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MATURATION OF BEER WITH α-ACETOLACTATE DECARBOXYLASE

by

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α-Acetolactate decarboxylase isolated from Enterobacter aerogenes strain 1033 has been applied for maturation of beer. Addition of the enzyme to freshly fermented beer effected removal of α-acetolactate and α-aceto-α-hydroxybutyrate in 24 hours at 10 °C to a level below the taste treshold of the corresponding volatile diketones, diacetyl and 2,3-pentanedione, without affecting other important properties of the beer. By comparison of organoleptic properties, the beer matured in the presence of α-acetolactate decarboxylase was judged to be of an equally satisfactory quality when compared with conventionally prepared beer. The evidence suggest that apart from removal of diacetyl, 2,3-pentanedione and the precursors of these compounds from fermented beer no other important events related to flavour development occur during maturation of the type of beer studied. Consequently, it is concluded, that application of enzymes may allow flavour maturation of beer to be carried out in less than 24 hours.

1. INTRODUCTION

Adoption of enzyme technology in the brewing industry has been prompted mainly by the persistent demand for increased flexibility, simplicity and efficiency of the various steps in the beer production process. The aim has been for example to provide a higher degree of freedom in choice of starting materials used in beer production, to allow for use of a simplified equipment, to reduce process time or to make possible a higher control over product characteristics. So far, these important objectives have made various enzymes well established tools in the brewing industry, for example for stabilization of beer (3) or for direct conversion of barley into wort (9).

Rather surprisingly, relatively little attention has apparently been devoted to the possibility of applying enzyme technology to increase the efficiency of early parts of the beer maturation process in spite of the fact that essential biochemical features of this process are well understood and that, potentially, the economic advantages to be gained by intensifying this

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process are considerable. Beer maturation, which can be defined as all steps in the beer production process which take place after the main fermentation or, more precisely, from the point of cessation of yeast growth to the final filtration of the beer (10) usually comprises storage of the beer for extended periods of time at low temperatures – typically, the beer is stored for about three weeks at temperatures varying from 13 to −1 °C.

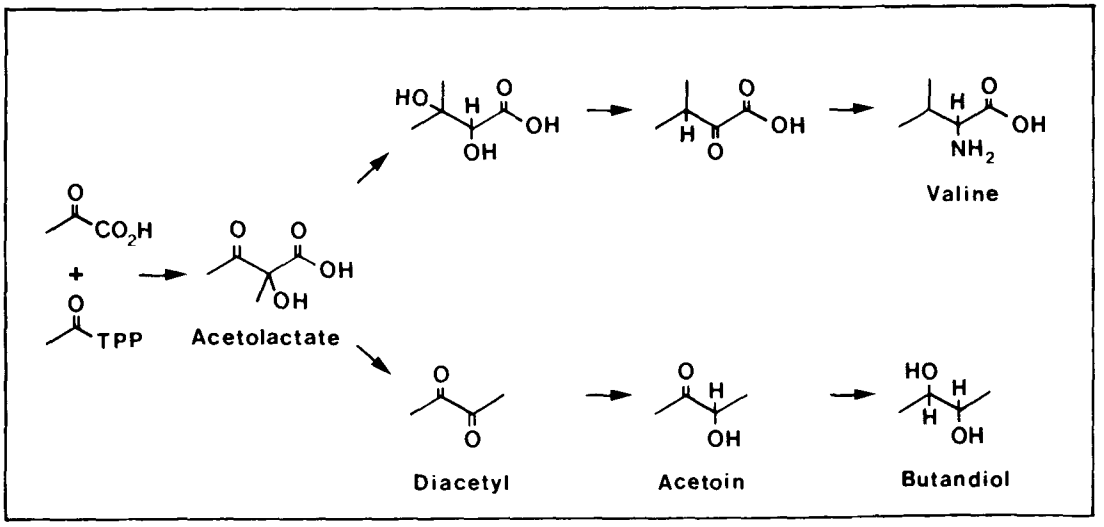
During beer maturation a number of important objectives are achieved (10). The beer becomes saturated with carbon dioxide, insoluble material is removed, the colloidal stability of the beer is improved, and the flavour of the beer is allowed to reach its final level. Apart from flavour maturation these various objectives can be achieved within a short process time by means of known technology (8). As a consequence, flavour maturation can be considered the rate limiting factor in beer maturation and, accordingly, any measure which reduces the time necessary for development of the desired flavour of the beer makes possible a reduction of the total time required for maturation.

The flavour of beer and related fermented products is due mainly to volatile esters, alcohols, ketones, aldehydes and sulphur compounds; among these the diketone, diacetyl, plays a major role. The precise adjustment of the concentration of this compound in beer is one of the most important objectives of the beer maturation process since diacetyl in small concentrations contributes to the desired flavour of beer but conveys an objectionable character to the beer if present in too large concentration. The optimal content of diacetyl varies from beer type to beer type but in most lagers it is preferable to adjust the level of diacetyl in the final beer to approximately 0.1 ppm.

In beer diacetyl is a product of yeast metabolism and is derived in part from α-acetolactate (12) which serves as an intermediate in the biosynthesis of valine (Scheme 1) (17). In the course of amino acid biosynthesis α-acetolactate, generated by enzyme catalyzed condensation of pyruvate and the thiamine pyrophosphate of acetaldehyde, is reductively isomerized, dehydrated, and finally transaminated (Scheme 1). Being a β-ketoacid, α-acetolactate may, however, also readily decarboxylate in a non-enzyme catalyzed fashion to provide acetoin or, in the presence of oxygen, diacetyl. Quite similarly, the higher homologoue 2,3-pentanedione, likewise a volatile α-diketone, is generated by oxidative decarboxylation of α-aceto-α-hy-

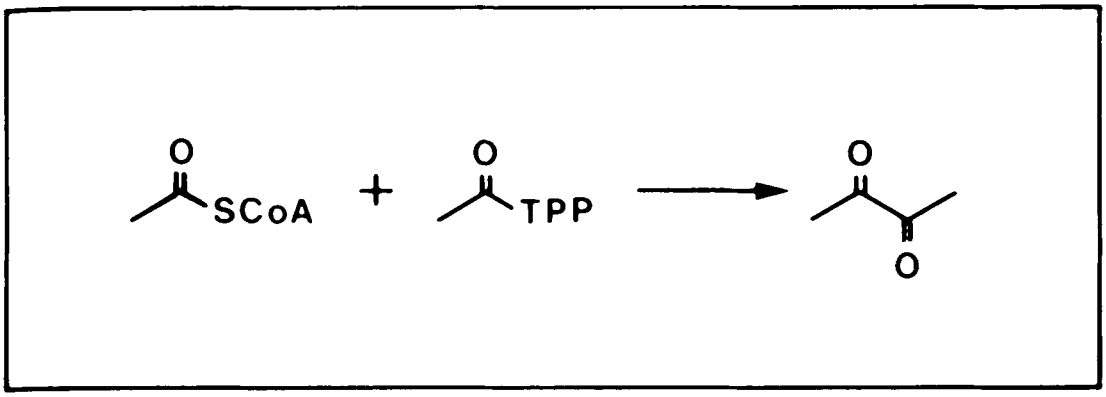
Scheme 1

Key reactions involved in the formation of α-acetolactate from pyruvate and the thiamine pyrophosphate (TPP) of acetaldehyde and its conversion either to valine or diacetyl.



Scheme 2

Biosynthesis of diacetyl in yeast from acetaldehyde as its thiamine pyrophosphate (TPP) and acetyl CoA.



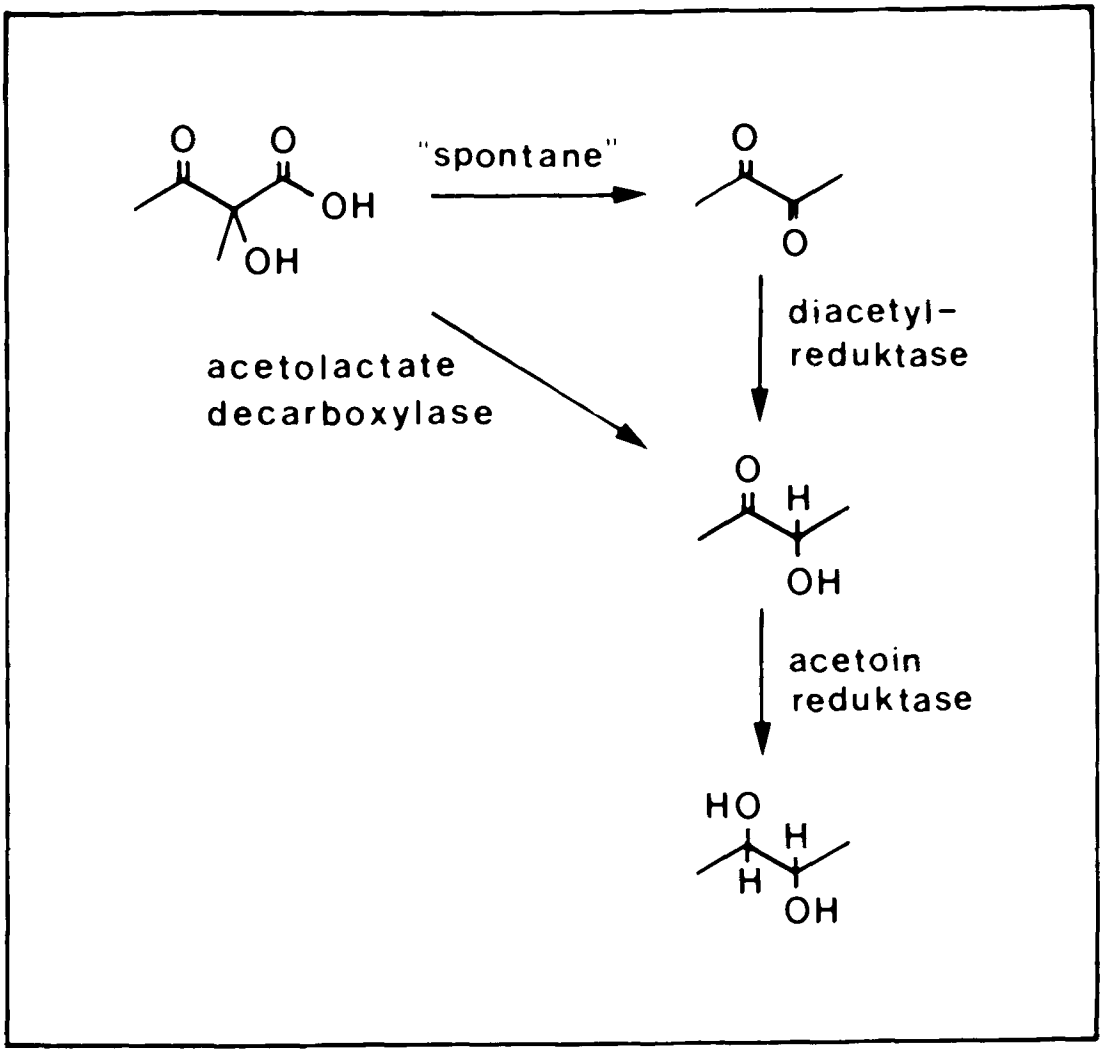
droxybutyrate, which is an intermediate in the biosynthesis of isoleucine (17). Diacetyl in beer may also be derived from the condensation of acetaldehyde as its thiamine pyrophosphate and acetyl CoA. (Scheme 2) (2). Since, as mentioned below, virtually no free diacetyl can be detected in maturing beer, the extent to which this pathway contributes to the diacetyl content of beer is clearly insufficient to be of any practical consequences for the course of the beer maturation process. The key reactions in removal of diacetyl and its precursor, α-acetolactate, from beer involve the oxidative non-enzyme catalyzed decarboxylation of α-acetolactate to diacetyl and the subsequent enzyme catalyzed conversion of this compound to acetoin and 2,3-butanediol by reductases in yeast cells present during beer maturation (Scheme 3). Usually, maturing beer contains α-acetolactate in concentrations up to about 2 ppm but virtually no free diacetyl since this compound is rapidly reduced by the yeast present. The decarboxylation of α-acetolactate is, in other words, a slow reaction compared to the enzyme catalyzed removal of its degradation products and it follows, therefore, that the rate limiting step in the removal of α-acetolactate and diacetyl from beer is the non-enzyme catalyzed decarboxylation of the diacetyl precursor.

In accordance with the mechanism of diacetyl formation and degradation in beer the methods applied so far to accelerate removal of these compounds from beer aim at accelerating the conversion of α-acetolactate to diacetyl. One approach which has been pushed to its extreme (1) is to maintain the beer at an elevated temperature during the maturation process while another consists in lowering the pH of the α-acetolactate containing beer (11). Relatively little attention, however, has been given to the possibility of applying enzymes for accelerating the removal of α-acetolactate and diacetyl from beer. The use of diacetyl reductases has been suggested (14, 15) although these enzymes do not catalyze the rate limiting reaction but, so far, no attempts have been made to accelerate the beer maturation process by applying α-acetolactate converting enzymes even though one would expect this approach to be particularly fruitful. By using, for example, α-acetolactate decarboxylases which catalyze the conversion of α-acetolactate into acetoin it might thus be possible to effect selective removal of the diacetyl precursor from beer under very mild conditions. Furthermore, use of this enzyme would be expected to circumvent formation of free diacetyl since less α-acetolactate will be available for diacetyl formation by oxidative decarboxylation (scheme 3) and, finally, application of this enzyme would not be expected to affect the composition of the finished beer since acetoin is also produced from α-acetolactate during conventional beer maturation.

α-Acetolactate decarboxylases are known to occur in a variety of bacteria as e.g. strains of

Scheme 3

Reactions involved in the removal of α-acetolactate from beer during conventional and enzymatic beer maturation.



Enterobacter, Aeromonas, Streptococcus, Serratia, Leuconostoc and Bacillus (5, 7, 16). These organisms depend at least to some extent on such enzymes for the formation of acetoin and 2,3-butanediol. In the cheese industry, the ability of Streptococcus diacetylactis and strains of Leuconostoc to synthesize acetoin, 2,3-butanediol and diacetyl is utilized for aroma development in cheese, while strains of Bacillus polymyxa, Enterobacter aerogenes and Aeromonas hydrophila have been utilized for production of 2,3-butanediol on an industrial scale (13). α-Acetolactate decarboxylase was first described by JUNI as a constituent of Aeromonas aerogenes (5) and an α-acetolactate decarboxylase from Enterobacter aerogenes strain 1033 has later been purified and characterized by LØKEN & STØRMER (17). α-Acetolactate decarboxylases from other species have not been properly characterized and, unfortunately for the brewer, the enzyme has not been detected in yeasts.

To our knowledge, no attempts have previously been reported regarding the possibility of utilizing α-acetolactate decarboxylases to accele-

rate flavour maturation of beer. However, as described in the following, it appears that addition of the enzyme to fermented beer can effect removal of the diacetyl precursor and α-aceto-α-hydroxybutyrate from beer in less than 24 hours under conditions normally used during beer maturation. As a consequence, the time required for beer maturation may be correspondingly reduced since the beer matured in the presence of α-acetolactate decarboxylase seems to be of an equally satisfactory quality as conventionally matured beer (18).

2. EXPERIMENTAL SECTION

2.1. Preparation of α-acetolactate

α-Acetolactate was prepared essentially as described by KRAMPITZ (6): Ethyl 2-methyl-acetoacetate (8.6 g) was dissolved in benzene (28 ml) and lead tetraacetate (26 g) was added in small portions to the stirred solution at room temperature. The reaction mixture was left at 40 °C for 5 hours and subsequently stirred for 40 hours at 35 °C. Solid material was then removed by filtration and washed thoroughly with benzene. The combined benzene solutions were washed with water, dried over calcium chloride and concentrated in vacuo. The resulting residue was distilled at approximately 1 torr through a short column to provide the desired ethyl-O-acetyl-acetolactate as a colourless oil (3.0 g, bp. app. 97 °C). The purity of the product was ensured by GC analysis on a Carbowax column. The ^{1}H-NMR spectrum of the product exhibited the following signals: δ 1.2 (3H, t), δ 1.69 (3H, s), δ 2.19 (3H, s), δ 2.34 (3H, s) and δ 4.22 (2H, q) (chemical shifts are given downfield relative to tetramethylsilane as internal reference in 10% $CDCl_3$, s = singlet, t = triplet, q = quartet). Prior to use ethyl-O-acetyl-acetolactate was converted into α-acetolactate by dissolving 10 µl of the precursor pr. ml of 0.1 M-sodium hydroxide and by gently shaking the reaction mixture until all of the starting material had dissolved.

2.2. Assay for α-acetolactate decarboxylase

The assay was carried out essentially as described by LØKEN & STØRMER (7) by mixing 200 µl of an α-acetolactate containing solution prepared as described above with 200 µl phosphate buffer (pH 6.2, 1 M), an enzyme containing solution and water to a total volume of 1 ml. The mixture was incubated for 30 minutes at 37 °C, sodium hydroxide (1 ml, 2.5 M) was added to stop the enzymatic reaction and a portion (100 µl) of the solution was then transferred to a mixture of water (900 µl), α-naphtol (200 µl, 1 g in 20 ml 2.5 M-NaOH) and creatine (200 µl, 1 g in 200 ml of water). The resulting reaction mixture was left for 1 hour at room temperature before its absorbance at 540 nm was measured.

1 unit of enzyme is defined as the amount of enzyme which liberates 1 µmol of acetoin in 1 hour under the conditions of the assay (7).

2.3. Measurement of diacetyl, α-acetolactate, 2,3-pentanedione, and α-aceto-α-hydroxybutyrate

Vicinal diketones and their precursors were measured by gas-liquid chromatography as described by HAUKELI and LIE (4).

2.4. Growth of Enterobacter aerogenes

The organism, Enterobacter aerogenes strain 1033, was a gift from F. C. STØRMER and was grown at 37 °C with aeration in a medium containing K_2HPO_4 (7.0 g), KH_2PO_4 (3.0 g), Na_3-citrate · $3H_2O$ (0.5 g), Mg SO_4 · $7H_2O$ (0.1 g), $(NH_4)_2SO_4$ (1.0 g), D-(+)-glucose (10 g), yeast extract (5.0 g), tryptose (5.0 g), $FeSO_4$ (0.1 mg), $Na_2B_4O_7$ · $10H_2O$ (0.2 mg), $CoSO_4$ · $7H_2O$ (0.1 mg), $CuSO_4$ · H_2O (0.01 mg), $MnSO_4$ (0.01 mg), $(NH_4)_6$ Mo_7O_{24} (0.1 mg) and $ZnSO_4$ (0.2 mg) per liter of distilled water. All chemicals used were of analytical grade.

2.5. Preparation of partially purified α-acetolactate decarboxylase for beer maturation

Cells of Enterobacter aerogenes 1033 were isolated from 40 liters of growth medium by centrifugation in an IEC centrifuge (4850 g, 30 min) and suspended in phosphate buffer (pH 6.5, 50 mM, 1250 ml). 200 ml of a dextran T_{500} (Pharmacia) containing solution (20% w/w in phosphate buffer pH 6.5, 25 mM, 200 ml) and

200 ml of a solution containing polyethylene glycol (PEG 6000, 25% w/w in phosphate buffer pH 6.5, 25 mM) was added to the suspension and the mixture which was kept cold by addition of ice was subsequently homogenized three times in a Manton Gaulin homogenizer at a pressure of 700 atm. All subsequent operations were carried out in the cold. Disrupted cells were removed by centrifugation using a Sorval RC-5 centrifuge (27500 g, 10 min) and ammonium sulfate (1 kg) was added to the clear supernatant (2500 ml). The precipitated material was collected by centrifugation (27500 g, 20 min) and suspended in phosphate buffer (pH 6.5, 25 mM, 300 ml), undissolved material was removed by centrifugation and the clear solution containing 1900 kU α-acetolactate decarboxylase was desalted on a column of Sephadex G-75 (5 · 50 cm) previously equilibrated with phosphate buffer (pH 6.5, 25 mM). α-Acetolactate decarboxylase was eluted with the same buffer, the enzyme containing fractions (1500 kU) were pooled and the combined fractions were subjected to chromatography on a column of DEAE cellulose (2.5 50 cm, Whatmann DE-52) equilibrated with phosphate buffer (pH 6.5, 25 mM). The α-acetolactate decarboxylase was eluted by applying a linear gradient of phosphate buffer ranging from 25 to 250 mM at pH 6.5 to the column. α-Acetolactate decarboxylase containing fractions (600 ml) were pooled and the enzyme (1340 kU) was precipitated by addition of ammonium sulfate (240 g). Before use for beer maturation an appropriate amount of this stock material was suspended in phosphate buffer (pH 6.5, 25 mM) and dialyzed against the same buffer.

2.6. Beer maturation with α-acetolactate decarboxylase

Portions (20 l) of freshly fermented lager (green beer) from the Carlsberg Brewery was transferred under an atmosphere of carbon dioxide to casks containing the desired amount of α-acetolactate decarboxylase in a small quantity of phosphate buffer (pH 6.5, 25 mM, approximately 100 ml). After mixing, the beer was stored for 24 hours at 10 °C whereupon the tempera-

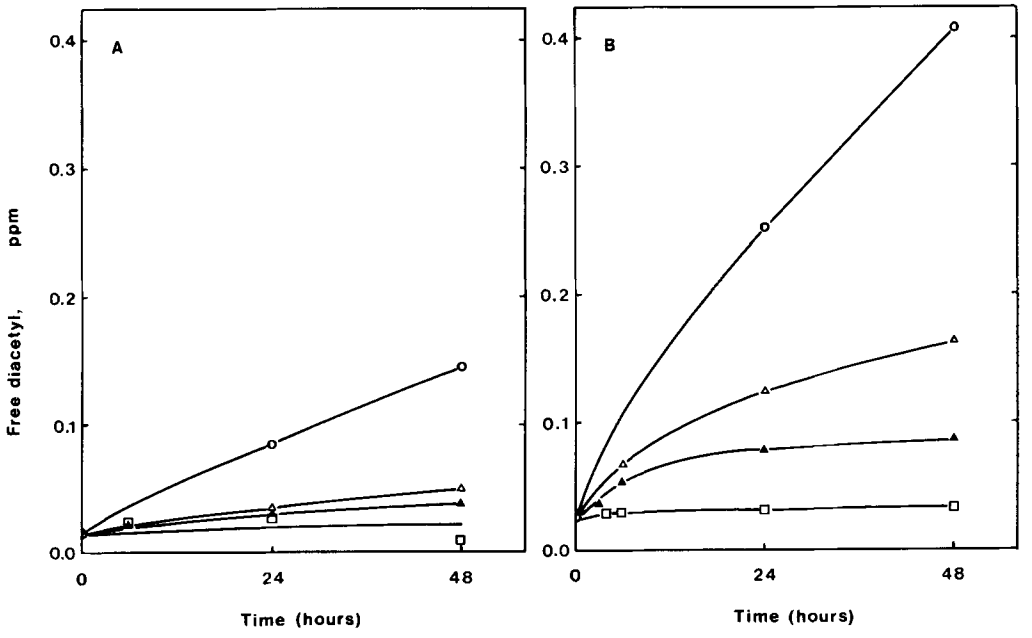


Figure 1. Formation of free diacetyl at 10 °C in the presence of 0 (○---○), 4.3 (△---△), 21 (▲---▲) and 90 (□---□) kU/l α-acetolactate decarboxylase in phosphate buffers at pH 5.80 (A) and pH 4.31 (B) containing an authentic specimen of α-acetolactate in an initial concentration corresponding to 2.0 ppm diacetyl.

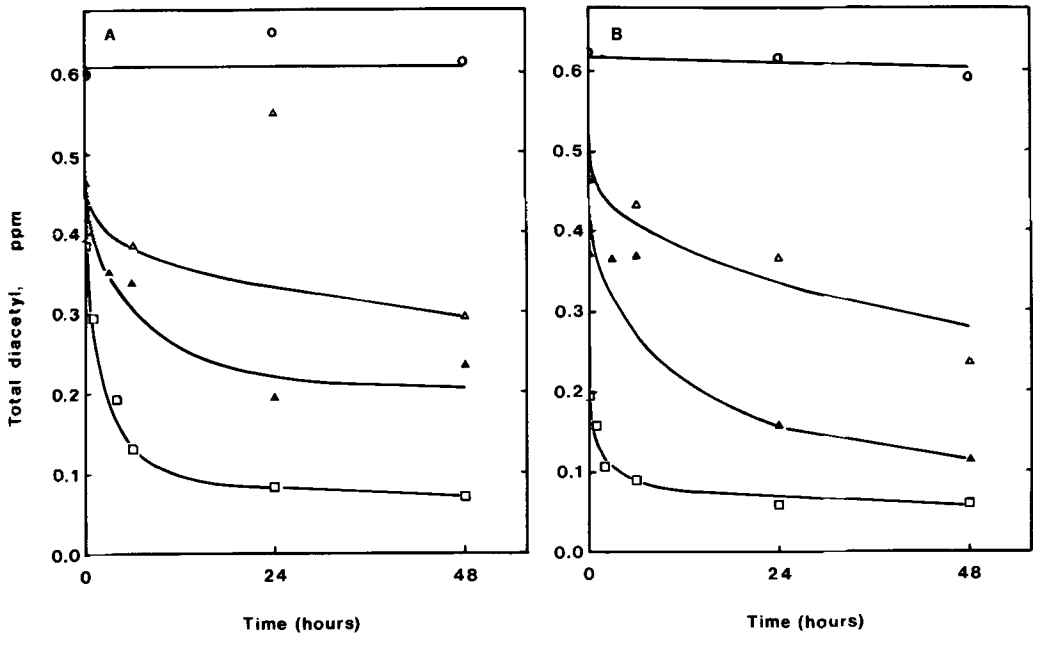


Figure 2. Changes in total diacetyl content at 10 °C in phosphate buffers of pH 5.80 (A) and pH 4.31 (B) containing 0 (O---O), 4.3 (△---△), 21 (▲---▲) and 90 (□---□) kU/l α-acetolactate decarboxylase and α-acetolactate in an initial concentration corresponding to 2.0 ppm diacetyl.

ture of the beer was lowered to −1 °C. The beer was left for 48 hours at this temperature and then filtered, bottled and pasteurized.

3. RESULTS AND DISCUSSION

The fate of α-acetolactate in buffers at pH 5.8, which is close to the pH optimum of the enzyme, and pH 4.2, which is close to the pH of maturing beer, containing varying amounts of α-acetolactate decarboxylase isolated from Enterobacter aerogenes strain 1033 is depicted in Figures 1 and 2. In solutions devoid of enzyme α-acetolactate is seen to decompose slowly to diacetyl which accumulates in enzyme free solutions (Figure 1) while the sum of the concentrations of α-acetolactate and diacetyl (total diacetyl) in these solutions remain constant (Figure 2). On the other hand, in the presence of enzyme the total diacetyl content of the solutions is seen to decrease with time (Figure 2) while the formation of free diacetyl (Figure 1) is correspondingly suppressed. This pattern is to be expected since, in the presence of enzyme, less α-acetolactate is available for diacetyl formation because of its conversion to acetoin. It is apparent from the figures that the presence of 90 $kU \cdot l^{-1}$ α-acetolactate decarboxylase suffices for reducing the total diacetyl content to a level below the taste treshold of diacetyl in less than 24 hours and that free diacetyl is not generated in concentrations exceeding the taste treshold of the diketone under these conditions.

These findings suggest that it may be possible to carry out beer maturation with respect to removal of diacetyl and α-acetolactate in less than 24 hours and, consequently, that it will be possible to carry out flavour maturation of beer within this period of time by applying an α-acetolactate decarboxylase if, apart from removal of these compounds, no important events related to flavour maturation of beer take place.

Table I indicates the concentration of free and total diacetyl, 2,3-pentanedione, α-acetolactate and α-aceto-α-hydroxybutyrate in the filtered and pasteurized beer prepared by maturation of freshly fermented wort (green beer) for 24 hours

Table I

Content of free and total diacetyl and 2,3-pentanedione in beer matured for 24 hours at 10 °C in the presence of 90 kU/l α-acetolactase decarboxylase and in beer matured conventionally for 24 hours.

		α-Acetolactate decarboxylase matured beer	Beer conv. matured 24 hours
Free diacetyl	ppm	0.01	0.12
Total diacetyl	–	0.07	0.47
Free pentandione	–	0.00	0.10
Total pentandione	–	0.05	0.41

at 10 °C and subsequently for 48 hours at −1 °C in the presence of 90 kU·l^{-1} α-acetolactate decarboxylase. Also indicated is the content of diacetyl, 2,3-pentanedione and their precursors in a reference sample of freshly fermented wort matured for similar periods of time in the absence of enzyme. It is seen that in the presence of α-acetolactate decarboxylase the total diacetyl content of the beer has been reduced to 0.07 ppm while the total diacetyl content of the reference sample is 0.47 ppm. Moreover, the content of 2,3-pentanedione and its precursor has been reduced to a level of 0.05 ppm in the enzyme matured beer in contrast to a value of 0.41 in the reference sample. The removal of diacetyl as well as 2,3-pentanedione from the enzyme matured beer is in accordance with the observation that both α-acetolactate and α-aceto-α-hydroxybutyrate serve as substrates for α-acetolactate decarboxylase (7).

Table II includes a more extensive comparison of the two beers and a reference sample of beer of the same batch matured conventionally for three weeks. It is apparent that, with respect to the parameters listed in the Table, the three beers are virtually identical and possess properties within the normal specification range of the beer. Finally, in Table III, the three beers are compared with respect to some volatiles, other than diacetyl, of importance for the flavour of the beer. It can be noted that the difference between the measured amounts of the listed esters, alcohols and carbonyl compounds is too small to be of any consequence for the flavour of the beers. Last, the enzyme matured beer was compared with a conventionally prepared beer with respect to organoleptic properties. By these tests the beer matured in the presence of α-acetolactate decarboxylase was judged by the taste panel to be of an equally satisfactory quality as the reference sample of conventionally prepared beer.

The findings summarized above indicate that it is possible to remove diacetyl and its precursor α-acetolactate as well as their congeners 2,3-pentanedione and α-aceto-α-hydroxybutyrate selectively from freshly fermented beer without affecting any other of the important properties and volatile constituents of the beer listed in

Table II

Properties of beer matured for 24 hours at 10 °C in the presence of 90 kU/l α-acetolactate decarboxylase, of beer matured conventionally for 24 hours and of beer matured conventionally for 3 weeks.

		α-Acetolactate decarboxylase matured beer	Beer matured 24 hours	Beer conv. matured 3 weeks
Original extract, P (p)	ppm	10.51	10.80	10.79
Alcohol, % by weight (a)	–	3.78	3.91	3.90
Real res. ferm. extract, °P	–	3.12	3.17	3.18
App. res. fer. extract, °P	–	1.38	1.37	1.39
Real degree of Ferm., % (Fg°r)	–	70	71	71
Colour. EBC units	–	6.4	6.0	6.0
pH	–	4.29	4.18	4.31
Bitterness. B.U./EBU	–	21	23	23
Head retention, Blom, sec.	–	89	85	84

Table III

Head space profiles of beer matured for 24 hours in the presence of 90 kU/l α-acetolactate decarboxylase, of beer matured conventionally for 24 hours and of beer matured conventionally for 3 weeks.

		α-Acetolactate decarboxylase matured beer	Beer matured 24 hours	Beer conv. matured 3 weeks
Acetaldehyde	ppm	3.4	3.3	3.0
Ethyl formate	–	0.70	0.59	0.66
Ethyl acetate	–	15.0	14.9	16.9
Isobutyl acetate	–	0.022	0.024	0.025
1-Propanol	–	16.0	16.0	18.4
2-Methyl-1-propanol	–	4.7	4.6	5.0
3-Methylbutyl acetate	–	1.8	1.9	2.1
3-Methyl-1-butanol	–	58	56	62
Ethyl hexanoate	–	0.30	0.34	0.32
N-Hexylacetate	–	0.005	0.015	0.018
Ethyl octanoate	–	0.11	0.12	0.18

Table II and Table III by adding α-acetolactate decarboxylase to the beer under conditions normally applied for beer maturation. Taken together with the result of the organoleptic test these findings seem to indicate that, apart from removal of diacetyl, 2,3-pentanedione, α-acetolactate and α-aceto-α-hydroxybutyrate, no important events related to flavour development take place during maturation of the type of beer investigated. If a more thorough comparison of beer matured in the presence of α-acetolactate decarboxylase and conventionally matured beer substantiates these findings it follows that it will be possible to carry out flavour maturation of beer in less than 24 hours by adding α-acetolactate decarboxylase to maturing beer and thereby to reduce the time required for beer maturation considerably since technology is presently available for achieving objectives of beer maturation other than flavour maturation within a short period of time. Moreover, the possibility of selectively removing α-acetolactate and α-aceto-α-hydroxybutyrate from fermented wort will open an interesting opportunity for studying other factors of the beer maturation process. However, it is obvious that the technical application of α-acetolactate decarboxylase in the brewing industry will depend, among other things, on the possibility of producing a suitable preparation of α-acetolactate decarboxylase at a reasonable cost on a technical scale.

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18. The subject of the present work is covered by patent applications: Europe – No. 81303606.8; International – No. PCT/DK81/0076, and applications in other countries.