it was also shown that 3-DG has an influence on the cell activity of the yeasts in incubations in a minimal medium and that beer yeasts are more stress-tolerant to the MRP [20]. In order to investigate this phenomenon under brewing-relevant conditions, the dead cell count of the 24 h incubation samples was determined considering the yeast blanks (YB) in Pilsner wort. Figure 3 shows the dead cell counts of each yeast strain, divided into beer yeast strains and yeasts isolated from natural habitats, additionally adding the wine yeast "Steinberg" (white triangle) and the reference yeast BY4741 (striped triangle), after incubation with 0.25 mM of 3-DG, subtracting the respective YB. With the exception of the beer yeast strain DSM 1848, all yeast samples showed a higher dead cell count following incubation with 3-DG. While for the beer yeast strains, an average of 2.3 ± 3.1% dead cells was measured, and corresponding data for the yeasts isolated from natural habitats were 13.2 ± 3.0% after 24 h incubation. For the reference yeast BY4741, the highest relative amount of dead cells with 16.0 ± 4.0% was found, indicating that this cell strain is the most stress-sensitive strain towards 3-DG. Therefore, as already shown for minimal medium, also in Pilsner wort significantly fewer dead cells were found for the beer yeast strains when compared with the yeasts isolated from natural habitats ($\rho < 0.0005$), thus indicating that the technologically relevant yeast strains are more resistant towards carbonyl stress in the complex medium wort. The individually determined percentages of dead cells after 24 h incubations of the samples with added 3-DG as well as the YB of all yeast strains are listed in Supplements Table S4.

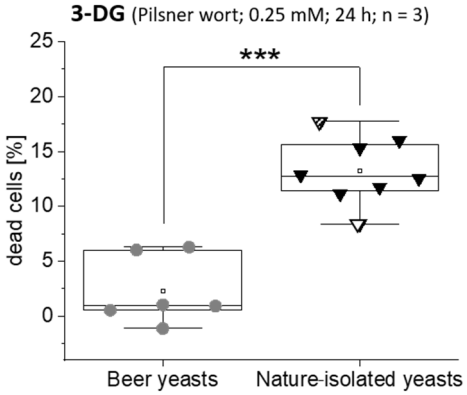

Figure 3. Dead/live assay of the *Saccharomyces* yeast strains, separated into beer yeast strains (grey dots) and yeasts isolated from natural habitats (black triangles; wine yeast "Steinberg", white triangle; reference yeast BY4741, striped triangle), after 24 h incubation in Pilsner wort at 30 °C with 0.25 mM of 3-DG. Shown are the mean values of the percentages of dead cells in the incubation mixtures after subtraction of dead cells in the YB without substrate addition (n = 3). Data are presented as box plots. The square indicates the mean value, and the line indicates the median. Significance: *** $\rho < 0.0005$.

To investigate a possible influence of 3-DG on the growth of the *Saccharomyces* yeasts, growth curves of selected yeasts strains were additionally recorded. For this, varying amounts of 3-DG ranging from 0 to 10 mM were added to Pilsner wort, and the OD at 600 nm was measured in intervals of 20 min for 72 h with a constant orbital shaking. Figure 4 shows the growth curves in Pilsner wort with different concentrations of 3-DG (0.25 mM, 1 mM, 5 mM, 10 mM) as well as the growth curves without 3-DG addition of 4 different yeasts (the beer yeast strains *S. pastorianus* w34/70 and *S. cerevisiae* T-58, the nature-isolated yeast *S. paradoxus* S445-OA and the reference yeast *S. cerevisiae* BY4741) at 30 °C. With an increasing concentration of 3-DG, an increasing delay of growth was

observed. This means that the yeast cultures take longer to enter the exponential growth phase in the presence of 3-DG. Furthermore, at high concentrations of 3-DG, a complete growth inhibition can be observed over the period of 72 h. The different sensitivities of the *Saccharomyces* yeasts tested for the dicarbonyl compound are particularly striking. While w34/70 could enter the exponential growth phase even at high 3-DG concentrations and a growth inhibition at a concentration of 10 mM of 3-DG was observed for T-58, the nature-isolated yeast S445-OA and the reference yeast BY4741 did not grow at a 3-DG concentration of 5 mM and higher. In summary, the two beer yeast strains showed a higher resistance to 3-DG than the yeasts isolated from natural habitats.

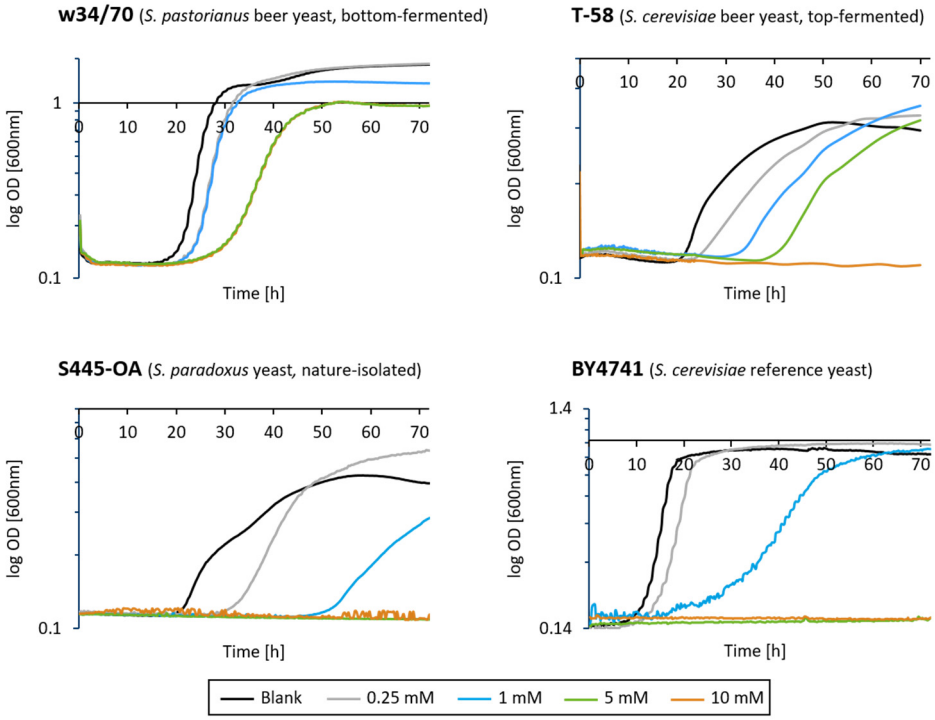

Figure 4. Growth curves of the selected *Saccharomyces* yeast strains w34/70, T-58, S445-OA and BY4741 incubated in Pilsner wort with different concentrations of 3-DG (0.25 mM, 1 mM, 5 mM, 10 mM) and without 3-DG (Blank) for 72 h at 30 °C with detection of the optical densities at 600 nm in intervals of 20 min.

To point out these differences in more detail, the growth delay of the growth curves in relation to the blank is additionally shown as a heat map for 30 °C and for 20 °C as a temperature relevant for brewing (see Figure 5) [27]. Here, blue means no influence on growth compared to the blank, and in dark red, no growth following in the presence of 3-DG after 72 h.

This figure illustrates the clear differences between the beer yeast strains and yeasts isolated from natural habitats in their 3-DG sensitivity and confirms the assumption that beer yeasts are significantly more stress-resistant to 3-DG in Pilsner wort. These results can also be reproduced at technologically relevant temperatures of 20 °C and should therefore be relevant for real brewing conditions.

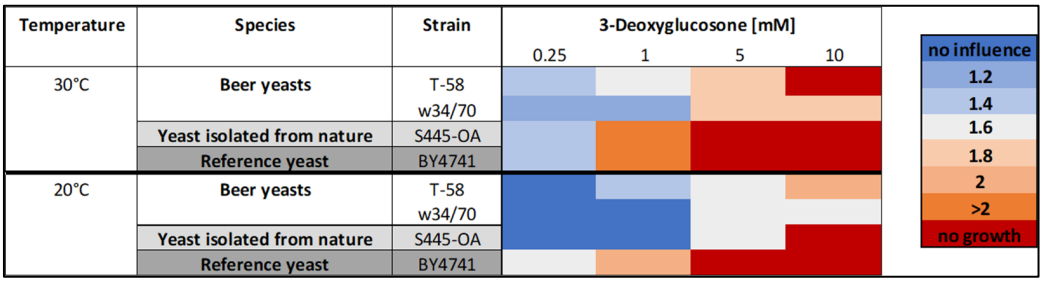

Figure 5. Heat map representation of the influence of different 3-DG concentrations on the microbial growth in Pilsner wort at 30 °C and 20 °C of the selected *Saccharomyces* yeasts, depending on the yeast blanks. The lag phase delay compared to the respective yeast blank is shown according to the color-coding on the right (blue, no influence; red, no growth).

3.3. Degradation and Metabolization of 3-DG and 3-DM during Wort Fermentation by Saccharomyces Yeasts

In order to transfer the results from the incubation experiments to technologically relevant brewing conditions, fermentation experiments were carried out with the selected yeast strains *S. cerevisiae* T-58 (top-fermenting beer yeast strain), *S. pastorianus* w34/70 (bottom-fermenting beer yeast strain), *S. paradoxus* S445-OA (strain isolated from a natural habitat) and *S. cerevisiae* BY4741 (reference yeast) for 7 days at 21 °C (primary fermentation) and for another 7 days at 6 °C (secondary fermentation). For the experiments, two different worts (Pilsner wort and dark beer wort) were used, containing different concentrations of 3-DG and 3-DM (Pilsner wort: 254.7 ± 4.6 µmol/L of 3-DG and 24.9 ± 1.7 µmol/L of 3-DM; dark beer wort: 564.7 ± 21.0 µmol/L of 3-DG and 251.6 ± 22.2 µmol/L of 3-DM). To monitor the fermentation process, the extract content of the worts was determined throughout the experiment. Furthermore, 3-DM and 3-DG degradation and potential metabolite formation of 3-DF and 3-DGA during fermentation were measured. As a reference, the worts without addition of yeasts were used.

Figure 6 shows the results of the fermentation in Pilsner wort. The wort had an initial extract content of 13 °P, which was stable throughout the entire experiment without yeast addition, indicating that no contamination with wild yeasts occurred. For the samples with yeasts added, a significant decrease in extract content over the fermentation time was measured, which differed depending on the yeast strain (Figure 6A). The two beer yeast strains efficiently fermented the wort to a final extract content of 2.8 °P, which was reached by the top-fermenting yeast T-58 on day 5 and by the bottom-fermenting yeast w34/70 on day 7. Due to the non-optimal fermentation temperature of 21 °C, the yeast *S. pastorianus* w34/70 possibly needed more time to start the fermentation process [27]. The two non-industrial yeasts were similar in their low fermentation performance, showing a residual extract content of 9.5 °P, which was not reached before day 14. This indicates that the yeasts isolated from natural habitats less efficiently utilized the carbohydrates maltose and/or maltotriose as well as other substrates, which are dominant in the wort. In Pilsner wort, a 3-DG content of 254.7 ± 4.6 µmol/L and a 3-DM content of 24.9 ± 1.7 µmol/L was determined, which agrees with existing literature [11,12]. The yeasts were able to significantly degrade 3-DG and 3-DM during the fermentation, whereby the differences in the degradation rates between beer yeast strains and yeasts from natural habitats were observable for both dicarbonyl compounds, with the beer yeast strains showing a faster degradation rate (Figure 6B,C). Furthermore, 3-DF was present in the fermentation samples, which was formed in concentrations up to 20 µM by the reference yeast as well as the strain isolated from nature and about 80 µM by the beer yeast strains (Figure 6B). These contents are comparable to existing literature data [18].

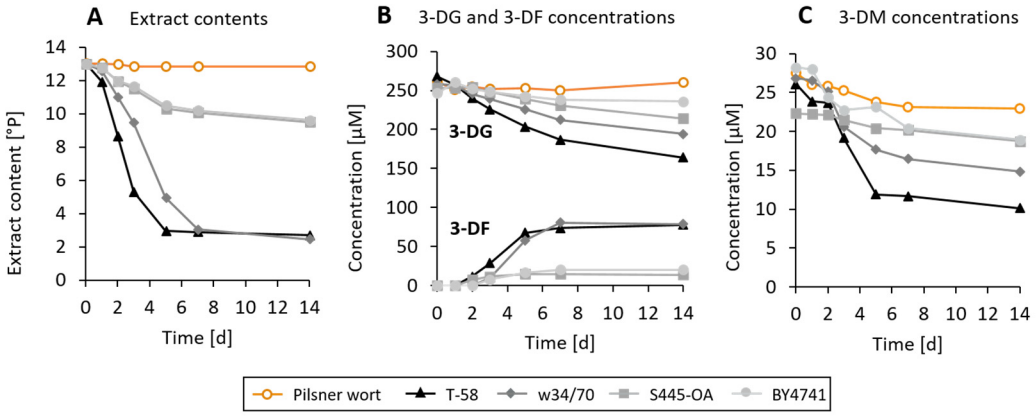

Figure 6. Fermentation results in Pilsner wort at RT (days 0–7) and 6 °C (days 7–14): (**A**) Extract contents of days 0, 1, 2, 3, 5, 7 and 14 in °P, (**B**) 3-DG and 3-DF concentrations and (**C**) 3-DM concentrations on days 0, 1, 2, 3, 5, 7 and 14 in µM of the yeast strains *S. cerevisiae* T-58 (beer yeast strain, top-fermented), *S. pastorianus* w34/70 (beer yeast strain, bottom-fermented), *S. paradoxus* S445-OA (isolated from nature), *S. cerevisiae* BY4741 (reference yeast) and Pilsner wort without yeast addition as a stability blank. Data are given as mean value (n = 2).

In order to elucidate the dicarbonyl utilization, the intracellular contents of 3-DG as well as those of the formed metabolites 3-DF as well as 3-DGA were quantified at day 14 after cell disruption (Figure 7). 3-DG as well as its metabolites could be detected intracellularly in all yeast samples. To exclude a possible adsorption of 3-DG to the yeast cells, the fermentation was repeated with autoclaved main cultures. Here, no adsorption effects could be observed in any batch. The accounting of dicarbonyl concentrations at day 0 and day 14 showed the mentioned differences in the utilization of the dicarbonyl compounds between beer yeast strains and yeasts isolated from natural habitats regarding the degradation rates of 3-DG and 3-DM and the formation rate of 3-DF.

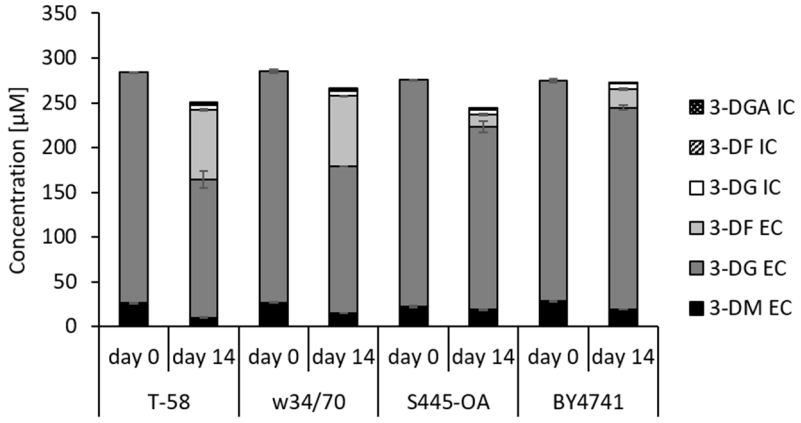

Figure 7. Accounting of dicarbonyl utilization at days 0 and 14 of fermentation in Pilsner wort including the amounts in µM of 3-DM, 3-DG and its metabolites 3-DF and 3-DGA in the extracellular supernatants (EC) and the intracellular concentrations (IC) after cell disruption of the yeast strains *S. cerevisiae* T-58 (beer yeast strain, top-fermented), *S. pastorianus* w34/70 (beer yeast strain, bottom-fermented), *S. paradoxus* S445-OA (isolated from nature), *S. cerevisiae* BY4741 (reference yeast) and Pilsner wort without yeast addition as a stability blank. Data are given as mean value (n = 2).

Following fermentation of the dark beer wort, similar trends with regard to the extract contents were observed, resulting in a final extract content of 4 °P for the beer yeast strains and 9–10 °P for the yeasts isolated from natural habitats (Figure 8A). This is slightly higher compared to Pilsner wort and probably due to a higher proportion of unfermentable components in the dark beer wort. However, the utilization of 3-DG in the dark beer

wort by the yeasts showed a different result when compared to the results of the Pilsner wort fermentation experiments. A 3-DG content of 564.7 ± 21.0 µmol/L was quantified in dark beer wort, which is a 2-fold higher concentration than in Pilsner wort. In contrast to Pilsner wort, where the starting amount of 3-DG was almost completely metabolized by the yeasts, it was observed for the dark beer wort that the 3-DG concentration only very slightly decreased by 10 to 20% over 14 days of fermentation in the presence of all the yeasts studied. Significant differences between the yeasts with regard to the degradation rate of the dicarbonyl compound could not be observed here. Despite this small reduction in the concentration of 3-DG, however, 40 µM of 3-DF could be determined following fermentation for 14 days with the yeasts isolated from natural habitats and even up to 180 µM of 3-DF for the beer yeast strains. This confirms the differences between the technologically relevant yeasts and the yeasts isolated from natural habitats. However, in contrast to the Pilsner wort, no or only a slight 3-DG degradation rate was found for the dark beer wort (Figure 8B). On the first view, the yeasts, especially beer yeasts, thus appear to produce more 3-DF than 3-DG available for metabolization at the starting point of the fermentation. This phenomenon can be explained by the concentration of the dicarbonyl compound 3-DM, which is present in a 10-fold higher concentration in dark beer wort than in Pilsner wort (251.6 ± 22.2 µmol/L). It was found that 3-DM significantly decreased for all yeasts examined during fermentation. Compared to 3-DG, 3-DM is thus degraded significantly faster and also to a higher extent by the yeasts examined. Slight differences between the beer yeast strains and the yeasts isolated from natural habitats were found with respect to 3-DM degradation. While the final concentration of 3-DM was approximately 40 µM following 14 days of fermentation with beer yeast strain samples, corresponding concentrations were approximately 70 µM following fermentation samples with the yeasts isolated from natural habitats (Figure 8C). This indicates again a faster utilization of 3-DM by technologically used yeasts. An adsorption of 3-DG or 3-DM to the yeast cells could additionally be excluded by repeating the experiment with inactivated yeast cells.

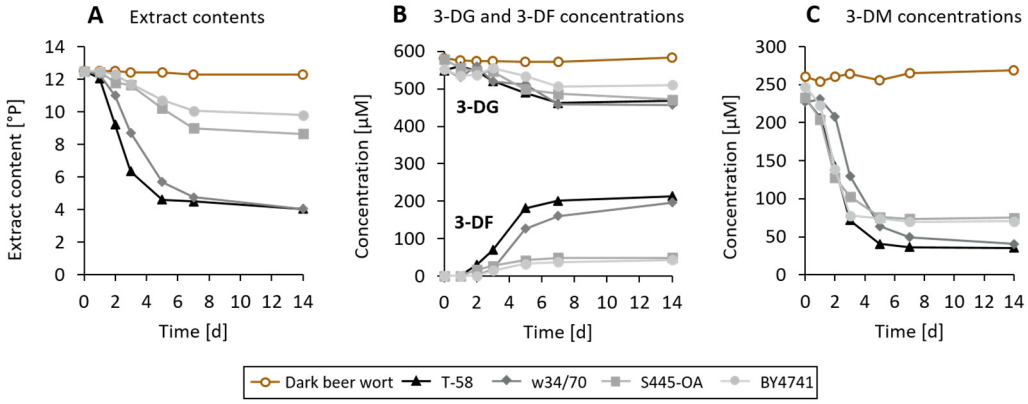

Figure 8. Fermentation results in dark beer wort at RT (days 0–7) and 6 °C (days 7–14): (**A**) Extract contents of days 0, 1, 2, 3, 5, 7 and 14 in °P, (**B**) 3-DG and 3-DF concentrations and (**C**) 3-DM concentrations on days 0, 1, 2, 3, 5, 7 and 14 in µM of the yeast strains *S. cerevisiae* T-58 (beer yeast strain, top-fermented), *S. pastorianus* w34/70 (beer yeast strain, bottom-fermented), *S. paradoxus* S445-OA (isolated from nature), *S. cerevisiae* BY4741 (reference yeast) and dark beer wort without yeast addition as a stability blank. Data are given as mean value (n = 2).

When considering the dicarbonyl utilization by the yeasts after cell disruption of the fermentation samples on day 14 on a molar basis, a particularly noticeable difference between beer yeast strains and yeasts isolated from natural habitats in the formation of the metabolite 3-DF was evident (see Figure 9). By these yeasts, in total, only around 40 µM of 3-DF were formed, but 3-DG and 3-DM have declined in total by approximately 250 µM. Thus, around 210 µM of dicarbonyl metabolization was not explainable by 3-DF formation. In the case of the beer yeast strains, around 220 µM of 3-DF were formed, and 3-DG and

3-DM have declined in total by approximately 300 µM, indicating that the decrease in dicarbonyl compounds can be explained by 3-DF formation. As this phenomenon did not occur in the Pilsner wort, the high levels of 3-DM in dark beer wort and the rapid degradation of this dicarbonyl compound must be responsible for this "dicarbonyl gap" in the molar accounting and the varying levels of 3-DF formation by the yeasts.

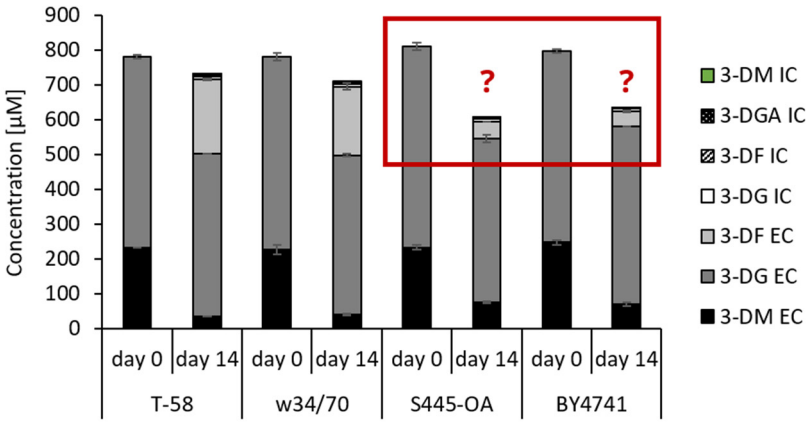

Figure 9. Accounting of dicarbonyl utilization at days 0 and 14 of fermentation in dark beer wort including the amounts in µM of 3-DM, 3-DG and its metabolites 3-DF and 3-DGA in the extracellular supernatants (EC) and the intracellular concentrations (IC) after cell disruption of the yeast strains *S. cerevisiae* T-58 (beer yeast strain, top-fermented), *S. pastorianus* w34/70 (beer yeast strain, bottom-fermented), *S. paradoxus* S445-OA (isolated from nature), *S. cerevisiae* BY4741 (reference yeast) and Pilsner wort without yeast addition as a stability blank. Data are given as mean value (n = 2).

It can be assumed that, due to its structural similarity to maltose, 3-DM is taken up analogously by corresponding permeases into the yeast cells and is subsequently cleaved intracellularly into glucose and 3-DG [32]. 3-DG then can subsequently be metabolized to 3-DF. As maltose is the main carbohydrate in beer wort, increased expression of the corresponding transport systems in beer yeast strains is conceivable for an effective energy production, and therefore, this metabolization of 3-DM is possible [15]. Since for the yeasts isolated from natural habitats only a low level of 3-DF formation was observed, a different degradation pathway of 3-DM might be assumed. One possibility would be a direct reduction in the disaccharide to the corresponding alcohol, which could occur both intracellularly and extracellularly. This must be clarified in further studies. Detailed concentrations of 3-DG, 3-DM and 3-DF during the fermentation experiments as well as the extracellular and intracellular percentages of the accountings on day 14 are shown in the Supplements Tables S5 and S6.

Overall, the results show that high levels of 3-DM in beer wort influence the utilization of 3-DG by the yeasts, and thus higher levels of 3-DG remain in the wort, which can have a substantial impact on the flavor of the final beer due to further subsequent reactions such as the Strecker degradation [33]. The metabolization of 3-DM by yeasts should therefore be a focus of further studies.

4. Conclusions

In summary, we could show that *Saccharomyces* yeasts differ in their handling of the dicarbonyl compounds 3-DG and 3-DM. During beer fermentation, beer yeast strains showed a faster degradation of 3-DG compared to strains isolated from natural habitats and were more resistant to a "dicarbonyl stress" induced by 3-DG, as demonstrated by the dead/live assay. For dark beer wort, it was found that the high concentrations of 3-DM significantly influence the formation of 3-DF. While the beer yeast strains quantitatively metabolize 3-DG and 3-DM to 3-DF, for yeasts isolated from natural habitats only low 3-DF formation, despite high degradation of 3-DM, was found. What these yeasts do with the remaining levels of 3-DM in the beer wort could not be clarified in this study. By

continuous contact of the beer yeast strains with 3-DG and 3-DM and the high plasticity of the yeast genome, an adaptation of the yeasts to an efficient handling of the dicarbonyl compounds is conceivable and could be an indication of domestication [16,34]. To support this hypothesis, further fermentation experiments with variation in malt, mashing process, wort boiling (input of 3-DG and 3-DM into the wort) would be interesting. To identify how yeasts isolated from natural habitats utilize 3-DM in comparison to beer yeast strains, additional incubation experiments in minimal medium with isotopically labelled 3-DG and 3-DM would be essential. Corresponding studies are currently underway in our laboratory.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10060281/s1, Table S1: Specifications of the yeast strains used in this study; Table S2: Cell counts at the initial optical density for the incubations and determination of yeast growth during the incubation with 3-DG in Pilsner wort; Table S3: Mean values and standard deviations of the HPLC-UV and GC-MS results after the 24 h incubations in Pilsner wort with 3-DG; Table S4: Mean values and standard deviations of the Dead/Live assay with all yeast strains after the 24 h incubations with 3-DG in Pilsner wort; Table S5: Mean values and standard deviations of the 3-DG, 3-DM and 3-DF concentrations during fermentation experiments in Pilsner wort and dark beer wort; Table S6: Mean values and standard deviations of the intracellular concentrations of 3-DG, its metabolites 3-DF and 3-DGA, and 3-DM following fermentation experiments in Pilsner wort and dark beer wort; Figure S1: Chromatograms of the 3-DG degradation measured with HPLC-UV and the formation of 3-DF and 3-DGA measured with GC-MS.

Author Contributions: Conceptualization, A.-L.K. and T.H.; methodology, A.-L.K.; software, A.-L.K.; validation, A.-L.K.; formal analysis, A.-L.K.; investigation, A.-L.K.; resources, A.-L.K., M.B.-H., K.O. and T.H.; data curation, A.-L.K.; writing—original draft preparation, A.-L.K.; writing—review and editing, A.-L.K., M.B.-H., K.O. and T.H.; visualization, A.-L.K.; supervision, A.-L.K., M.B.-H., K.O. and T.H.; project administration, A.-L.K. and T.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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