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Yeast dehydrogenase activities in relation to carbonyl compounds removal from wort and beer

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Descriptors
Alcohol free beer, carbonyl compound, dehydrogenase, flavour formation, metabolism, yeast analysis

SUMMARY
Carbonyl compounds of wort and beer are well-known off-flavours. Their removal by yeast contributes to improved beer flavour and to less susceptibility to staling. Carbonyl marker compounds have been followed simultaneously with deshydrogenase activities of yeast: acetoin/diacetyl (diacetyl pathway), hexanal/nonenal (lipid route), 3-methyl-butanal (Strecker pathway) and acetaldehyde (glycolysis). Potential use of deshydrogenase activities measurements has been investigated with regard to yeast performances for the removal of carbonyls during fermentation and for the production of non-alcoholic beer using an immobilized yeast bioreactor.

LES ACTIVITES DESHYDROGENASES DE LA LEVURE EN RELATION AVEC L'ELIMINATION DES COMPOSES CARBONYLES DU MOUT ET DE LA BIERE

Descripteurs
Analyse de levure, bière sans alcool, composé carbonyle, déshydrogénase, formation de la flaveur, métabolisme

RESUME
Il est bien connu que les composés carbonylés du moût et de la bière sont les principaux responsables de l'altération du goût de la bière finie. Leur élimination par la levure contribue à améliorer le goût de la bière et sa susceptibilité au vieillissement. Des composés carbonylés marqueurs et les activités déshydrogénases de la levure ont été suivis simultanément. Il s'agit de: acétoïne/diacétyle (voie du diacétyle), hexanal/nonénal (voie lipidique), 3-méthyl-butanal (voie De Strecker) et acétaldéhyde (glycolyse). L'utilisation potentielle des activités déshydrogénases de la levure dans le but d'éliminer les composés carbonylés en fermentation ou de produire une bière sans alcool en bioréacteur sur cellules immobilisées, a été étudié.

AKTIVITAETEN DER HEFEDEHYDROGENASE IM ZUSAMMENHANG MIT DEM ABBAU VON CARBONYLVERBINDUNGEN IN WUERZE UND BIER

Deskriptoren
Alkoholfreies Bier, Aromabildung, Carbonylverbindung, Dehydrogenase, Hefeanalyse, Stoffwechsel

ZUSAMMENFASSUNG
Carbonylverbindungen sind als Fehlaromen in Würze und Bier gut bekannt. Ihre Beseitigung durch Hefe trägt dazu bei, den Biergeschmack zu verbessern und die geschmackliche Alterung zu verringern. Markierte Carbonylverbindungen wurden gleichzeitig mit der Dehydrogenaseaktivität der Hefe verfolgt: Acetoin/Diacetyl (Diacetylabbau), Hexanal/Nonenal (Fettstoffwechsel), 3-Methyl-Butanal (Streckerabbau) und Acetaldehyd (Glykolyse). Es wurde der potentielle Nutzen der Messung der Dehydrogenasenaktivität untersucht, im Hinblick auf die Hefeleistung bei der Beseitigung des Carbonyls während der Gärung und für die Produktion alkoholfreier Biere unter Verwendung eines Hefebioreaktors mit immobilisierter Hefe.

INTRODUCTION

Many carbonyls have very high off-flavour potential (low threshold) (3). This property makes them an important group of beer volatiles. Depending on origin, beer carbonyls may be divided into different groups : EMP pathway (acetaldehyde), amino acid metabolism (ILV pathway)(diacetyl, acetoïn), Strecker degradation of amino acids (branched carbonyls such as isobutanal, 3-methyl butanal, 2-methyl butanal), lipid oxidation (linear carbonyls such as pentanal, hexanal, heptanal, nonenal).

Yeast metabolism is known to bring about the chemical reduction of wort carbonyl compounds during fermentation (5). However, the importance of this reduction should depend on yeast deshydrogenase activities and on the structure of the carbonyls. Moreover, sulfite which leads to the formation of bisulfite-carbonyl adducts prevents or slows down the removal of the corresponding carbonyl(1).

The present work focus on the follow-up of an immobilized yeast bioreactor (2) to produce alcohol-free beer, with special attention to ethanol control and reduction of carbonyls.

MATERIAL AND METHODS

Analysis of carbonyls

"Purge and trap" injector (Chrompack). The chromatographic injection was achieved in three steps :

- precooling cold trap time : the cold trap was cooled during 1 min by a stream of liquid nitrogen issued from a Dewar vessel;
- purge time : during 15 minutes, a nitrogen purge flow of 10 ml/min passed through the sample (9 ml of beer + 25 µl IST + 50 µl of a saturated PEG 2000 solution as antifoam) heated to 70°C in order to sweep both the water vapour and most of the volatiles. The first one was stopped by a condensor maintained at -15°C by means of a cryostat. The volatile flow was led through an oven at 200°C and further captured by the cold trap at -95°C;
- desorption time : the trap cooling was stopped and the surrounding metal capillary was immediately heated to 220°C during 5 minutes. The carrier gas sweeped the trapped compounds into the analytical column.

Gas chromatography conditions. A Hewlett Packard Model 5890 GC equipped with a flame ionisation detector and an automatic integrator was used. Separations were carried out on a 50m x 0.32mm, wall coated, open tubular (WCOT) apolar CP-SIL5 CB capillary column. The oven, initially kept at 30°C for 15 min, was temperature programmed from 30 to 100°C at 2°C/min, and eventually held at the maximum temperature for 15 min. Nitrogen carrier gas was used at the flow rate of 1.5 ml/min. Injection and detection temperatures were 200°C and 220°C, respectively.

Gas chromatography - mass spectrometry analysis. A gas chromatography device, equipped with the same column, was directly coupled to a HP 5988 quadrupole mass spectrometer. Electron impact mass spectra were recorded at 70 eV. Spectral recording throughout elution was automatically performed by a computer. The oven temperature was programmed as previously described. Carrier gas was helium. Mass spectral assignments were carried out by injection of pure compounds and by use of the PBM library.

In vitro measurements of yeast carbonyl reducing activity. Yeast cell free extract was prepared as described by Tuduri et al. (8). Carbonyl reducing activity was measured according to the procedure of Strecker and Harary (7) for the reduction of diacetyl and acetoïn or based on the methods described by Molina et al. (4) for the reduction of acetaldehyde, 3-methyl butanal and trans-2-nonenal. ADH activity was measured as previously described (6).

RESULTS

Typical GC chromatogram of non-alcoholic beer (outflow of the reactor) is illustrated in Figure 1. A large number of compounds are distinctively separated. These molecules are distributed among five major groups : alcohols (**I-IV**), aldehydes (**V-X**), esters, ketones and sulfur compounds.

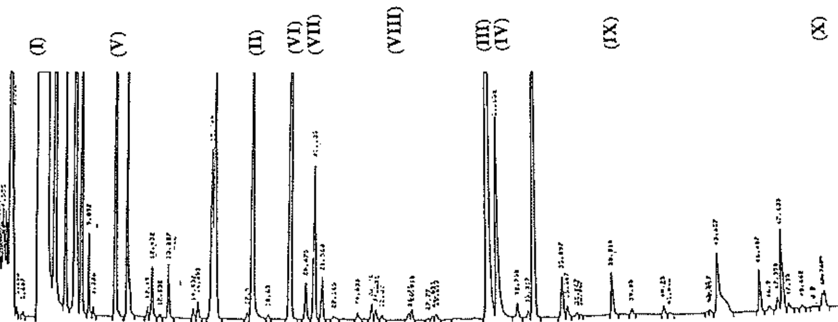

Figure 1. Typical GC chromatogram of non-alcoholic beer (**I** ethanol, **II** isobutanol, **III**, 3-methyl butanol, **IV** 2-methyl butanol, **V** isobutanal, **VI** 3-methyl butanal, **VII** 2-methyl butanal, **VIII** pentanal, **IX** hexanal, **X** heptanal).

Optimalization of the operating conditions for long term use of the immobilized yeast bioreactor.

Yeast cells count and ethanol level in the outflow were followed during 43 days of continuous operation of an experimental reactor (Figure 2 a and b). Yeast cells count (millions/g d.m. of carrier) was relatively constant during the first two weeks but exponentially increased thereafter (Figure 2a). Reactor was running out of control. Concomitant with the uncontrolled yeast growth, there was an increase in

ethanol production with levels exceeding the set-point (0.1 %) (Figure 2b).

Carefull conditioning of wort (lipid content, oxygen level) together with a tight control of operating parameters (temperature, flow rate...) allowed to maintain the limited fermentation under control. As a consequence, ethanol level was always below the upper limit. Under these conditions, non alcoholic beer was successfully produced using the immobilized yeast bioreactor during six months (Figure 3).

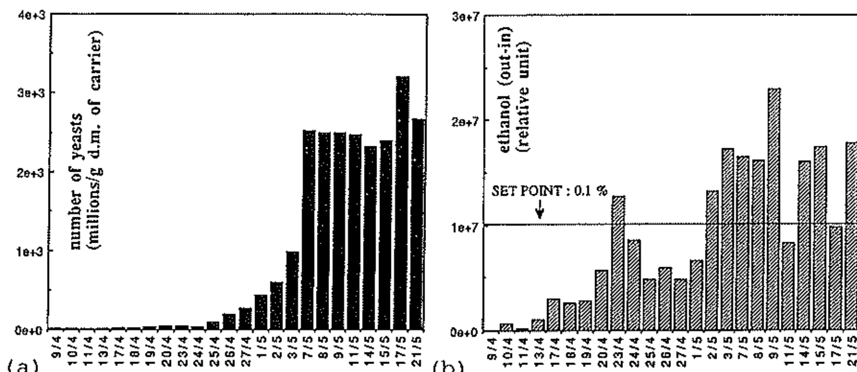

Figure 2. Evolution of yeast cells count (a) and ethanol (b) for the immobilized yeast bioreactor working under non-optimum conditions

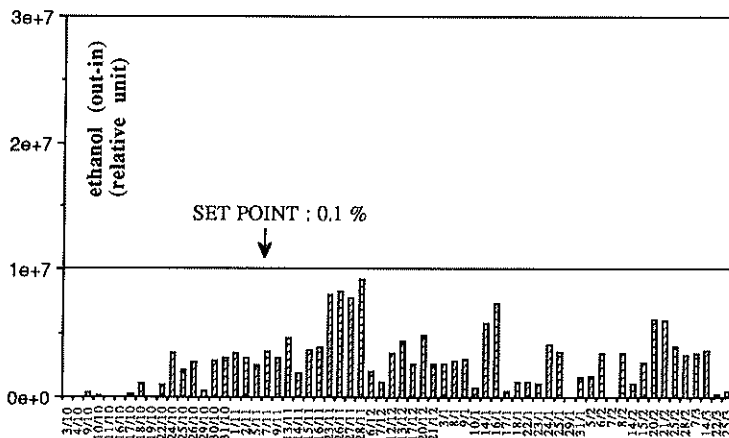

Figure 3. Evolution of ethanol for the immobilized yeast bioreactor working under optimum conditions.

<u>Study of wort carbonyl reduction by yeast.</u>

The above mentioned analytical system allowed us to demonstrate the effective reduction of several volatile carbonyls present in the wort. Typical data are illustrated

in Table I for two Strecker aldehydes and one linear aldehyde (issued from the lipid oxidation).

Table 1. Carbonyl reducing activity of yeast.

Strecker degradation						Lipid route		
2-methyl butanal			3-methyl butanal			heptanal		
in (ppb)	out (ppb)	R* (%)	in (ppb)	out (ppb)	R* (%)	in (ppb)	out (ppb)	R* (%)
90.4	23.4	74.2	372.1	113.8	69.4	7.9	2.8	64.6
106.5	8.8	91.7	432.2	39.4	90.9	6.7	3.4	49.3
93.7	34.6	63.1	358.5	196.2	45.3	8.6	4.3	50.0
79.5	14.0	82.4	302.6	75.4	75.1	8.2	4.1	50.0
66.9	14.7	78.0	258.4	81.4	68.5	6.8	4.7	30.9
mean								
87.4	19.1	77.9	344.0	101.2	70.0	7.6	3.9	49.0

*R : [(in-out)/in] x 100

As shown in Figure 4, aldehydes from the same origin demonstrated similar behaviour as far as yeast reduction was concerned.

Alcohol deshydrogenase (ADH) activity has been suggested to participate to the reduction of wort carbonyls. Attempt was made to correlate carbonyl reducing activity of yeast with ADH level. Figures 5 and 6 illustrate the evolution of ADH activity of immobilized yeast and the removal of 2-methyl butanal from wort. A similar behaviour was obtained for the reduction of wort diacetyl (from Maillard reaction) and linear carbonyls (data not shown). Although the trends indicated some similarities, the absolute values did not show any significant correlation ($r^2 < 0.1$) between reduction of the carbonyl and ADH. Lack of correlation is presumably attributable to the involvement of distinct enzymes for the reduction of the different kinds of carbonyls.

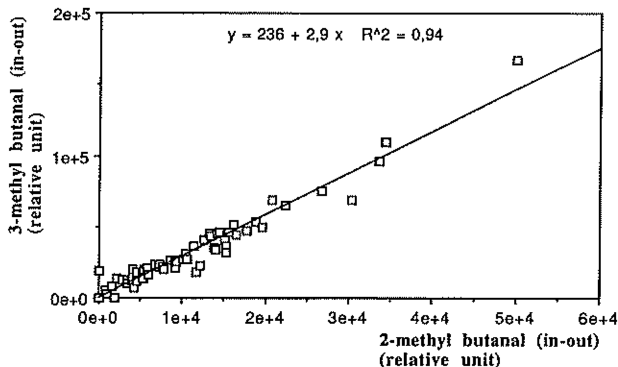

Figure 4. Relationship between 2-methyl butanal and 3-methyl butanal reductions (inflow minus outflow).

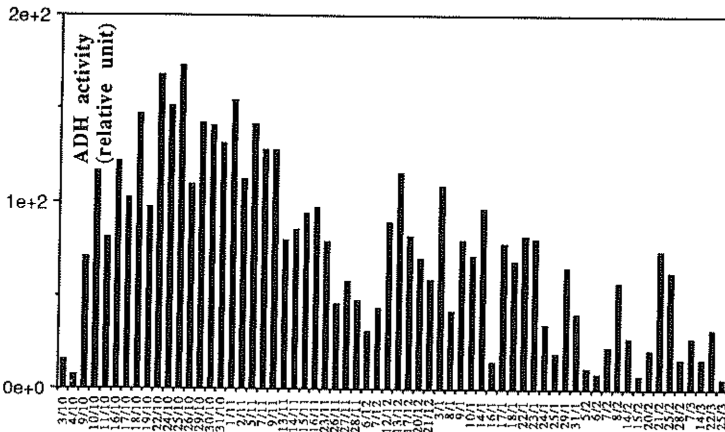

Figure 5. Evolution of ADH activity

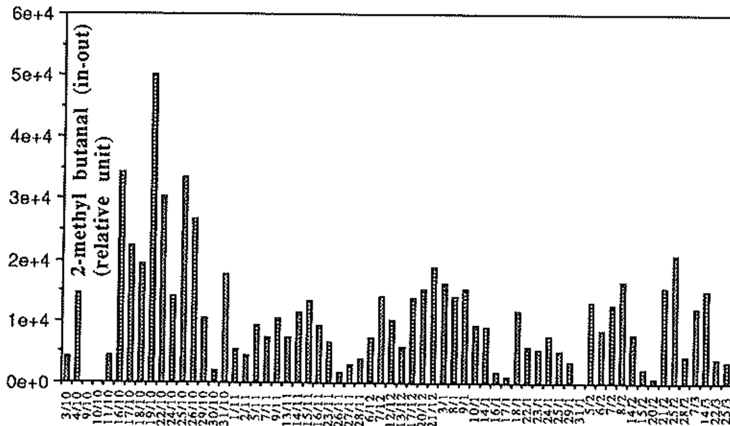

Figure 6. Removal of 2-methyl butanal (inflow minus outflow).

In vitro measurements of yeast carbonyl reducing activity were carried out on yeast cell-free extract using different carbonyl substrates : acetaldehyde (glycolysis), 3-methyl butanal (Strecker degradation), trans-2-nonenal (lipid oxidation), diacetyl and acetoïn (diacetyl pathway). For each reaction, the requirement for the cofactor NADH or NADPH was evaluated. Specificity for the cofactor and relative specific enzyme activity (to acetaldehyde reductase) are summarized in Table II. The data suggested that several "reductases" are involved. At least three "reductase" activities have been proposed in yeast : acetaldehyde reductase, diacetyl reductase and acetoïn reductase. The involvement of these enzymes in the reduction of Strecker carbonyls and lipid oxidation carbonyls must await their purification and characterization. In vitro

measurements of carbonyl reduction indicated also a large variation in the reduction rate between the different kinds of carbonyls. Except for the reduction of trans-2-nonenal, all other carbonyl reductions proceed very slowly.

Table 2. Carbonyl reducing activity in yeast cell free extract

Substrate	Cofactor	Relative specific activity
acetaldehyde	NADH	100
3-methyl butanal	NADPH	0.2
trans-2-nonenal	NADH	7.0
diacetyl	NADPH/NADH	0.5
acetoïn	NADH	2.2

Interestingly, although trans-2-nonenal/3-methyl butanal reduction rate ratio was 35 for in vitro measurements, the corresponding ratio heptanal/3-methyl butanal was 0.01 for in vivo measurements, suggesting that other factors should be considered, such as the uptake of carbonyl by yeast.

CONCLUSIONS

1.Well-controlled operating conditions allowed long term use of immobilized yeast bioreactor ($\geq$ 6 months) to produce non-alcoholic beer.
2.ADH activity has been tentatively used as a sensor of reducing capacity of yeast. Although there was a parallel between the evolution of ADH measurements and carbonyl reduction by yeast, the correlation was poor. Improvement should be observed by measuring specific "reductases".
3.In vitro carbonyl reduction measurement indicated low reducing activity for most carbonyls compare to acetaldehyde.
4.In vivo carbonyl reduction measurement suggested that the uptake of carbonyls by yeast could be the limiting step in the reduction of some carbonyls.

REFERENCES

1. Dufour, J.P., Proceedings of the European Brewery Convention Congress, Lisbon, 1991, this volume
2. Louvain Brewing Letters, 1991, 4, 48-49
3. Meilgaard, M, MBAA Technical Quarterly, 1975, 12, 151-168
4. Molina, I., Nicolas, M. & Grouzet,J., American Journal of Enology and Viticulture, 1986, 37, 169-173
5. Peppard, T.L. & Halsey, S.A., Journal of the Institute of Brewing, 1981, 87, 386-390
6. Racker, E., Methods in Enzymology, 1955, 1, 500-503
7. Strecker, H.J. & Harary, I., Journal of Biological Chemistry, 1954, 211, 263-270
8. Tuduri, P., Nso, E., Dufour, J.P. & Goffeau, A.(1985), Biochemical Biophysical Research Communications, 133, 917-922