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Conversion of green note aldehydes into alcohols by yeast alcohol dehydrogenase

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Abstract

'Green note' aldehydes were successfully reduced into their corresponding alcohol by commercial yeast alcohol dehydrogenase. Among different yeasts tested for their ability to convert (*Z*)-3-hexenal into (*Z*)-3-hexenol, *Pichia anomala* gave the best results. Conversion yields higher than 90% were also obtained by directly conducting the reaction in the medium where (*Z*)-3 -hexenal is produced by the action of lipoxygenase and hydroperoxide lyase on linolenic acid.

Keywords : green note aldehydes ; yeast alcohol dehydrogenase ; (*Z*)-3-hexenal ; (*Z*)-3-hexenol

Introduction

'Green note' aldehydes and alcohols (C-6 and C-9) are high value molecules widely used in the aroma industry. The natural compounds are extracted from plants where they are present at very low concentration. The aim of our research is to study the biosynthesis of natural 'green note compounds' and particularly the production of (*Z*)-3-hexenol so called 'leaf alcohol', a key component in aromas destined to the food industry. In plants, 'green note compounds' are produced by a cascade of enzymatic reactions: lipoxygenase transforms polyunsaturated fatty acids (linoleic and linolenic acid) into fatty acids hydroperoxides which are cleaved by hydroperoxide lyase into aldehydes. Aldehydes can further be reduced in alcohols by alcohol dehydrogenase (ADH) (Hatanaka 1993). In previous researches, we studied the production of fatty acid hydroperoxides using soybean lipoxygenase (Fauconnier & Marlier 1996) and the cleavage of hydroperoxides by an hydroperoxide lyase extracted from tomato leaves (Fauconnier *et al.* 1997). This paper is dedicated to the study of the last step of the reaction: the conversion of the aldehydes in alcohols. The reaction was first conducted using commercial ADH from baker yeast and pure 'green note' aldehydes (hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenal, (*E*)-2-nonenal, (*E*)-2, (*Z*)-6-nonadienal). We particularly focussed on the synthesis of (*Z*)-3-hexenol by determining optimum synthesis parameters. To avoid the costly use of co-factor (NADH), commercial ADH was replaced by yeast cells. Different species were tested for their efficiency in reducing (*Z*)-3-hexenal in (*Z*)-3-hexenol. Among the tested yeasts, *Pichia anomala* gave the best reaction yields. The experiments revealed substrate inhibition but no product inhibition. Finally, the reduction reaction was directly conducted in the reaction medium where (*Z*)-3-hexenal is produced by the action of lipoxygenase and hydroperoxide lyase on linolenic acid. Two methods were considered: conducting the reduction reaction after action of the hydroperoxide lyase or at the same time.

Materials and methods

Materials

ADH (alcohol dehydrogenase E.C. 1.1.1.1. from baker yeast, 90% protein, 300-500 units mg^{-1} protein) was obtained from Sigma. The enzyme activity was systematically tested in standardised conditions before use. One unit of activity is defined as the amount of enzyme that convert 1.0 μmol of ethanol to acetaldehyde per minute at pH 8.8 at 25 °C. NADH was obtained from Sigma, hexanal, (*E*)-2-hexenal, (*E*)-2-nonenal, (*E*)-2, (*Z*)-3-nonadienal were obtained from Aldrich, the purity of the aldehydes was checked by GC analysis and was higher than 99%. (*Z*)-3-Hexenal was obtained from Fontarôme (France) at 50% (V/V) in glyceryl triacetate. (*Z*)-3-Hexenal in glyceryl triacetate was distilled under vacuum just before use in order to obtain a GC purity higher than 99%. All other reagents were of analytical grade. The different species of yeasts were obtained from the

'Centre Wallon de Biologie Industrielle' (Prof Thonart). Tested species: *Yarrowia lipolytica* CBS 6303 (diploid), *Pichia anomala* isolated from apple (diploid), *Saccharomyces cerevisiae* 1278b (haploid and diploid).

Reduction reaction using commercial ADH and NADH

The reduction reaction was directly conducted in 3 ml quartz cuvettes. A typical reaction consisted of 2.78 ml of sodium phosphate buffer (0.1 M, pH 7.0), 100 μl 15 mM NADH, 100 μl commercial ADH (85 units ml^{-1}) and 20 μl 0.1 M aldehyde in acetonitrile. The reaction was monitored by following the decrease of absorbance at 340 nm (oxydation of NADH in NAD+). All experiments were realised at 20 °C in triplicate using a blank to take into account spontaneous oxidation of NADH. Initial rate was systematically determined (V_0 in μmol l^{-1} min^{-1}). Optimum reaction conditions of the (*Z*)-3-hexenal reduction was determined in the same manner varying the reaction temperature, the reaction pH by adding diluted HC1 or NaOH (the final pH of the reaction was carefully controlled in each experiment), the enzyme, the substrate or the cofactor concentration.

Reduction reaction using living yeast cells and commercial (Z)-3-hexenal

Yeasts were cultivated at 30 °C in 250 ml flasks with 150 ml medium (1% W/V yeast extract, 2% W/V glucose and 1% W/V caseinate peptone). The growth rate was determined every 2 h; when the yeast dry weight content reached 1.5 mg ml^{-1} of culture medium, the medium was centrifuged (15,600 g, 15 min at 4 °C), the residue was rinsed two times with 10 ml of peptonised water and resuspended in 25 ml medium (0.175% W/V yeast nitrogen base (Difco), 0.5% W/V $(NH4)_2SO_4$, 2% glucose in sodium acetate buffer (0.05 M, pH 5.5). The cells were transferred in 50 ml flasks. The reaction reduction, conducted in triplicate at 30 °C under agitation, was initiated by introducing variable amounts of substrate of 0.1 M (*Z*)-3-hexenal in acetonitrile. Reduction reaction was monitored by measuring the (*Z*)-3-hexenol formed by GC after extraction by diethyl ether according to Fauconnier *et al.* (1997) The identification of (*Z*)-3-hexenal and (*Z*)-3-hexenol was performed by injecting the pure compounds in the same conditions (GC) and by GC-MS analysis.

Reduction reaction using living yeast cells and (Z)-3-hexenal produced enzymatically

13-hydroperoxide of linolenic acid was synthesised using lipoxygenase extracted from soybean seeds according to Fauconnier & Marlier (1996).

(a) *Reduction reaction after action of hydroperoxide lyase*

Hydroperoxide lyase extracted from tomato leaves obtained according to Fauconnier *et al.* (1997) was added to the previous reaction medium (linolenic acid 13-hydroperoxide solution). The cleavage reaction was conducted during 10 min at 15 °C. The amount of (*Z*)-3-hexenal formed, was determined by GC analysis after extraction with diethyl ether. This reaction medium containing the (*Z*)-3-hexenal (for a final concentration of 0.68 mM in aldehyde) was directly added the *Pichia anomala* cells obtained as described before. The reaction was carried out at 30 °C during 2 h. Aliquots were regularly taken to determine the concentration in (*Z*)-3-hexenol by GC analysis.

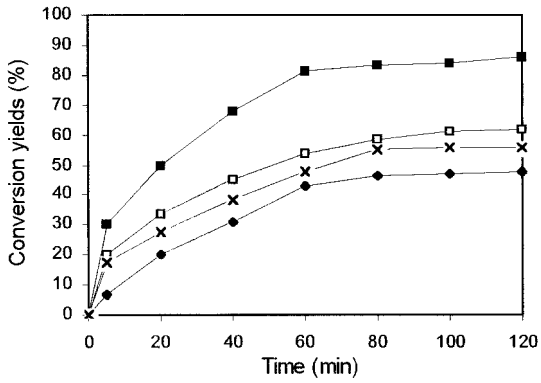
(b) *Reduction concomitant with hydroperoxide lyase reaction*

The reaction medium containing the 13-hydroperoxide of linolenic acid (for a final concentration of 1.36 mM in hydroperoxide) was directly added to the *Pichia anomala* cells obtained as described above. Hydroperoxide lyase extracted from tomato leaves obtained according to Fauconnier *et al.* (1997) was added to initiate the synthesis of (*Z*)-3-hexenal. The reaction was carried out at 30 °C during 2 h. Aliquots were regularly taken to determine the concentration in (*Z*)-3-hexenol by GC analysis. The decrease in linolenic acid 13-hydroperoxide was followed by measuring the absorbance at 234 nm after dilution in ethanol.

Table 1. *Initial rates of reduction of different aldehydes (0.67 mM) by commercial ADH (8.5 units ml of reaction medium) in presence of NADH (0.5 mM) at pH 7.0 and at 20°C.*

Substrate	V_0 (µmol ml^{-1} min^{-1})
Hexanal	0.105 ± 0.011
(*E*)-2-hexenal	0.072 ± 0.007
(*Z*)-3-hexenal	0.057 ± 0.005
(*E*)-2-nonenal	0.131 ± 0.014
(*E*)-2, (*Z*)-6-nonadienal	0.108 ± 0.011

Fig. 1. *Conversion yields of (Z)-3-hexenal in (Z)-3-hexenol by different species of living yeast cells at 30 °C (initial substrate concentration: 0.067 mM). (■) P. anomala, (□) S. cerevisiae diploid, (×) S. cerevisiae haploid, (♦) Y. lipolytica.*



Results and discussion

Reduction reaction using commercial ADH and NADH

The initial rates of reduction of different green note aldehydes were determined using commercial ADH and NADH. The results, average of the three repetitions and the standard deviation are presented in Table 1.

As shown in Table 1, enzymatic activity decreases, for a same number of carbons, with aldehydes insaturation: hexanal is converted more efficiently than (*E*)-2-hexenal or (*Z*)-3-hexenal; ADH activity is higher with (*E*)-2-nonenal than with *(E)*-2, (*Z*)-6-nonadienal. Enzymatic activity increases with the length of the hydrocarbon chain: ADH activity is higher for C-9 aldehydes than for C-6 ones. Finally, the (*E*) form is preferred than the (*Z*) form. Those results are in agreement with results obtained by Pietruzko *et al.* (1973) and Alexander *et al.* (1982) with other aldehydes.

Optimum synthesis parameters were determined using (Z)-3-hexenal as substrate. Enzymatic activity is high between pH 5 and 7 while maximal activity is observed at pH 5.5. Between 30 °C and 50°C, ADH activity increases gradually. For higher temperature, the enzyme undergoes a rapid denaturation. At pH 5.5 and at 45 °C, we determined the yield of transformation of (*Z*)-3-hexenal into (*Z*)-3-hexenol. After 20 min, maximum transformation yield is reached and 82% of the initial aldehyde are reduced in alcohol. We also checked the potentially inhibitory effect of the reaction product on the reduction reaction by adding increasing concentrations of (*Z*)-3-hexenol in the reaction medium at the beginning of the reaction. We could not notice any inhibitory effect in the range of the tested concentrations in (*Z*)-3-hexenol (0.04 mM to 4.0 mM).

Reduction reaction using living yeast cells and commercial (Z)-3-hexenal

To avoid the use of the costly cofactor NADH, we replaced commercial ADH by living yeast cells. Different species were tested to determine their ability to transform (*Z*)-3-hexenal into (*Z*)-3-hexenol. The reaction was monitored by determining the amount of alcohol formed by GC because the UV determination used before based on the oxidation of NADH is not applicable with living cells. Figure 1 presents the results obtained with the different species tested. The initial substrate concentration was 0.067 mM. The results presented are the average of three replicates.

All the tested species are able to reduce (*Z*)-3-hexenal into (*Z*)-3-hexenol but *Pichia anomala* is the most efficient strain furnishing the highest initial rate and conversion yield.

Further experiments were undertaken with *Pichia anomala* cells. Kinetic parameters (K_m and V_{max}) were determined by measuring initial rates for different initial substrate concentrations (Figure 2).

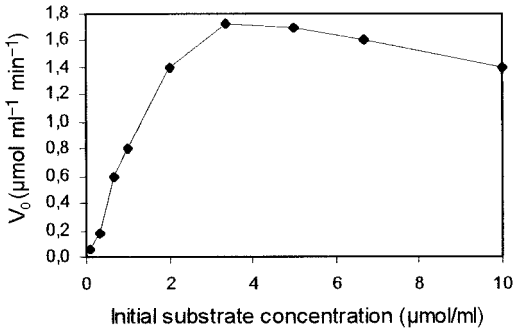
From Figure 2, it can be deduced that K_m and V_{max} values are respectively 0.9 μmol ml^{-1} and 1.7 μmol ml^{-1} min^{-1}. The curve pattern is typical of an enzymatic reaction inhibited by its substrate because V_0 decreases for high substrate concentrations. As observed with commercial ADH, the reduction reaction realised with living yeast

cells is not inhibited by the reaction product. Indeed, reactions conducted with initial addition of (*Z*)-3-hexenol for concentrations ranging from 1 to 10 μmol ml^{-1} of reaction medium revealed no inhibitory effect.

Table 2. *Conversion yields of (Z)-3-hexenal (A) or of linolenic acid 13-hydroperoxide (B) into (Z)-3-hexenol by P. anomala.*

Test	Reduction yields (%) for different reaction times (min)					
	0.5	1	5	10	30	120
A	34 ± 1.1	60 ± 1.5	73 ± 0.8	84 ± 1.4	86 ± 1.6	90 ± 1.4
B	14 ± 0.8	28 ± 0.9	41 ± 1.1	44 ± 1.3	47 ± 2.0	48 ± 1.0

Fig. 2. *Initial rate of reduction of (Z)-3-hexenal into (Z)-3-hexenol for different initial substrate concentrations.*



Reduction reaction using living yeast cells and (Z)-3-hexenal produced enzymatically

The last experiments were realised to integrate the reduction reaction in the complete production scheme of green note compounds. Two different procedures were used. In the first one (A), the substrate containing the (*Z*)-3-hexenal was added to the reaction medium resulting from action of lipoxygenase and hydroperoxide lyase on linolenic acid. This last substrate was used without further extraction or purification of (*Z*)-3-hexenal. In the second experiment (B), the *P. anomala* cells were introduced at the same time as the hydroperoxide lyase in the reaction medium containing the linolenic acid hydroperoxides (obtained by the enzymatic reaction of lipoxygenase on linolenic acid). In the two cases, experiments were carried out in triplicate, the results are presented in Table 2. The reduction yields are the percentages of initial (*Z*)-3-hexenal transformed in (*Z*)-3-hexenol (case A) or the percentages of linolenic acid 13-hydroperoxide transformed in (*Z*)-3-hexenol (case B). In the case B, it is impossible to determine the amount of (*Z*)-3-hexenal formed by the hydroperoxide lyase because this last compound is rapidly reduced in the corresponding alcohol. Moreover, it is possible to follow the decrease in hydroperoxide concentration by UV measurements. The reduction reaction is very rapid and reaches its maximum almost in 10 min. In the case A, the final transformation yield is very high meaning that the reaction is not inhibited by the complex composition of the reaction medium in which lipoxygenase extracted from soybean, hydroperoxide lyase extracted from tomato leaves and linolenic acid 13-hydroperoxides which have not reacted are present. In the case B, the reaction yields seem to be smaller but they are calculated from the amount of initial linolenic acid 13 -hydroperoxide transformed. The UV measurements revealed that 45% of the initial hydroperoxides were not transformed after 120 min of hydroperoxide lyase reaction. If the final reaction yield of formation of (*Z*)-3-hexenol is calculated taking into account that only 55% of the hydroperoxides are transformed in aldehyde, the final yield reaches 88% of substrate transformed. Therefore, reduction reaction conducted in the same time that the hydroperoxide lyase one is not inhibited by the reaction conditions. The low transformation yield of hydroperoxide by hydroperoxide lyase indicated here are due to two reasons: the reaction pH which is not optimal for the hydroperoxide lyase and mainly because of the irreversible inhibition of the enzyme by its substrate previously demonstrated (Fauconnier *et al.* 1997, Matsui *et al.* 1992). As ADH reduces different green note aldehydes, the procedure described for the biosynthesis of (*Z*)-3-hexenol can adapted to the production of other green note alcohols by using another source of fatty acid (linoleic acid) and/or another source of lipoxygenase and hydroperoxide lyase.

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