Flavour enhancement of beer and related beverages by increasing flavour precursors in raw materials by enzymes

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Abstract

In the past two decades, the brewing industry has witnessed the growing consumer demands for new beer types. Meanwhile, responsible drinking and health concern fuel the non-alcoholic and low-alcohol beer market. Towards this end, a plethora of literature has been dedicated to fermentation by non-conventional yeast strains, producing fruity flavours in beer and related beverages [1]. In parallel with this, it is feasible to achieve flavour improvement by using raw material to its fullness. This approach focuses on releasing more flavour precursor amino acids – leucine, isoleucine, valine, and phenylalanine – by enzymes during the beer manufacturing process. These branched-chain and aromatic amino acids participate in the Ehrlich pathway in yeast cells during fermentation, which accounts for the characteristic higher alcohols and their ester derivatives in beer [2]. Lab scale lager beer production was performed to demonstrate this method. GC-MS with stir bar sorptive extraction (Twisters) was applied to characterise the resulting products.

Keywords: beer, enzymes, flavour, non-alcoholic and low-alcohol beer

Introduction

In recent decades, health concern and responsible drinking propel new consumers to pursue non-alcoholic and low-alcohol (NABLAB) beer [1]. There are two main methods to produce this beer type: the physical method involving dealcoholisation and the biological method employing limited fermentation or non-*Saccharomyces* yeast strains. Nonetheless, NABLAB usually associates with taste deficiency compared to related normal strength beer [3]. Dealcoholisation compromises aroma compounds while evaporating alcohol or filtering through a membrane. Limited fermentation results in worty off flavour. Non-*Saccharomyces* yeasts oftentimes introduce novel fruity or floral aromas that can stand as a new beer category, as opposed to the low-alcohol version of the more conventional lager [1, 3]. In order to fulfil the criterion of NABLAB being 'as close as possible' to regular lager beer [4], the present study probes the possibility of using proteases to boost the yeast production of higher alcohols and esters relating to conventional lager. These volatiles are mainly from the Ehrlich amino acid degradation pathway. The compounds of interest are 2-methylpropyl alcohol and acetate from valine (Val), 2-methylbutyl alcohol and acetate from isoleucine (Ile), 3-methylbutyl alcohol and acetate from leucine (Leu), and 2-phenylethyl alcohol and acetate from phenylalanine (Phe). In brewing practice, free amino acids come from proteolysis during malting and mashing [5]. Here, we first compared the aforementioned higher alcohols and acetate esters between NABLAB and the related regular lager beer; and then studied the evolution of these compounds in fermentation of wort with added amino acids Val, Ile, Leu, and Phe. Finally, we tested the possibility to apply proteases in mashing to produce more flavoursome lager beer.

Experimental

Reagents and materials

Two commercial alcoholic beer and their corresponding non-alcoholic beer from two Danish manufacturers were used for gas chromatography-mass spectrometry (GC-MS) comparison. All amino acids were purchased from Sigma-Aldrich (St. Louis, MO, USA), and the amino acid stock solutions were sterile filtered before use. Pilsner malt from 2-row spring barley (Sophus Fuglsang Maltfabrik ApS, Haderslev, Denmark) was used in all mashing experiments. All enzymes were obtained from Novozymes A/S (Kongens Lyngby, Denmark).

Mashing and fermentation

High gravity mashing using a liquor/grist ratio of 3 was conducted in a mashing device LB12 (Lochner, Berching, Germany) to imitate industrial process. An infusion mashing program was employed: 45 °C (30 min) → 63 °C (30 min) → 72 °C (20 min) → 78 °C (10 min). Two serine proteases (SP1 and SP2) and two metalloproteases (MP1 and MP2) were added respectively at the beginning of the mashing experiment. Each mashing experiment was carried out in duplicate. At the end of the experiment, wort was obtained by gravity

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filtration. A portion of wort (2.000 ± 0.020 mL) was measured by the alpha-amino nitrogen *O*-phthaldialdehyde (NOPA) method for free amino nitrogen content in a photometric analyser Gallery™ Plus Beermaster Discrete Analyzer (Thermo Scientific, Waltham, MA, USA). This resulting wort was boiled in 100 °C water bath for 60 min and sterile filtered before subsequent fermentation. A commercial lager yeast strain (Saflager-34/70, Fermentis, FR) was pitched at 1.5 × 10^6 viable cells/mL and fermentation was carried out with 14 °Plato wort at 14 °C for 7 days.

GC-MS analysis

The polydimethylsiloxane coated stirring bars, Twister® of 10 mm length and 0.5 mm film thickness (Gerstel, Mülheim an der Ruhr, Germany) were used for sorptive extraction sampling. Volatile compounds were thermally desorbed and analysed using an Agilent 5977B GC/MSD single quadrupole system (Agilent Technologies, Santa Clara, CA, USA). The evolution of volatiles from fermentation of wort containing spiked-in amino acids, was studied using dynamic headspace sampling. The samples were purged with nitrogen and volatiles were trapped on Tenax-TA before thermally desorbed and analysed using an Agilent 5975C GC/MSD Triple-Axis Detector system (Agilent Technologies, Santa Clara, CA, USA).

Results and discussion

In general, the volatiles derived from the Ehrlich amino acid degradation pathway appeared less in NABLAB (F1 and F2, Figure 1) compared to the corresponding regular strength lager beer (B1 and B2, Figure 1). This confirmed the aroma deficiency issue in NABLAB, and thus the shift of focus to the generation of these higher alcohols and acetate esters with amino acid addition. Higher alcohols and acetate esters are produced by yeasts via a three-step catabolic pathway [2]. After amino acid uptake, transamination leads to an α-ketoacid intermediate; an irreversible decarboxylation of this intermediate results in an aldehyde that undergoes reduction to the corresponding alcohol. Further, the alcohol couples with an acetyl coenzyme A to produce the acetate ester. Examination of higher alcohols and acetates evolution with spiking samples at Day 2, Day 4, and Day 6 confirmed such metabolism. For instance, aldehyde intermediates detected in spiking trials in Figure 2 reached maxima at Day 2. This was due to the incomplete reduction of this intermediate at an early fermentation stage. This may partially contribute to aldehyde off-flavours in NABLAB produced by limited fermentation, besides the aldehydes from enzymatic reaction – lipid oxidation, and biochemical reaction – Strecker degradation of amino acids, during malting and mashing. Higher alcohols, on the other hand, increased over the first 4 days and either reached a plateau or decreased afterwards, which could be caused by esterification consuming these higher alcohols. Again, esterification was evidenced by the increase of acetate esters at the end of fermentation in Figure 2. In summary, spiking in these chosen amino acids led to the increase of corresponding higher alcohols and acetates from controls.

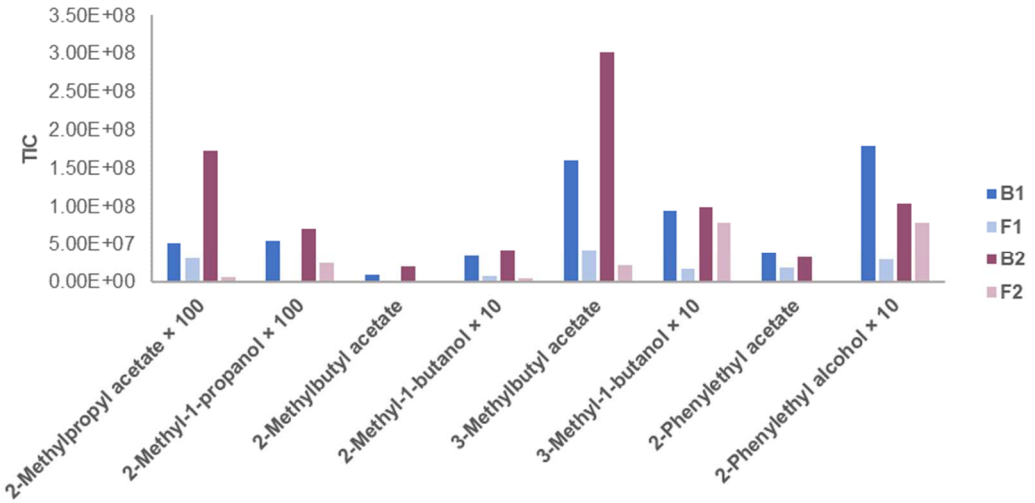

Figure 1*: Higher alcohols and corresponding acetate esters in NABLAB (F1 and F2) vs. normal strength beer (B1 and B2). Some volatiles are multiplied by 10 or 100 to improve visibility.*

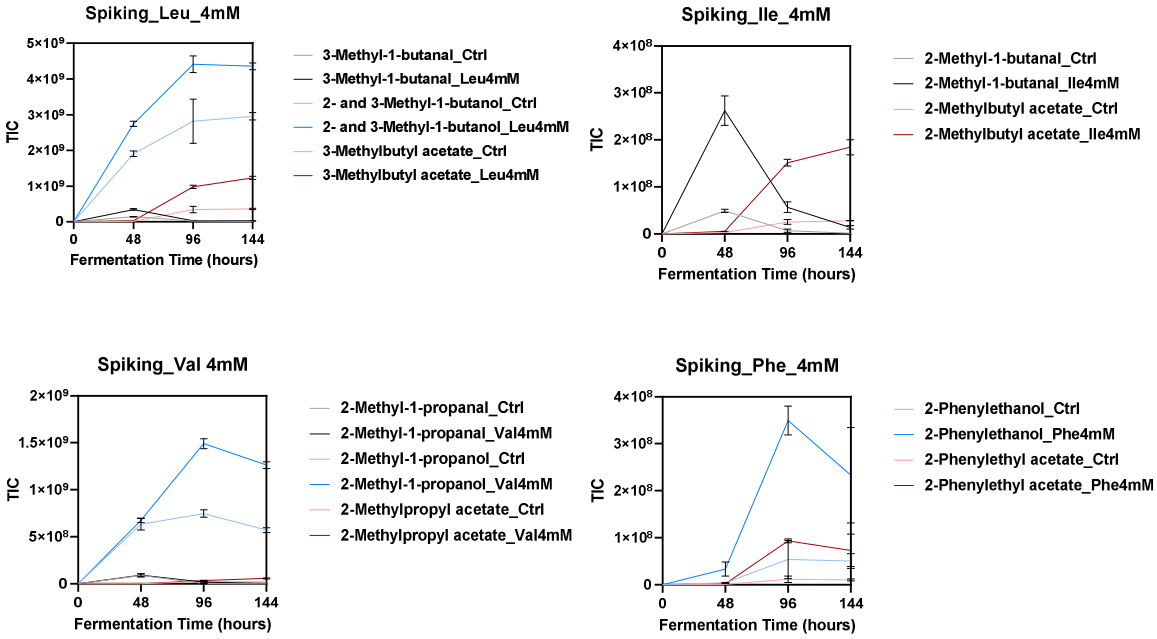

Figure 2*: Fermentation of 14 °Plato wort with spiked-in Leu 4mM (upper left), Ile 4mM (upper right), Val 4mM (lower left), and Phe 4mM (lower right). It was noted that 2- and 3-methylbutanol from Ile and Leu respectively, could not be separated.*

With these observations in hand, fermentation was conducted with wort derived from protease-treated mash to examine the impact of protease on amino acids (Val, Ile, Leu, and Phe) in wort, and relevant higher alcohols and acetates after fermentation. Figure 3 shows that wort from SP1, MP1, and MP2-treated mashing led to more acetate esters, i.e. 2-methylpropyl acetate from Val, 2- and 3-methylbutyl acetates respectively from Ile and Leu, and 2-phenylethyl acetate from Phe. On the other hand, higher alcohol productions varied. Fermentation following protease-treatment appeared at similar or even lower levels of 2-methylpropyl alcohol, 2-methylbutyl alcohol, and 3-methylbutyl alcohol, compared to control; but 2-phenylethyl alcohol was higher after enzyme treatment. This could be explained by esterification consuming these higher alcohols. It might be that these four higher alcohols were increased to different extent after fermentation (of wort derived from enzyme-treated mash), with 2-phenylethyl alcohol being higher than the rest. Esterification led to lower concentrations of higher alcohols but higher concentrations of acetates. A high 2-phenylethyl alcohol level resulted in more 2-phenylethyl acetate but also retained more starting material afterwards, which was observed in Figure 3.

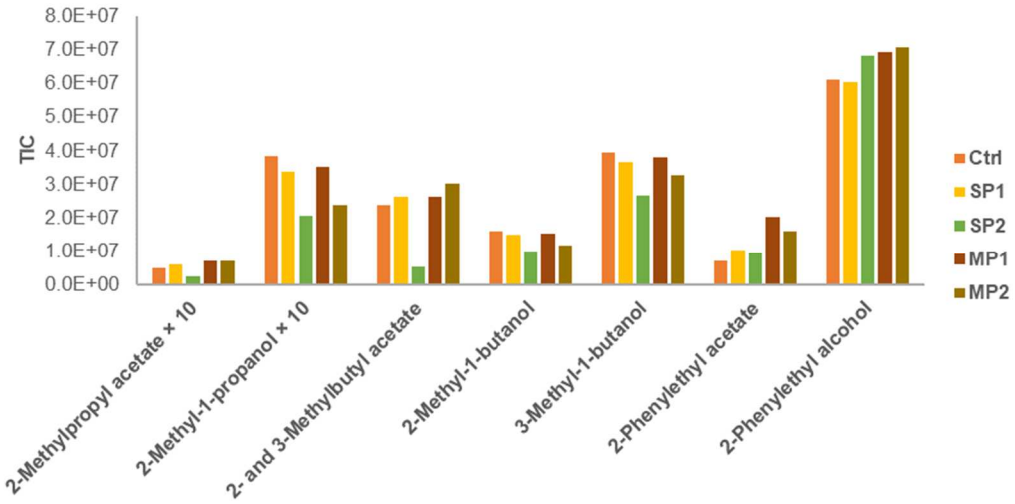

Figure 3*: Volatiles from the Ehrlich amino acid degradation pathway after fermentation of wort derived from protease-treated mash vs. control. Some volatiles are multiplied by 10 to improve visibility.*

Application of proteases usually result in more free amino nitrogen (FAN) that comprise of free amino acids and peptides, which was the case in this mashing experiment (Figure 4). Nonetheless, the increase of FAN did not necessarily correlate to the increase of these higher alcohols or acetates after fermentation, as was shown by the results from SP2. SP2 produced similar level of FAN as that by SP1 (Figure 4), and yet 2-methylpropyl alcohol and acetate, 2-methylbutyl alcohol and acetate, and 3-methylbutyl alcohol and acetate after fermentation of wort from SP2-treated mash differed from those from SP1 (Figure 3). Further quantification of free amino acid composition in the wort can better elucidate the impact of protease on the generation of these higher alcohols and acetates.

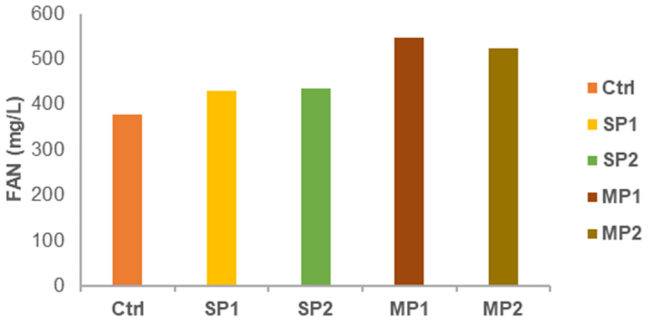

Figure 4*: Free amino nitrogen after mashing.*

Conclusion

This work focused on aromas derived from the Ehrlich pathway in yeasts. GC-MS comparison of NABLAB and related regular lager beer confirmed the aroma deficiency in the former beer type. Further investigation in aroma evolution from Val, Ile, Leu, and Phe in the spiking trials confirmed the effects of these amino acids on the formation of their corresponding higher alcohols and acetate esters. Finally, increases in acetate esters were observed in protease-treatment and showcased the potential of this method to increase aromas via the Ehrlich pathway.

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