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Investigating on the fermentation behavior of six lactic acid bacteria strains in barley malt wort reveals limitation in key amino acids and buffer capacity

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ABSTRACT

Understanding lactic acid bacteria (LAB) fermentation behavior in malt wort is a milestone towards flavor improvement of lactic acid fermented malt beverages. Therefore, this study aims to outline deficiencies that may exist in malt wort fermentation. First, based on six LAB strains, cell viability and vitality were evaluated. Second, sugars, organic acids, amino acids, pH value and buffering capacity (BC) were monitored. Finally, the implication of key amino acids, fructose and wort BC on LAB growth was determined. Short growth phase coupled with prompt cell death and a decrease in metabolic activity was observed. Low wort BC caused rapid pH drop with lactic acid accumulation, which conversely increased the BC leading to less pH change at late-stage fermentation. Lactic acid content ($\leq$3.9 g/L) was higher than the reported inhibitory concentration (1.8 g/L). Furthermore, sugars were still available but fructose and key amino acids lysine, arginine and glutamic acid were considerably exhausted ($\leq$98%). Wort supplementations improved cell growth and viability leading to conclude that key amino acid depletion coupled with low BC limits LAB growth in malt wort. Then, a further increase in organic acid reduces LAB viability. This knowledge opens doors for LAB fermentation process optimization in malt wort.

1. Introduction

Although their tremendous reported health benefits and possibilities for technological innovation, lactic acid fermented cereal-based beverages are scarce on the market. Low consumer's acceptance and limitations in the flavor profile are the shortcomings (Nsogning Dongmo et al., 2016; Yu and Bogue, 2013). It is now known that limitations in the flavor profile of lactic acid fermented malt-based beverages (LAFMB) are not due to the aroma composition but to an insufficient concentration of important aroma compounds. The fermenting LAB strain has a significant impact on the concentration of key aroma compounds, the flavor, and acceptance of the resulting beverages (Nsogning Dongmo et al., 2017; Salmerón et al., 2015). However, the question of what causes insufficient aroma compound formation by LAB during malt wort fermentation is fundamental for further attempts aiming to improve the flavor profile of LAFMB.

The central issue that could be addressed is an adequate fermentation performance. For that, good cell physiological conditions and nutrient availability are key determinants. Nutrient deficiency or low viable and vital cell could impair fermentation performance resulting undoubtedly to short growth and low aroma yield. The basics on how is the fermentation performance of LAB in malt wort remained partially elucidated. Studies have described the growth behavior of LAB, change in fermentable sugars, free amino nitrogen, and lactic acid and acetic acid accumulation in cereal-based substrates fermentation. LAB growth phase in cereal-based substrates is generally very short ranging from 6 to 48 h depending on the substrate type, fermentation temperature, inoculation rate and LAB strain (Charalampopoulos et al., 2002, 2003; Peyer et al., 2015; Salmerón et al., 2014). Furthermore, studies on the evaluation of limitations that may exist in lactic acid fermentation of malt wort do not exist yet.

LAB are strictly fermentative but require fermentable sugars, amino acids, fatty acids, vitamins and purines for their development. As such, they are auxotrophic to several amino acids and unable to grow in a simple media containing carbon sources and minerals (Hebert et al., 2000). Any lack of nutrients could,

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therefore, be a real challenge for LAB growth.

Besides, medium composition changes such as pH drop, consequent to organic acid accumulation occurring in lactic acid fermentation, cause acidic stress that may also affect LAB viability and metabolic activity. The toxic effect of the undissociated lactic acid on LAB cells has been described (Adamberg et al., 2003; Pieterse et al., 2005). To overcome the acidic stress they are subject to in their fermentation environment, LAB cells exploit four main systems: the glutamate decarboxylase (GAD), lysine decarboxylase, arginine deiminase (ADI) and F1Fo ATPase multisubunit (Azcarate-Peril and Klaenhammer, 2010). However, whether to increase the intracellular pH or to intensify protons extrusion from the cytoplasm, they all rely on proteins or amino acids. Furthermore, amino acids are known to be strongly involved in enzymatic functions and as aroma compound precursors. Lack of some amino acids could, therefore, result to low acidic resistance, cell death, and impaired metabolic activity leading too low aroma formation.

Thus, nutrient composition of malt wort is of major importance to LAB cell fermentation performance and by extension to aroma compound yield. Barley malt substrate is a complex rich nutrient medium that was described to support LAB growth and aroma compound formation (Charalampopoulos et al., 2003; Nsogning Dongmo et al., 2016; Peyer et al., 2015) than other considered cereals like wheat and oat. But whether malt wort is an optimum medium to meet LAB nutritional requirement for efficient and long-lasting LAB growth is still to be determined.

To understand the limitations occurring in lactic acid fermentation of malt wort, this study aims at investigating on the viability, vitality and death rate of six LAB strains, medium physical and compositional changes regarding residual nutrients, organic acids, buffering capacity and pH value change in barley malt wort fermentation. In the end, the implication of key amino acids, fructose, and wort buffering capacity in LAB growth was evaluated. This knowledge will orientate in the optimization of malt wort composition and fermentation process for improved LAB growth and will bring a light to the understanding of the reported low aroma yield of lactic acid fermented cereal-based beverages as well.

2. Material and methods

2.1. Wort preparation, strains, and fermentation

Wort at 14% concentration was prepared from 72% standardized unhopped Bavarian pilsner barley malt extract from Weyermann (Bamberg, Germany) using distilled water and autoclaved at 110 °C for 10 min. Hot break materials were separated after cooling. Six selected and previously identified strains of *Lactobacillus plantarum* Lp.758, Lp.765 and Lp.725, *Lactobacillus brevis* Lb.986, and *Lactobacillus amylolyticus* La.TL3 and La.TL5 were obtained from the strain collection of the chair for Brewing and Beverage Technology (Technical University of Munich, Germany). Cultures were propagated twice in MRS broth (Sigma Aldrich, Germany) for 24–36 h and pre-cultured in wort for 12 h at 28 °C for *L. brevis* and *L. plantarum* and at 48 °C for *L. amylolyticus* before use in the experiments. Cells were washed thrice with sterile quarter strength Ringer's solution (Sigma Aldrich) at 4000 rpm, 4 °C for 10 min. Fermentation was carried out in triplicate at laboratory scale in 500 mL wort volume under static conditions for 72 h. The inoculation rate was $5.8 \pm 1.1 \times 10^6$ CFU (colony forming unit)/mL. Fermented samples were immediately used for viability, vitality and staining procedures whereas those for pH, buffering capacity and analytical measurements were stored at 4 °C and −20 °C, respectively until analysis.

2.2. Viability and death kinetics

Cell viability was evaluated using plate counting method whereas fluorescence microscopic counting after cell labeling with carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) fluorescent probes was considered for death rate determination.

2.2.1. Plate count

The viability was measured by colony forming unit counting method. Serial decimal dilutions were prepared in sterile quarter strength Ringer's solution and 0.1 mL diluted samples were plated on MRS agar in triplicate using pour-plate procedure and incubated at 28 °C and 48 °C for *L. plantarum*/*L. brevis* and *L. amylolyticus*, respectively for 48 h. Viable colonies were counted and recorded as the number of CFU/mL.

2.2.2. Cell fluorescence labeling with CFDA and PI

A solution of CFDA (10 mM) was prepared by dissolving 4.6 mg CFDA in 1 mL dimethyl sulfoxide (DMSO) and stored in aliquots of 1 mL at −20 °C in the dark and was diluted to 1 mmol using DMSO before use. PI was supplied as a 1 mg/mL solution and was stored in darkness in the refrigerator. Cells were washed thrice with Ringer's solution and resuspended into an OD_{600nm} of 1.2 prior to staining. 30 μL of CFDA was added to 940 μL bacteria suspension and kept in the darkness at 30 °C for 10 min. Thereafter, 30 μL of PI was added and kept in the darkness at 30 °C for 10 min (Bunthof et al., 1999). Cells were washed out twice to remove excess probes and re-suspended in Ringer's solution; aliquots were then stored on ice until analysis within 1 h. Red-labelled and green-labeled cells of 10 visual fields were counted at 40x objective using a Zeiss Axioskop epifluorescence microscope equipped with three filter sets for DAPI/PI/CFDA and a camera (Carl Zeiss, Germany). Each sample was counted in triplicate. The death percentage was calculated as the ratio of the red-labeled cells to the total of red- and green-labeled cells.

2.3. Vitality assessment – acidification power (AP) test

Vitality was assessed by means of acidification power test, which measures the glycolytic activity through the ability of cell to lower the pH value of 0.1% glucose solution (Riis et al., 1995; Sigler, 2013). The applied method was adapted from previously reported methodology (Bunthof et al., 1999). Cells were washed thrice (4000 rpm, 4 °C, 10 min) and re-suspended in quarter strength Ringer's solution at pH 6.5 to a final OD_{600nm} of 1.0 ± 0.1 corresponding to $8 \pm 1 \times 10^7$ cells/mL. After equilibration at 25 °C, 200 μL glucose was added to 20 mL cell suspension to a final concentration of 6 mM. Medium acidification was assessed after 10 min by pH value measurement. The acidification power was calculated as the pH difference between the initial pH value (6.5) and the pH value after 10 min. The test was done in triplicate.

2.4. Analytical determinations

2.4.1. Sugar analysis

Extracellular sugars were analyzed by high-performance liquid chromatography (HPLC) using a pulsed amperometric detector (PAD) and a Dionex Carbopac PA10 carbohydrate column (Thermo Scientific, Germany). External commercial reference compounds were used for the calibration.

2.4.2. Organic acid analysis

Specific enzymatic kits (Megazyme, Ireland) were used to determine the concentrations of extracellular L-lactic acid (kit k-late 07/14), acetic acid (kit k-acetrm 07/12), L-malic acid (kit k-lmal-58A