Volume 34, Number 2 • 1997 • Pages 115-118

Technical Quarterly

Flavor Evolution of Top Fermented Beers

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ABSTRACT

With regard to the overall flavor-evolution of top-fermented beers, acetate esters play an important role. A significant decrease of mainly isoamyl acetate has been observed during storage of these beers. This hydrolysis is partly chemical, partly enzymatic, the latter only for unpasteurized and/or bottle conditioned beers. The enzymatic activity towards isoamyl acetate can have a profound impact on the consumers appreciation of the beer. Moreover, this activity is strongly strain-dependent. Our to-fermenting strain showed two esterases, active towards paranitrophenyl acetate, from which only one was active towards isoamyl acetate. Immunological studies suggest that this enzyme is not actively secreted but released after cell-lysis during fermentation, maturation and bottle conditioning (for beers refermented in the bottle).

SINTÉSIS

Con respecto a la evolucion global de sabor en de cervezas de fermentacion superior, acetatos de esteres jugan un papel importante. Un decremento significativo principalmente en acetato isoamil ha sido observado durante el almacenamiento de estas cervezas. Esta hidrolisis es parcialmente quimica, parcialmente encimatica, la ultima unicamente para cerveza no pasteurizada y/o acondicionada en la botella. La actividad encimatica con respecto a acetato isoamil puede tener un profundo impacto en la apreciacion de los consumidores de la cerveza. Mas aun, esta actividad es fuertemente dependiente de la ceta. Nuestra eta de fermentacion superior mostro dos esterases, activos hacia acetato paranitrophenil, del cual solo uno fue activo con respecto a acetato isoamil. Estudios inmun ologicos sugieren que esta encima no es una secrecion activa pero es liberada despues de cellysis durante la fermentacion, maduracion y condicionamiento en la botella (para cervezas refermentadas en la botella).

INTRODUCTION:

Top fermented beers represent only a small percentage of the total beer consumption. However, taking into consideration the growing popularity of these beers in several European countries, and the phenomenon of micro- and pub-breweries all around the world, these top-fermented beers cannot be left aside.

Since most of these beers are advised to be consumed within a period of two years (or even more) after bottling, we were very interested in the evolution of the product's aroma during this period, particularly in the evolution of esters and fusel alcohols which make these beers so different from typical lager beers.

Besides work carried out by Derdelinckx and co-workers,[1] little references are found with regard to the aroma evolution of of special beers. This is presumable partly due to confidentiality reasons. With regard to the esterase activity in the genus *Saccharomyces*, several authors indicate that this activity is not present in filtered beers. According to Schermers,[9] esterase activity is present in brewer's yeast but this activity is m likely to survive in the surrounding medium after cell-lysis. Suomalainen and Parkkinen[6,7,8,12] reported the presence of an esterase activity in baker's yeast against various substrates but acetate esters were hydrolyzed only very slowly or not at all Toshimitsu[13] reported some esterase properties purified from baker's yeast. Higuchi[3] reported the ester-decomposing activity of an esterase present in *Aspergillus* species in soy sauce. Interesting here was that they obtained by gel-filtration a koji esterase able to decompose isoamyl acetate. Inoue[4] believes that esterases of *Hansenula mrakii* are involved in the production of esters in sake brewing. Spaepen[11] reported the presence of esterase activity in *Brettanomyces*.

G.S. Derdelinckx, Prof. Dr. Ir. (1954), is active in the field of Belgian special beers since 1979 and acts as a scientific advisor of several well known Belgian brewers of "White beers," "Wheat beers," "Sour beers," "Ales" and typical brands as "Trappist beers" and so-called "Abbey beers."

In 1992, he joined the Brewing Research Group at the Catholic University of Louvain (K.U. Leuven - Belgium) and started together with Dr. Ir. H. Neven research activities in the field of maturation and aging of beers carbonated by bottle and keg refermentation. Their activity was (is) focused especially on the evolution of esters and highlights the chemical and enzymic aspects involved. Besides several publications in the field of special beers, both authors are actually writing, together with Prof. Dr. Ir. F. Delvaux, a textbook on "Traditional and Actual Belgium Beer Technology" (publication scheduled in December1997).

EXPERIMENTAL

Different Yeast Strains

A standard 16°P wort (25% adjunct) was fermented by two different yeast strains: one top-fermenting strain (CMB 64) and one bottom fermenting strain (CMB 11). Standard fermentations were carried out until end gravity, at 24°C for the top-ferment-

ing strain and at 12°C for the bottom-fermenting strain. The beers were chilled to -1°C and transferred to maturation.

After a maturation of one week, they were clarified by a single pass diatomaceous earth filtration. Before bottling, the beers were corrected with ethylacetate, isoamyl acetate, acetic acid and isoamyl alcohols in order to start our comparison at the same level. Also the pH was equal for both beers.

The beers were subsequently stored at 24°C. Esters and fusel alcohols were determined weekly by headspace gas-chromatography. Results with regard to the acetate esters are presented in figures 1 and 2.

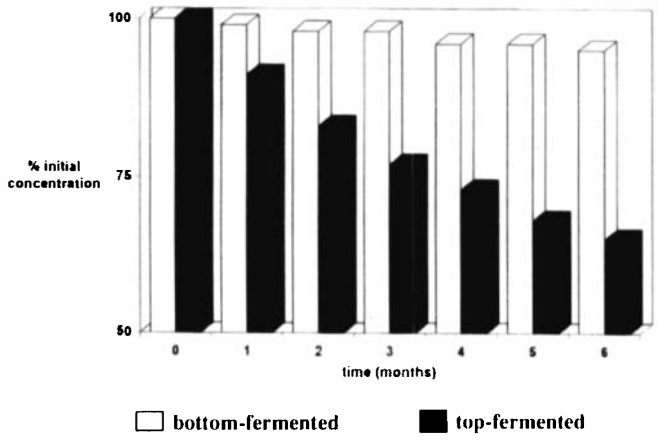

FIGURE 1
Evolution of Isaomyl Acetate During Storage at 24°C

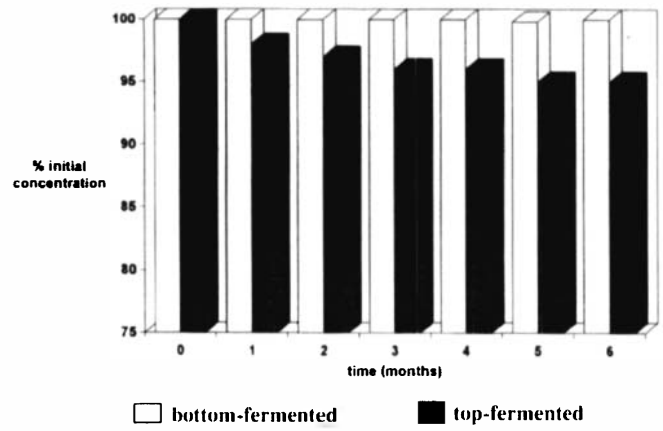

FIGURE 2
Evolution of Ethyl Acetate During Storage at 24°C

We see clearly in figure 1 that after a period of 6 months, the top-fermented beer lost about 35% of its original isoamyl acetate concentration whereas the bottom-fermented beer only lost about 10%. It needs little explanation that this hydrolysis can have a profound impact on the consumers appreciation of the product. With regard to the results for ethylacetate presented in figure 2, the difference is not as sharp but still present: a drop to 95% of the original concentration after six months storage at 20°C for the top-fermented beer whereas the bottom-fermented beer remained fairly stable.

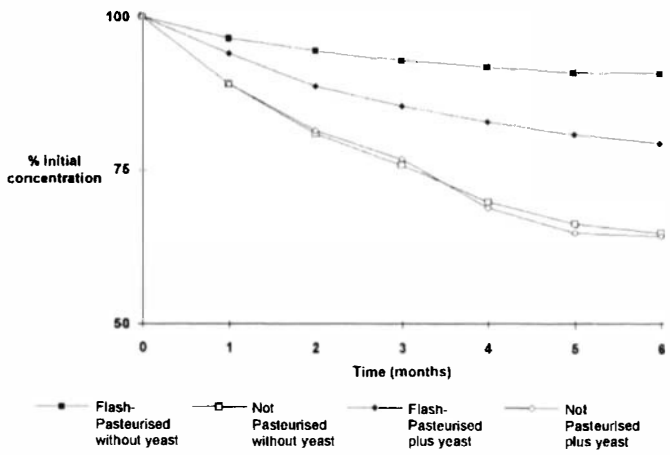

FIGURE 3
Influence of Heat Inactivation and Bottle Conditioning on the Hydrolysis of Isoamyl Acetate in Top Fermented Beers

Heat Inactivation of the Enzymatic Activity Towards Isoamyl Acetate

In this experiment, the above described top-fermented beer (yeast strain CMB 64) was partly flash-pasteurized, partly not. Part of the beer was brought in contact with yeast cells (CMB 64) to a final concentration in the bottle of 2.10^6 cells per ml (so finally we started our observations with four 'different' beers). The beers were kept at 24°C for 6 months. The hydrolysis of isoamyl acetate in the different beers is given in figure 3.

Non-pasteurized beers showed an identical evolution of acetate esters, whether yeast was present in the bottle or not. The decrease in concentration of isoamyl acetate made a small drop to 90% of the initial concentration after six months of storage at 24·C. In the presence of yeast cells, the concentration drop is more pronounced, i.e. to 72%.

From these results we can state that the breakdown of acetate esters is partly chemical (acid hydrolysis) and partly enzymatic (esterase activity). These enzymes are presumably liberated by yeast cells during fermentation/maturation and remain active in filtered beer.

Immunological Evidence

Polyclonal antibodies were raised against a commercial available carboxylesterase as described by Schoofs.[10] Since complete Freund's adjuvant was used for immunization of the rabbits, non-specific cell wall directed antibodies had to be removed. This was done in a procedure using intact yeast cells of *Hansenula polymorpha*. In a further stage, the immune and pre-immune (serum before immunization) serums were extensively purified by adsorption on a CNBr-Activated Sepharose 4B column on which the native protein was coupled. The purified serums were used for further tests.

a) Addition of the Antibodies to Non-Pasteurized Beer

In a first test we added the antibodies to the previously described top-fermented beer. During one month of storage at 24°C, acetate esters were determined twice every week and these results are shown in figure 4. In this figure, a comparison

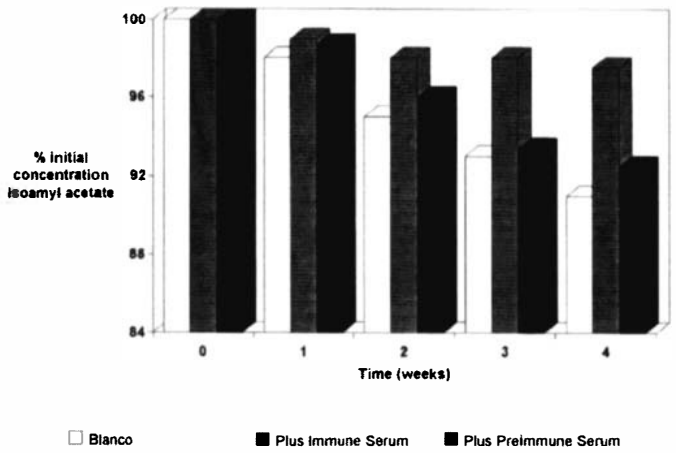

FIGURE 4
The Inhibition of Esterases by Purified Antibodies

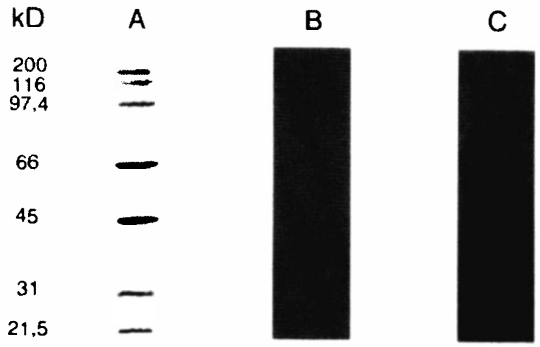

FIGURE 5
Immunoblotting of a Crude Cell-free Protein Extract
Lane A: Molecular Weight Markers, Coomassie Blue;
Lane B: Crude Protein Extract, Pre-immune Serum;
Lane C: Crude Protein Extract, Immune Serum.

is made with the non-pasteurized beer to which we added the pre-immune serum

Obviously, we succeeded in raising antibodies against the esterase responsible for the hydrolysis of (for one) isoamyl acetate. Addition of the immune serum resulted in an almost complete inhabitation of the enzymatic activity towards acetate esters. The degree of hydrolysis fell to the same level as for the pasteurized beer.

The addition of the pre-immune serum on the other hand, did not inhibit the enzymatic hydrolysis of isoamyl acetate.

b) Specificity

In order to determine whether the immunoglobulines of the immune serum are specific towards this enzyme, proteins of a crude protein extract of brewer's yeast (CMB 64) were fractionated according to Laemmli.[5] Western blotting was carried out according to the method of Towbin.[14] Immunoblotting (dilution 1:2000) of the membrane afterwards showed two distinct bands (figure 5) having a molecular weight of approximately 56 and 79 kD. These values correspond exactly with earlier findings obtained via gel-filtration of the same yeast protein-extract. However, in this experiment it was demonstrated that only one of the two peaks which were active against p-nitrophenyl acetate, showed an esterolytic behavior towards isoamyl acetate, namely, the 56 kD protein.

c) Immunocytochemical Localization

From the above-mentioned results, we can state that we can obtain a specific reaction towards esterase enzymes, present in yeast and responsible for the hydrolysis of isoamyl acetate. Non-specific immunoglobulines are likely to be still present in the purified immune serum but it was shown in figure 5 that an adequate dilution of the 'antiserum' suppresses the background signal of these non-specific antibodies in such a way that only the specific sites are spotted because of the higher presence of specific immunoglobulines.

Immunolabeling was performed on ultrathin sections, essentially as described by Douma.[2] Observations were made using

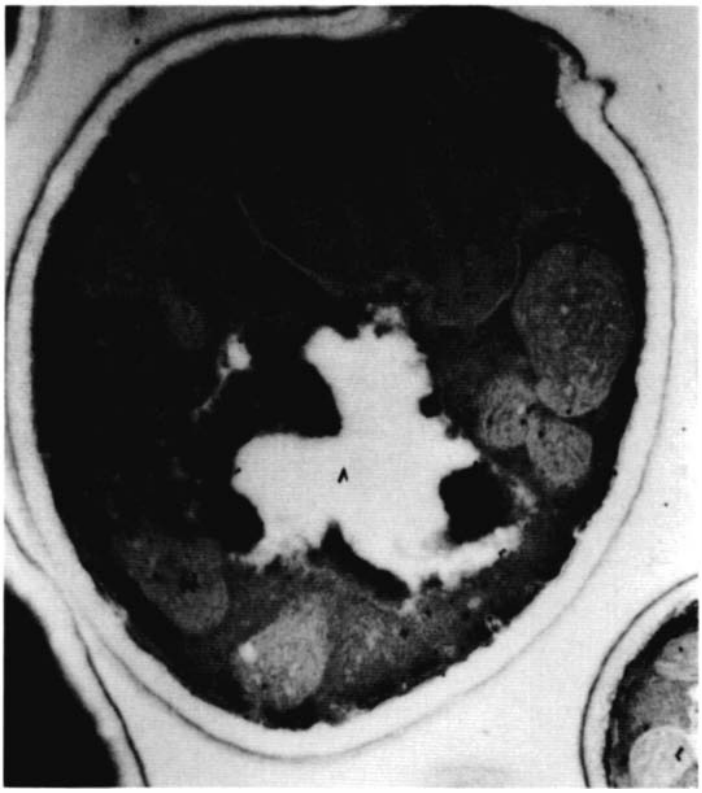

FIGURE 6
Colloidal Gold Staining Inside the Yeast Cell.

electron-microscopy (scope Philips EM 300) (figure 6). Staining was observed inside the yeast cell. Based on the blots, the active enzymes are apparently not glycosylated, which makes the secretion theory very unlikely. Since we were able to make a positive correlation between total dead cell count and the amount of isoamyl acetate hydrolyzed after 2 months (Neven, unpublished data), we suggest that the enzymes are liberated after cell lysis. Certain hydrolases, linked to the endoplasmatic reticulum become soluble after cell lysis and these enzymes are generally very stable, which could explain the observed activity.

CONCLUSIONS

Acetate esters and especially isoamyl acetate, play an important role in the fruity flavor perception of top-fermented beers. Isoamyl acetate is hydrolyzed both chemically and enzymatically, an enzymatic hydrolysis which greatly depends on pH, storage temperature and fermentation/maturation conditions. The molecular weight of these esterases are respectively 56 and 79 kD. Both esterases are active against para-nitrophenyl acetate but only the 56 kD protein is capable of hydrolyzing isoamyl acetate.

Immunological studies have shown that these enzymes are most likely released after cell-lysis during fermentation/maturation and bottle conditioning (for beers refermented in the bottle). Further work will be undertaken to reveal the metabolic function of the enzyme which is active against isoamyl acetate. It is our aim to modify the yeast strain biologically in such a way that the strain becomes esterase deficient.

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QUESTION AND ANSWER

Q. Is there any effect of adsorption by the bottle crown liner of isoamyl acetate since there is still some decrease after pasteurization?

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A. No, because a proportional increase is observed in the concentration of isoamyl alcohols.