



## Optimizing the production of wheat beer by recycling the yeast biomass: perspective for cost reduction with quality maintenance

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### Abstract

A considerable investment is being experienced worldwide in the production of artisanal beers of different styles, such as wheat-based Weizen beer. The challenge is to reduce production costs while maintaining the commercially accepted quality. Thus, the improvement of the production process involves the possibility of shortening the fermentation time by reusing the yeast biomass without affecting the yeast vitality and the quality of the end product. In this study, the fermentation parameters of a Weizen beer were measured directly from a scaled-up process in a microbrewing in order to identify the optimum point at which the process might be ended without compromising the final characteristics. The results were confirmed by laboratory studies that compared three different yeast strains in a wheat-based industrial substrate. The results led to the conclusion that the fermentation time was reduced by more than 50% from 7 to 3 days, with less variation in the concentration of the investigated metabolites. By reducing the time of fermentation, the yeast biomass was maintained active to be used up to six consecutive cycles of fermentation. All these improvements might help in reducing the cost of beer processing, which is of paramount interest to microbreweries worldwide.

**Keywords:** esters; high alcohols; microbrewery; sensorial quality; yeast recycling.

**Practical Application:** Improvement of the brewing process for wheat beer to reduce cost production.

## 1. INTRODUCTION

Group 10 of the Beer Judge Certification Program Inc. (BJCP) style guide refers to the German wheat beers, also known as Weizenbier or Weissbier. In German, “weizen” means wheat and “weiss” means white. Historically, this group is composed of beers that were originally produced in the north of the Danube River and contain at least 50% wheat (Meusinger, 2019). In terms of characterization, the current legislation established that wheat beer contains up to 80% of wheat malt and is fermented by the top-fermenting yeast *Saccharomyces cerevisiae* (Briggs, 1998). The group of Weissbier (10A) is a beer with fruity esters (usually with banana notes), vanilla, and phenols (BJCP, 2015; Yin et al., 2016). In a recent analytical challenge launched by Meusinger (2019), the phenolic derivative ferulic acid has been identified as the metabolite that gives the distinct flavor that gives clove notes to wheat beers (Meusinger, 2020). Besides ferulic acid, wheat malts also contain p-coumaric acid that is a precursor of the aroma-active compound 4-vinyl phenol produced by yeast metabolism (Kalb et al., 2020). Wheat, which is the main adjunct of this beer, has a constitution of 80% of carbohydrates, most

of them in the form of polysaccharides, with a predominance of starch. Other mono-, di-, tri-, and oligosaccharides together with traces of cellulose and hemicellulose are also found (Faltermaier et al., 2014). Non-carbohydrate components such as lipids, proteins, and phosphorus are also found in wheat starch, but in very small concentrations (Shewry et al., 2020). Generally, wheat beers have a low concentration of free nitrogen (79–139 mg/100 g) when compared to barley (120–150 mg/100 g), and this can cause some problems in fermentation, such as lower concentrations of higher alcohols and organoleptic compounds (Faltermaier et al., 2014). Regardless of its adjunct, beers have more than 450 different substances that can influence the characteristics of the final product. From the analytical point of view, there is less information of data in the scientific literature regarding wheat beers when compared to pure barley beers, being the revision by Faltermaier et al. (2014) the most recent record in this matter. Very recently, Byeon et al. (2021) compared two varieties of wheat malt in Korea with two commercial malts sold in the USA and Germany and found no difference in the sensorial parameters of the respective beers, despite the free amino nitrogen contents among them.

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The industrial beer production process can be divided into three major stages: wort production, where malt grinding, mashing, filtration, boiling, and clarification take place; the fermentation process, which involves fermentation and maturation, and, finally, the post-treatment of the beer, which may involve filtration, carbonation, alteration of aroma and flavor, color standardization, and pasteurization (Hardwick, 1994). Any changes in the ingredients and/or in the production process will result in a final product with different characteristics. Part of the organoleptic characteristics of wheat beer is produced by the yeast cells during fermentation. In this phase, in addition to the conversion of sugars into ethanol and carbon dioxide, other substances produced in lower concentrations are responsible for the flavor and aroma of the drink (Stewart, 2017). The metabolization of the carbon and nitrogen sources in the wort converge for the production of high alcohols and esters that contribute to the final taste of the product (Meier-Dörnberg et al., 2017; Narziss, 1984; Schneiderbanger et al., 2016). In this sense, interventions by medium supplementation and/or operational optimizations can be carried out at this stage to induce the production and maintenance of desirable flavors and aromas. The traditional recipe for making wheat beers involves fermenting at 17°C for 7 days or more, with the changing of the yeast biomass at every new batch. In the microbrewery studied in this article, the attempt to recycle the yeast biomass in the traditional operation was facing problems of product variability after the third batch. It was shown that the yeast cells accumulate changes in the cell surface structure, which could be used for the prediction of their fermentative state (Smart and Whisker, 1996). Later on, it was reported that serial recycles cause several damages to the yeast cells capable of triggering the general stress response, although it seems a strain-dependent behavior (Jenkins et al., 2003). On the other hand, Powell and Fischborn (2010) observed that recycled cells performed beer fermentation similar to active dried yeasts for five consecutive fermentations. However, these studies were performed for barley wort and no work on this subject is so far available in the scientific literature on wheat beer fermentation.

Unlike large breweries, Brazilian microbreweries face the challenge of increasing production scale to reduce processing costs and gain market share. It has been calculated that the production of Brazilian microbrewery in a year is equivalent to that of a large brewery produces in 10 min of its day. However, these microbreweries have been increasing their market share (Ramos & Pandolfi, 2019). With regard to wheat beer, its production has experienced an astonishing increase worldwide from 1970 to 2012 (Faltermaier et al., 2014). This is another variable to be added to the industrial equation which is the increment of the market. This imposes the challenge of increasing production, which often goes against the maintenance of the quality of the final product.

There are quite a number of publications reporting the importance of brewing wort composition and fermentation conditions for the production of secondary metabolites with desirable sensory characteristics for the production of the beverage (Pires et al., 2014). However, despite the practice of recycling yeasts for the production of beer, there are no scientific articles that portray the metabolic and organoleptic characteristics for the

production of beer using biomass, especially for a Weizen-style wheat beer. Besides, there is no information in the literature about the effect of the fermentation time on the maintenance of yeast vitality along the successive cycles of fermentation. Therefore, there is no experimental approach to fermentation process adjustments by the indication and management of yeast strains that aim to maintain product quality and reduce cost and production time for a Weizen-style wheat beer.

In view of this scarcity of information, this work aimed to propose adjustments in the processing of Weizen-style beer that allow increasing industrial efficiency without affecting the characteristics of the beverage. To achieve this goal, we evaluated the metabolic profile of two commercial beer yeast strains during six wheat beer fermentation cycles in a microbrewery and compared the results with laboratory fermentation. As a reference, we included a fuel-ethanol strain that is also used for the production of the Brazilian spirit cachaça, both products generated by the fermentation of the sucrose-rich sugarcane substrates (Vidal et al., 2013). This strain was not yet tested for beer production. Based on the results, this study proposes the management of the use of the yeast strain that obtained the best performance in terms of the production of desirable organoleptic substances for wheat beer in order to reduce the time and costs of beer processing without affecting the quality of the beverage.

## 2. MATERIALS AND METHODS

### 2.1. Yeasts

The brewing strains *S. cerevisiae* SAFBREW WB06 (Fermentis division, Lesaffre Brazil, Campinas, SP) was used by the microbrewery partner of this work (Debron Bier, Jabotão dos Guararapes, PE, Brazil). The brew strain *S. cerevisiae* Yeast-Lab YLB4000 — German Weizen 01 (YeastLab Biotecnologia, Franca, SP, Brazil) was also tested in laboratory fermentation. The fuel-ethanol strain *S. cerevisiae* JP1 is marketed for the production of fuel-ethanol and cachaça spirit in Brazil and under the brand Fermol Distiller MZ (AEB Biochemistry Latin America, São José dos Pinhais, PR, Brazil). This strain has been studied for the production of Brazilian spirit cachaça (Vidal et al., 2013, 2015) and used for comparison.

### 2.2. Cultivation conditions and beer fermentations in the microbrewery

The lyophilized cells of yeast WB-06 were hydrated in a volume of mineral water to 100 g/L at 25°C ( $\pm 3^\circ\text{C}$ ). After 20 min, the suspension was transferred to the fermentation tank containing the wheat brewing wort to an initial cell concentration of 0.75 g/L ( $1.5 \times 10^7$  cell/mL). The fermentation wort was prepared following the standard recipe with a 50%:50% mixture of Weyermann® wheat malt pale (Weyermann Specialty Malting, Bamberg, Germany) with Weyermann® Pilsner malt. The malts were analyzed by the Cooperativa Agraria Industrial (São José dos Pinhais, Paraná, Brazil) with the following characteristics:

- For wheat malt: 4.5% moisture content, 4.5 EBC (5.3 EBC after boiled), pH 6.12, Hartog index 35.9%, 11.9% of protein

(771 mg of soluble nitrogen/100 g MTs), and 40.4% of Kolbach index;

- For the base Pilsner malt: 10.6% of protein (497 mg of soluble nitrogen/100 g malt) and 3.9 EBC (European Brewing Convention) color units.

Fermentation took place at 18°C for 7 days (first cycle). At the end of fermentation, cell sediment was recovered and used for further cycles of fermentation of 5–7 days at 20–22°C. In the intervals between fermentation cycles, the active biomass remained stored together with the “trub” (sediment that settles in the fermenter during the fermentation process and that consists of insoluble material from the hops and inactive yeasts) at 4°C while preparing tanks and wort.

### 2.3. Cultivation conditions and beer fermentations in the laboratory

Lyophilized cells were re-hydrated with sterile distilled water equivalent to 100 g/L at 25°C ( $\pm 3^\circ\text{C}$ ) in 15 mL graduated tubes for 20 min, and the cell suspensions were mixed with the wheat brewing wort provided by the microbrewing company to an initial cell concentration of 0.15 g of lyophilized cells in 200 mL of fermentation wort (equivalent to 0.47 g wet biomass/200 mL) in 250 mL cylindrical glass tubes. Fermentation took place at 18°C for 7 days (first cycle). At the end of fermentation, cell sediment was recovered by centrifugation, re-suspended in sterile distilled water, and stored at 4°C. Further cycles of fermentation were performed at 20°C (second cycle) and 22°C (third to sixth cycles) using the biomass from the previous batch. All experiments were performed in three independent biological replicates. The cell density in the wort was determined by counting in an optical microscope (40x magnitude) with the aid of a Neubauer chamber.

### 2.4. Metabolite analysis

Wheat wort and fermented wort were analyzed for quantification of sugars (glucose, fructose, and maltose), glycerol, acetic acid, and ethanol by high-performance liquid chromatography (HPLC). The samples were diluted with deionized water and filtered using 0.22 mm filters. Quantification was done in an HPLC device equipped with a refractive index (IR) detector (Agilent Technologies 1200 Series) and HPX-87H<sup>+</sup> ion exchange column (Aminex<sup>®</sup> HPX-87H<sup>+</sup>, Bio-Rad, USA). The mobile phase of 5 mM H<sub>2</sub>SO<sub>4</sub> solution was pumped at 0.6 mL/min at 35°C. The metabolites were identified by their relative retention times and quantified using a standard curve made with each individual compound (Vidal et al., 2013, 2015).

Sensorial molecules (higher alcohols and esters) were quantified by gas chromatography (GC). The samples were prepared by liquid/liquid extraction with diethyl ether solution (50% v/v) pre-chilled at -20°C containing octanol (1 µg/mL) as the internal standard (Vidal et al., 2013, 2015). Extraction was performed by mechanical stirring in a Vortex-type agitator for 1 min, and the tubes were centrifuged at 14,000 rpm for 5 min to separate phases. The upper phase containing the extracted compounds went to

the analytical method. The GC device was equipped with a flame ionization detector (Agilent Technology 7890) and HP-5 column (30 m in length  $\times$  320 µm in diameter  $\times$  0.25 µm). Gas flow was done as follows: helium at 1.5 mL/min, argonium at 400 mL/min, nitrogen at 25 mL/min, and hydrogen at 30 mL/min. The initial temperature of oven was 40°C for 5 min, followed by the first ramping of 10°C/min until reaching 100°C and the second ramp of 34.5°C/min until reaching 250°C which was maintained for 1 min. The temperatures of the injector and detector were adjusted to 250°C. The metabolites were identified by their relative retention times, normalized with internal standard octanol, and quantified using a standard curve made with each individual compound (Vidal et al., 2013, 2015).

### 2.5. Statistical analysis

The difference in significance of mean metabolite concentrations was determined by Turkey's test using Microsoft Office Excel 365<sup>®</sup> at a 5% significance level ( $\alpha \leq 0.05$ ).

## 3. RESULTS AND DISCUSSION

### 3.1. Fermentation in the microbrewery lasts longer than needed

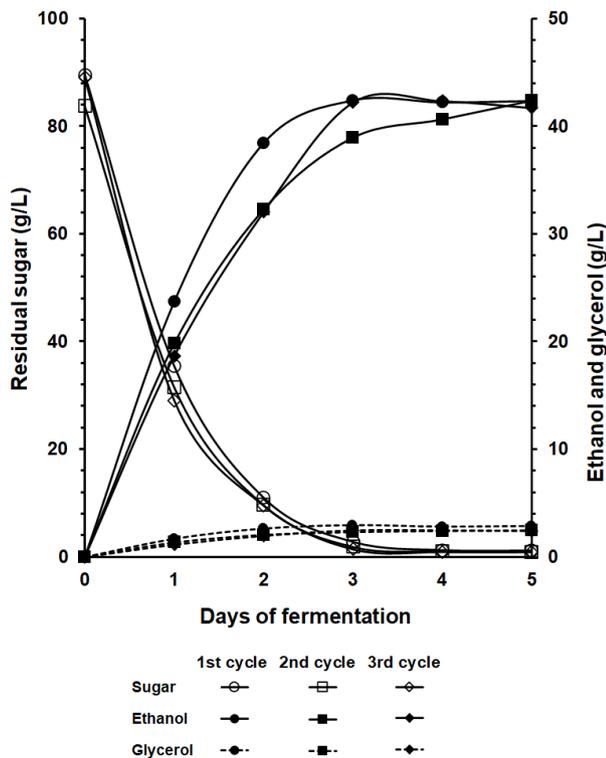
Fermentations were carried out in the microbrewery according to the standard manufacturing procedures, lasting 7 days of fermentation. Yeast biomass was re-used for up to three consecutive cycles and stored at 4°C in intervals. The results of fermentative parameters were compiled as the average of three independent industrial cycles, with each industrial cycle comprising 5 days of fermentation (Table 1). The initial carbohydrate content of the wort (including maltose and derivatives, glucose, and fructose) was in the range of 87.4 g ( $\pm 3.2$ )/L, which was in the range of the reported value of 88.9 g ( $\pm 9.4$ )/L (He et al., 2014). In general terms, 50% of the fermentation was taken at the end of the first day of fermentation indicated by sugar consumption and ethanol production and practically finished on the third day (Figure 1). The maximal ethanol yield reached 0.48 g/g on the third day of fermentation (Figure 2A), representing 95.4% of fermentation efficiency. Yeast population increased four times from 0.75 g/L ( $1.5 \times 10^7$  cell/mL) as initial inoculum to achieve almost 3 g/L ( $7 \times 10^7$  cell/mL) on the third day of fermentation, with less variation afterward. Reglitz et al. (2022) achieved around 80% of fermentation only on the sixth day of fermentation using kilned barley/wheat malt and WB06 yeast strain. Despite the use of roasted malt in that work, the comparison of final ethanol contents and similar ethanol yield indicated that both worts contained very similar amount of assimilable sugar. However, it should be taken into account that the initial concentration of cells in that work was 0.33 g/L, which is twice lower than used here. Moreover, Byeon et al. (2021) reported that sugars in the wheat malt were consumed for 7 days by the cells of the WLP300 strain, using almost ten times less cells as initial inoculum than used here. Moreover, about 30% of the sugars remained in the wort in that work.

Ethanol concentration reached 42 g/L on the third day of fermentation (Figure 1). It represents 5.3% (v/v) of ethanol, the

**Table 1.** Metabolic profile of wheat wort fermentation by SAFBREW WB06 beer strain in a scaled-up microbrewery in three successive batches.

Day	Total sugar (g/L)	Glycerol (g/L)	Ethanol (g/L)	2MP (mg/L)	3MB (mg/L)	2MB (mg/L)	2EP (mg/L)	EA (mg/L)	2EPA (mg/L)
<b>First cycle</b>									
0	89.5	0.00±0.00	0.00±0.00	0.00±00.00	0.00±0.00	0.00±00.00	0.00±00.00	0.00±00.00	0.00±00.00
1	35.4	1.67±0.08	23.70±1.43	24.00±00.00	28.00±02.83	0.00±00.00	104.00±02.83	8.00±01.41	80.50±00.71
2	10.95	2.60±0.13	38.42±1.25	64.00±04.24	102.67±06.51	60.50±00.71	153.00±01.41	63.00±19.00	80.00±00.00
3	2.76	2.91±0.22	42.39±1.46	39.00±00.00	103.00±17.06	61.50±06.36	125.00±16.97	68.50±24.75	72.00±00.00
4	1.21	2.78±0.09	42.20±0.79	33.50±03.54	145.33±10.69	73.50±02.12	144.50±10.61	40.50±09.19	64.00±01.41
5	1.14	2.84±0.04	42.33±0.94	41.50±00.71	191.00±14.14	87.50±03.54	174.50±7.78	69.33±04.51	70.50±02.12
<b>Second cycle</b>									
0	83.75	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
1	31.51	1.33±0.10	19.80±1.73	19.50±7.78	37.00±4.24	43.50±0.71	109.00±2.83	31.00±0.00	65.50±3.54
2	9.61	2.03±0.26	32.26±1.04	19.50±13.44	84.50±09.19	49.00±0.00	138.00±26.87	31.00±2.83	93.00±2.83
3	1.84	2.29±0.15	38.92±1.36	22.00±9.90	105.50±6.36	51.50±9.19	119.00±11.31	37.00±2.08	74.50±10.61
4	1.04	2.39±0.03	40.61±0.90	36.00±1.41	117.00±7.07	53.00±12.73	114.50±21.92	51.00±7.78	76.00±00.00
5	0.93	2.45±0.04	42.35±1.34	35.00±4.24	126.50±14.85	68.00±0.00	139.50±26.16	61.00±9.90	85.50±30.41
<b>Third cycle</b>									
0	89.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
1	29.07	1.13±0.06	18.67±2.23	14.33±3.06	28.67±9.07	43.00±2.83	108.00±9.90	14.00±4.00	70.50±0.71
2	9.54	1.94±0.08	32.03±1.70	22.33±3.06	83.33±4.04	51.50±0.71	118.00±1.41	31.00±3.00	69.50±3.54
3	1.4	2.43±0.02	42.22±2.19	23.67±3.51	93.67±4.73	56.00±1.41	130.50±9.19	44.67±4.16	89.50±10.61
4	0.91	2.44±0.13	42.34±2.40	31.00±13.08	93.67±04.73	58.50±00.71	120.00±1.14	47.50±0.71	70.50±3.54
5	0.91	2.37±0.11	41.71±1.96	19.00±6.56	102.50±7.78	62.50±2.12	128.50±2.12	49.50±2.12	74.50±9.19

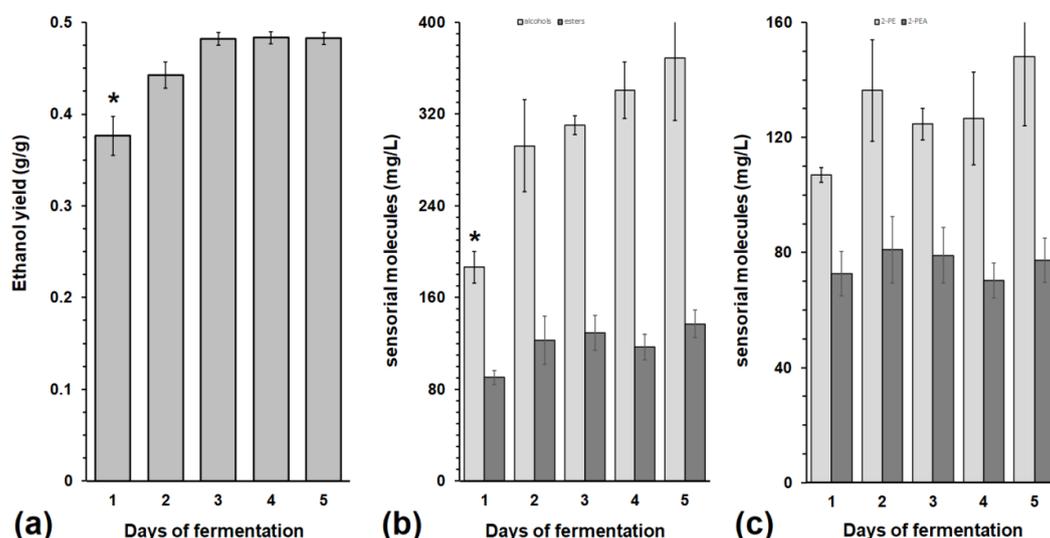
2MP: 2-methyl-1-propanol; 3MB: 3-methyl-1-butanol; 2MB: 2-methyl-1-propanol; 2EP: 2-ethylphenol; EA: ethyl-acetate; 2EPA: 2-ethylphenol acetate.



**Figure 1.** Fermentation profile of three independent wheat beer production in a scaled-up microbrewery using *Saccharomyces cerevisiae* var. *diastaticus* SAFBREW WB06 strain. The consumption of sugars (open symbols) and the production of ethanol (closed symbols straight lines) and glycerol (closed symbols dotted lines) were measured. No significant differences were observed for the different parameters tested among the cycles of fermentation ( $\alpha \leq 0.05$ ).

alcohol by volume (ABV) concentration that is in the range of 4–7% for this beer style. Less gain in terms of ethanol content was observed afterward which could compensate for the extension of fermentation periods and the costs of production. This ethanol content was reported for wheat beers using Korean wheat malt and higher than the values calculated for German and USA wheat malts (Byeon et al., 2021). The production of glycerol varied from 2.3 to 2.9 g/L (Figure 1), which was also similar to the glycerol content reported (Byeon et al., 2021).

The quality of the beer is defined by its chemical composition regarding the presence of sensorial molecules, mainly higher alcohols and esters. Their production is the consequence of the metabolic state of the yeast population. As there was no ethanol consumption after carbohydrate exhaustion (Figure 1), the yeast cells were kept in an anoxic environment after ending ethanol production on the third day. This is an important scenario, since even minimal oxygenation could result in the reduction of sensorial compounds in the wort due to the inhibition of flavor biosynthetic genes (Saerens et al., 2008). The production of sensorial metabolites was evaluated by the quantification of three aliphatic higher alcohols (i.e., active amylic/2-methyl-1-propanol: 2MB; isoamylic/3-methyl-1-butanol: 3MB; and isobutyllic/2-methyl-1-propanol: 2MP), one aromatic alcohol (i.e., 2-ethylphenol: 2EP), and two esters (i.e., ethyl-acetate: EA and 2-ethylphenyl acetate: 2EPA) in the course of beer fermentation (Table 1). These metabolites are the result of the catabolism of the excess of the branched-chain amino acids (BCAA) such as leucine (3MB), isoleucine (2MB), and valine (2MP) and the aromatic amino acids such as phenylalanine (2EP) (Dickinson et al., 2003; Hazelwood et al., 2008; Vidal et al., 2013, 2015;



\*Statistically different data were indicated ( $\alpha \leq 0.05$ ).

**Figure 2.** Fermentation parameters expressed as (A) ethanol yield, (B) total higher aliphatic alcohols (light gray columns) and their corresponding esters (dark gray columns), and (C) aromatic alcohol 2-ethylphenol (light gray columns) and its corresponding ester 2-ethylphenol acetate (dark gray columns) of wheat beer production in a scaled-up microbrewery using *S. cerevisiae* var. *diastaticus* SAFBREW WB06 strain. The values represent the average of three samples.

Vuralham et al., 2005). Therefore, the quantification of these alcohols may reflect the content of their respective amino acids free in the wort. Different from ethanol, the production of aliphatic higher alcohols by WB06 cells was increasing up to the fifth day of fermentation to reach around 370 mg/L (Figure 2B). From this total, isoamyl alcohol (3MB) accounted for 100–120 mg/L in each of the three batches. This was lower than reported for 3MB produced by WLP300 cells using different wheat wort that varied between 157 and 206 mg/L (Byeon et al., 2021). Interestingly, the production of these metabolites continued on days 4 and 5 even after the total consumption of sugars on day 3 (Figure 1). It might be related to the synthesis *de novo* of BCAA and their further catabolism (Vidal et al., 2015). This profile was mainly displayed by the huge increment of 3MB from the third day, which was the major metabolite among the aliphatic higher alcohols (Table 1). The production of these higher alcohols depends on the availability of reducing power as NADH for the last reductive reaction in the Ehrlich pathway catalyzed by an alternative pyruvate decarboxylase and alcohol dehydrogenase (Hazelwood et al., 2008; Vuralham et al., 2005). This reduced co-factor might be provided by the consumption of residual sugar in the wort (or from the storage carbohydrates) from day 3 to day 5 capable of providing glycolytic NADH, not connected to ethanol fermentation. Sugar consumption was quantified as 5.6 mmol of glucose equivalent per liter of wort, which would result in 11 mmol/L of produced NADH that ultimately would produce aliphatic higher alcohols at 11 mmol/L. This reducing power is 20 times higher than required for the additional production of 3MB detected in the range of 0.67 mmol/L from day 3 to day 5 (Table 1). However, that additional production was accompanied by a huge variation, as indicated by the standard deviation error bars (Figure 2B). A very small part of ethanol might be used for esterification reactions with the cytosolic acetyl-CoA to produce ethyl acetate ester, whose production

peak was achieved on the third day of fermentation and remained unaltered afterward (Figure 2B). All these results were a biochemical confirmation that 3 days were enough for the completion of the brewing process. Despite the production of aliphatic higher alcohols, their derivative acyl esters (2MB acetate, 3MB acetate, and 3MP acetate) were not detected in the industrial fermentation samples, or the metabolites were below the limit of detection. It leads to more bitter than fruity sensation to the final beer product.

The aromatic 2-EP alcohol was detected at a high concentration of 135 mg/L already on day 2 of fermentation (Figure 2C). It is the end product of the Ehrlich pathway in the degradation of phenylalanine in the wort, also requiring NADH for the last reductive enzyme reaction (Vuralham et al., 2005). The increment of 2EP from day 3 would follow the same metabolic explanation above by a surplus of glycolytic NADH produced from the consumption of residual sugar. Its derivative ester 2EPA is the result of an esterification reaction between one molecule of 2EP and one acetyl-CoA, meaning that both metabolites are metabolically connected. 2EPA was produced to its maximal concentration already on day 1 of fermentation (Figure 2C). The average molar ratio between the alcohol and its ester in the course of fermentation was calculated as 2:1, meaning that one-third of all 2EP produced from phenylalanine was converted to 2EPA. The balance between these metabolites is very important for the sensorial profile of the beverage, as they are responsible for the floral bouquet.

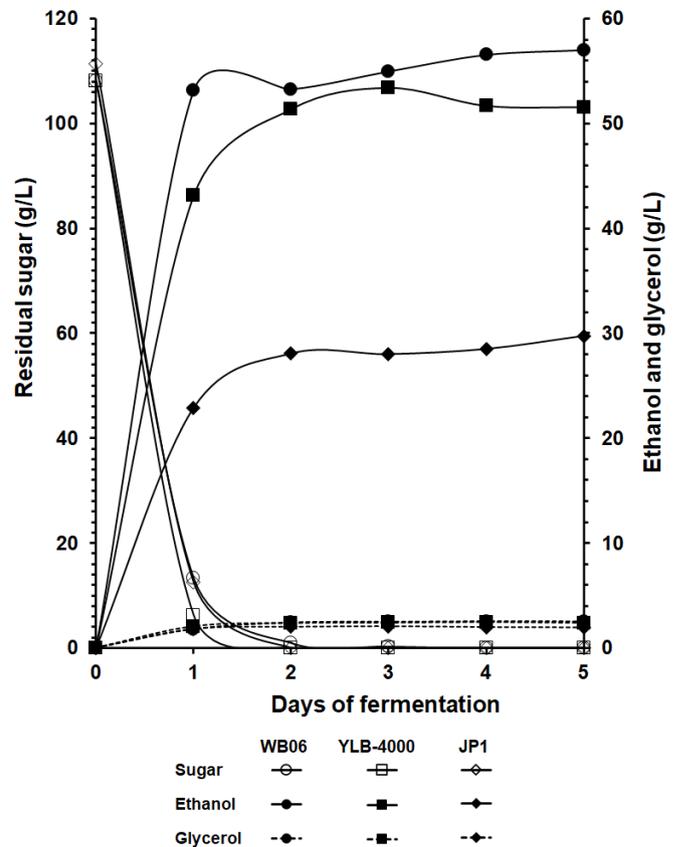
Altogether, these results showed that 3 days were enough to complete the fermentation of wheat wort and that the lack of additional gain in the sensorial panel of the beer after that period would not compensate for the operational costs of an additional 2 days of production. It is known that cells that spend a long time under anaerobic conditions reduce the synthesis of fatty acids that are important for plasma membrane maintenance,

with negative consequences to the yeast vitality (Snoek and Steensma, 2007). Thus, the shortage of fermentation time would benefit the cells by the refreshment of micro-aeration between the cycles and minimization of possible damages in different cellular components, as mentioned above (Jenkins et al., 2003; Smart and Whisker, 1996; Snoek and Steensma, 2007). In addition, there is a concern that long-term recycling could induce genetic and phenotype variation (Jenkins et al., 2009; Powell & Diacetic, 2007). However, the reduction in the fermentation time might also prevent this phenomenon and, therefore, increase the number of successive recycles without losing cellular performance. Besides, it was reported that the free amino nitrogen consumption is reduced with the decrease in the yeast pitching rate, and it has a direct relationship with higher alcohol production (Wang et al., 2019). Therefore, the maintenance of the yeast vitality along the successive recycling is the key to keep higher alcohol content at desirable levels. Therefore, the results presented in this study do not only refer to the reuse of the cell population for several cycles of fermentation. It deals with the benefits of decreasing the time of fermentation and so enabling the yeast population to perform efficient fermentation along the recycling. The reduction in the fermentation time would have, in addition to the clear economic advantage, the positive effect of reducing the appearance of genetic/phenotypic variations in the yeast population with a negative impact on the process. Experiments were done in laboratory conditions with two other yeast strains to test the advantages of this process improvement.

### 3.2. Laboratory fermentations confirmed the industrial data

Wheat wort fermentations were replicated in laboratory conditions by the same brewing yeast used in the microbrewery of the study. In addition, two other yeasts were added for comparison: the commercial brewing yeast YLB4000 and the industrial fuel-ethanol strain JP1 that is also used for cachaça fermentation (Figure 3). Fermentations with WB06 strain finished at 24 h of incubation, reaching the maximal theoretical ethanol yield. Afterward, ethanol concentration was maintained around 54–56 g/L (7.2% v/v). Fermentations with the YLB4000 strain approached the maximal theoretical ethanol yield on the third day of fermentation and kept 52–54 g of ethanol/L (6.9% v/v) thereafter. Sugar in the wort was completely consumed after 48 h of fermentation for both strains (Figure 3). Hence, these results confirmed that 3 days would be enough for fermentation indeed. Interestingly, the JP1 strain showed a lower ethanol yield of 0.25 g/g on the second day of fermentation, producing almost half of the ethanol compared to the two brewing strains (3.9% v/v). It can be explained by the fact that JP1 evolved for adaptation in high-sugar low-nitrogen substrates like sugar cane juice. Anyway, the results confirmed the wheat wort fermentation finished after 3 days of fermentation even for low-brewing yeast like JP1 (Figure 3). Cell population on the third day increased to  $8\text{--}9 \times 10^7$  cells/mL and underwent less change until the end of fermentation.

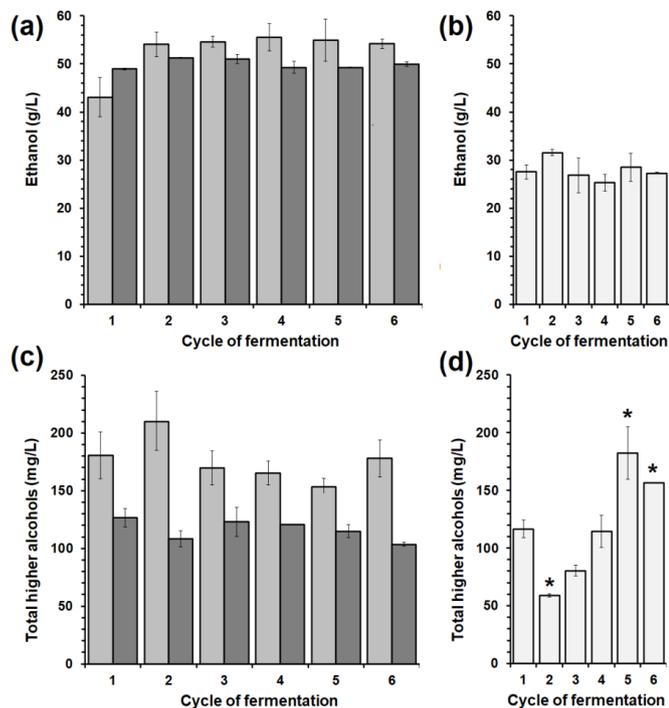
Glycerol production reached 2.5 g/L for all three strains (Figure 3), which was similar to glycerol concentration in industrial fermentation (Figure 1). Given that glycerol is a metabolic indicator of cell growth, this result indicated that lower ethanol



**Figure 3.** Fermentation profile of the brewing yeast strains WB06 (circles) and YLB-4000 (squares) and the fuel-ethanol yeast strain JP1 (diamonds) in laboratory conditions using wheat wort. The consumption of sugars (straight lines with open symbols) and the production of ethanol (straight lines with closed symbols) and glycerol (dotted lines with closed symbols) were quantified. The values represent the average of three independent fermentations. No significant differences were observed for the different parameters tested between the beer strains ( $\alpha \leq 0.05$ ).

production by JP1 was not associated with biomass increment, as expected from the excess of nitrogen in the wort. It also indicated that 2.5 g/L should be considered the threshold of glycerol for wheat wort fermentation.

The next step was to define the number of consecutive cycles of fermentation that might be possible with the re-cycled yeast biomass. The yeast cells were harvested on the third day of fermentation and used in successive 3-day batches. The relevant aspect of this approach was that cells were stored in water at 4°C in the intervals of fermentations. Both WB06 and YLB4000 maintained ethanol production in the range of 52–54 g/L up to six consecutive cycles (Figure 4A), while JP1 kept lower but stable ethanol production around 28 g/L (Figure 4B). In the beginning of each cycle, the wort was inoculated with the same initial number of cells between 1 and  $2 \times 10^7$  cells/mL, and at the end of each cycle, the population was calculated between 7 and  $9 \times 10^7$  cells/mL. It indicated that yeast biomasses maintained their highest fermentative capacities for long runs of use if collected at the right point of each batch of fermentation. This is a relevant industrial parameter for microbreweries by easing



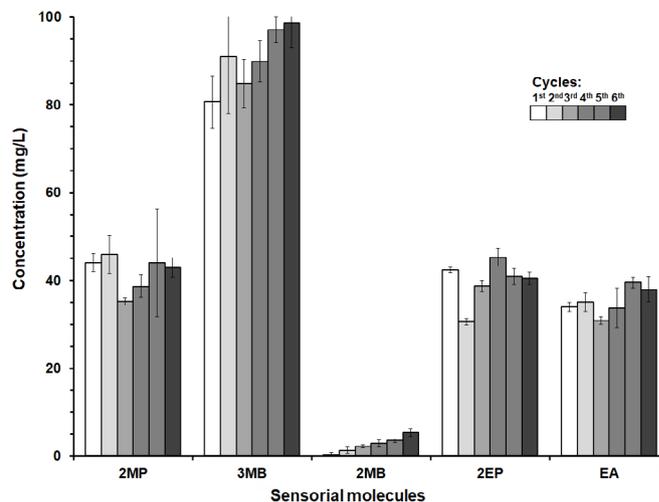
\*Statistically different data were indicated ( $\alpha \leq 0.05$ ).

**Figure 4.** Metabolite profile of three yeast strains in successive cycles of laboratory fermentations using wheat wort. On the third day, (A and B) ethanol and (C and D) high alcohols were quantified from the fermentation with (A and C – light gray columns) the strains WB06, (A and C – dark gray columns) YLB-4000, and (B and D) JP1. Cells were collected and used in the successive three-day cycle of fermentation. The values represent the average of three independent fermentations.

operational procedures and saving financial resources with biomass preparation.

Nevertheless, biomass re-pitching might last as long as it does not affect the sensorial qualities of the product. In this regard, the results showed that the WB06 strain produced a total of higher alcohols (2MP+2MB+3MB) in the range of 150–200 mg/L up to the sixth batch of fermentation (Figure 4C). This is a bit lower than the range detected by Wang et al. (2019) using top-fermenting yeast for wheat beer. This result confirmed that the WB06 cells maintained the metabolic vitality to produce the sensorial metabolites in successive batches, keeping the quality of the product.

The sum of all three branched-chain higher alcohols 2MP, 2MB, and 3MB compose the fruity bouquet of beverages. In view of this, a detailed view on the production of each higher alcohol by the WB06 strain showed that 3MB accounted for two-thirds and 2MP represented one-third of the total metabolites at the end of each fermentation cycle, while only traces of 2MB were detected (Figure 5). This is an important analysis since 3MB is a precursor to isoamyl acetate, which is an important aroma ester for a Weizen-style wheat beer, as it attributes a characteristic banana aroma to the beverage. The proportions of the three alcohols were maintained in the course of six batches of consecutive fermentations. Despite the drop in the 2EP concentration in the second round of fermentation, its final concentration was also



2MP: 2-methyl-1-propanol; 3MB: 3-methyl-1-butanol; 2MB: 2-methyl-1-propanol; 2EP: 2-ethylphenol; EA: ethyl-acetate; \*the values represent the average of three independent fermentations. No statistical differences were observed ( $\alpha \leq 0.05$ ).

**Figure 5.** End concentration of sensorial molecules by the brew strain WB06 in six consecutive 3-day cycles of laboratory fermentations using wheat wort\*.

constant in the range of 40 mg/L along the consecutive batches. The same stability was observed for the final concentration of EA in the same range of concentration (Figure 5).

The strain YLB-4000 showed lower production of higher alcohols than WB06 (Figure 4C). However, it is still above the minimal concentration (>100 mg/L) along the fermentation cycles that was proposed for quality beers (Hazelwood et al., 2008). In this case, the use of WB06 for the production of Wizen beer is advisable. On the other hand, the production of these metabolites was not stable in the strain JP1, varying from 50 to 150 mg/L in the course of six recycling (Figure 4D). Despite its low brewing capacity, the results with the JP1 strain confirmed that the yeast cells maintained their metabolic activity for successive batches when harvested on the third day of each cycle.

#### 4. CONCLUSION

The metabolic and organoleptic profiles obtained for the investigated analytes suggest that the fermentation period of wheat beer can be reduced to 3 days (>50% in a total of 7 days of fermentation) without large variations in concentration in the production of most of these compounds. Adjustment tests of the organoleptic compounds of WB06 can reveal whether the modifications carried out are capable of standardizing the formation of these compounds during the cycles of biomass utilization. Thus, the results obtained in this study indicate that the fermentation process for the production of Weizen beer in the microbrewery under study can last only 3 days without loss of product quality. This should decrease cellular stress during recycling and, consequently, increase industrial productivity.

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## REFERENCES

- Beer Judge Certification Program (BJCP). (2015). *2015 Style Guidelines: beer style guidelines*. Retrieved from [https://www.bjcp.org/docs/2015\\_Guidelines\\_Beer.pdf](https://www.bjcp.org/docs/2015_Guidelines_Beer.pdf)
- Briggs, D. E. (1998). *Malts and malting*. Blackie Academic & Professional.
- Byeon, Y. S., Lim, S.-T., Kim, H.-J., Kwak, H. S., & Kim, S. S. (2021). Quality characteristics of wheat malts with different country of origin and their effect on beer brewing. *Journal of Food Quality*, *2021*, 2146620. <https://doi.org/10.1155/2021/2146620>
- Dickinson, J. R., Salgado, L. E. J., & Hewlins, M. J. E. (2003). The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, *278*(10), 8028-8034. <https://doi.org/10.1074/jbc.M211914200>
- Faltermaier, A., Waters, D., Becker, T., Arendt, E., & Gastl, M. (2014). Common wheat (*Triticum aestivum* L.) and its use as a brewing cereal – a review. *Journal Institute of Brewing*, *120*(1), 1-15. <https://doi.org/10.1002/jib.107>
- Hardwick, W. (1994). *Handbook of Brewing*. CRC Press.
- Hazelwood, L. A., Daran, J. M., van Maris, A. J. A., Pronk, J. T., & Dickinson, J. R. (2008). The Ehrlich pathway for fusel alcohol production: A century of research on *Saccharomyces cerevisiae* metabolism. *Applied and Environmental Microbiology*, *74*(8), 2259-2266. <https://doi.org/10.1128/AEM.02625-07>
- He, Y., Dong, J., Yin, H., Zhao, Y., Chen, R., Wan, X., Chen, P., Hou, X., Liu, J., & Chen, L. (2014). Wort composition and its impact on the flavour-active higher alcohol and ester formation of beer - a review. *Journal of the Institute of Brewing*, *120*(3), 157-163. <https://doi.org/10.1002/jib.145>
- Jenkins, C. L., Kennedy, A. I., Hodgson, J. A., Thurston, P., & Smart, K. A. (2003). Impact of Serial Repitching on Lager Brewing Yeast Quality. *Journal of the American Society of Brewing Chemists*, *61*(1), 1-9. <https://doi.org/10.1094/asbcj-61-0001>
- Jenkins, C. L., Lawrence, S. J., Kennedy, A. I., Thurston, P., Hodgson, J. A., & Smart, K. A. (2009). Incidence and Formation of Petite Mutants in Lager Brewing Yeast *Saccharomyces Cerevisiae* (Syn. *S. Pastorianus*) Populations. *Journal of the American Society of Brewing Chemists*, *67*(2), 72-80. <https://doi.org/10.1094/asbcj-2009-0212-01>
- Kalb, V., Seewald, T., Hofmann, T., & Granvogl, M. (2020). Studies on the Impact of Malting and Mashing on the Free, Soluble Ester-Bound, and Insoluble Ester-Bound Forms of Desired and Undesired Phenolic Acids Aiming at Styrene Mitigation during Wheat Beer Brewing. *Journal of Agricultural and Food Chemistry*, *68*(44), 12421-12432. <https://doi.org/10.1021/acs.jafc.0c04835>
- Meier-Dörnberg, T., Hutzler, M., Michel, M., Methner, F.-J., & Jacob, F. (2017). The Importance of a Comparative Characterization of *Saccharomyces Cerevisiae* and *Saccharomyces Pastorianus* Strains for Brewing. *Fermentation*, *3*(3), 41. <https://doi.org/10.3390/fermentation3030041>
- Meusinger, R. (2019). Wheat beer challenge. *Analytical and Bioanalytical Chemistry*, *411*, 3699-3703. <https://doi.org/10.1007/s00216-019-01854-7>
- Meusinger, R. (2020). Solution to wheat beer challenge. *Analytical and Bioanalytical Chemistry*, *412*, 5. <https://doi.org/10.1007/s00216-019-02231-0>
- Narziss, L. (1984). The German beer law. *Journal of the Institute of Brewing*, *90*(6), 351-358. <https://doi.org/10.1002/j.2050-0416.1984.tb04288.x>
- Pires, E., Teixeira, J. A., Brányik, T., & Vicente, A. A. (2014). Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology Biotechnology*, *98*, 1937-1949. <https://doi.org/10.1007/s00253-013-5470-0>
- Powell, C. D., & Diacetis, A. N. (2007). Long Term Serial Repitching and the Genetic and Phenotypic Stability of Brewer's Yeast. *Journal of the Institute of Brewing*, *113*(1), 67-74. <https://doi.org/10.1002/j.2050-0416.2007.tb00258.x>
- Powell, C. D., & Fischborn, T. (2010). Serial Repitching of Dried Lager Yeast. *Journal of the American Society of Brewing Chemists*, *68*(1), 48-56. <https://doi.org/10.1094/asbcj-2010-0125-01>
- Ramos, G. C. B., & Padolfi, M. A. C. (2019). A evolução do mercado de cervejas artesanais no Brasil. *Revista Interface Tecnológica*, *16*(1), 480-488.
- Reglitz, K., Féchir, M., Mall, V., Voigt, J., & Steinhaus, M. (2022). The impact of caramel and roasted wheat malts on aroma compounds in top-fermented wheat beer. *Journal of the Institute of Brewing*, *128*(4), 138-149. <https://doi.org/10.1002/jib.701>
- Saerens, S., Delvaux, F., Verstrepen, K., Van Dijck, P., Thevelein, J., & Delvaux, F. (2008). Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Applied and Environmental Microbiology*, *74*(2), 454-461. <https://doi.org/10.1128/AEM.01616-07>
- Schneiderbanger, H., Koob, J., Poltinger, S., Jacob, F., & Hutzler, M. (2016). Gene expression in wheat beer yeast strains and the synthesis of acetate esters. *Journal of the Institute of Brewing*, *122*(3), 403-411. <https://doi.org/10.1002/jib.337>
- Shewry, P. R., Wan, Y., Hawkesford, M. J., & Tosi, P. (2020). Spatial distribution of functional components in the starchy endosperm of wheat grains. *Journal of Cereal Science*, *91*, 102869. <https://doi.org/10.1016/j.jcs.2019.102869>
- Smart, K. A., & Whisker, S. (1996). Effect of Serial Repitching on the Fermentation Properties and Condition of Brewing Yeast. *Journal of the American Society of Brewing Chemists*, *54*(1), 41-44. <https://doi.org/10.1094/asbcj-54-0041>
- Snoek, I. S. A., & Steensma, H. I. (2007). Factors involved in anaerobic growth of *Saccharomyces cerevisiae*. *Yeast*, *24*(1), 1-10. <https://doi.org/10.1002/yea.1430>
- Stewart, G. (2017). The production of secondary metabolites with flavour potential during brewing and distilling wort fermentations. *Fermentation*, *3*(4), 63-91. <https://doi.org/10.3390/fermentation3040063>
- Vidal, E. E., de Billerbeck, G. M., Simões, D. A., Schuler, A., François, J. M., & De Morais Jr., M. A. (2013). Influence of nitrogen supply on the production of higher alcohols/esters and expression of flavour-related genes in cachaça fermentation. *Food Chemistry*, *138*(1), 701-708. <https://doi.org/10.1016/j.foodchem.2012.10.147>
- Vidal, E. E., De Morais Jr., M. A., François, J. M., & de Billerbeck, G. M. (2015). Biosynthesis of higher alcohol flavour compounds by the yeast *Saccharomyces cerevisiae*: impact of oxygen availability and responses to glucose pulse in minimal growth medium with leucine as sole nitrogen source. *Yeast*, *32*(1), 47-56. <https://doi.org/10.1002/yea.3045>

- Vuralham, Z., Luttik, M. A. H., Tai, S. L., Boer, V. M., De Morais Jr., M. A., Schipper, D., Almering, M. J. H., Kötter, P., Dickinson, R., Daran, J.-M., & Pronk, J. T. (2005). Physiological characterization of the *aro10*-dependent, broad-substrate-specificity 2-oxo acid decarboxylase activity of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, 71(6), 3276-3284. <https://doi.org/10.1128/AEM.71.6.3276-3284.2005>
- Yin, H., Dong, J., Yu, J., Chang, Z., Qian, Z., Liu, M., Huang, S., Hu, X., Liu, X., Deng, Y., & Wang, D. (2016). A preliminary study about the influence of high hydrostatic pressure processing on the physicochemical and sensorial properties of a cloudy wheat beer. *Journal of the Institute of Brewing*, 122(3), 462-467. <https://doi.org/10.1002/jib.344>
- Wang, M., Sun, Z., Wang, Y., Wei, Z., Chen, B., Zhang, H., Guo, X., & Xiao D. (2019). The effect of pitching rate on the production of higher alcohols by top-fermenting yeast in wheat beer fermentation. *Annals of Microbiology*, 69, 713-726. <https://doi.org/10.1007/s13213-019-01463-w>
- Weyermann Specialty Malting (2023). *Weyermann Specialty Malts*. Retrieved from [https://www.weyermann.de/wp-content/uploads/2023/03/weyermann\\_brewery\\_us\\_pale-ale-malt.pdf](https://www.weyermann.de/wp-content/uploads/2023/03/weyermann_brewery_us_pale-ale-malt.pdf)
- Weyermann Pilsner Malt (2023). *Weyermann Specialty Malts*. Retrieved from [https://www.weyermann.de/wp-content/uploads/2023/03/weyermann\\_brewery\\_us\\_pilsner-malt.pdf](https://www.weyermann.de/wp-content/uploads/2023/03/weyermann_brewery_us_pilsner-malt.pdf)