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Unsaturated fatty acid bioconversion by apple pomace enzyme system. Factors influencing the production of aroma compounds

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Productions of volatile compounds (hexanal and 2,4-decadienal) obtained from polyunsaturated fatty acids by action of specific apple pomace enzyme system were quantitatively improved by increasing substrate and enzyme concentrations in the reaction medium. The importance of an exogenous supply of oxygen during bioconversion was also shown. Some physico-chemical factors involved in the pomace enzyme system expression were screened. A temperature of 25°C was favourable to the bioconversion. The control of alkaline or acidic conditions in the reaction medium may orientate the reaction preferentially to the production of one or other aldehyde. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

Apple pomace, solid and bulky waste derived from the apple juice industry (Givens & Barber, 1987) would appear from its chemical composition to be highly suitable for valorization (Hang, 1987).

Recently, we reported on the production of aroma compounds during bioconversion of polyunsaturated fatty acids by the endogenous enzyme system in apple pomace (Almosnino & Belin, 1991). These results were in agreement with previous works (Paillard, 1979; Ambid & Fallot, 1980; Berger & Drawert, 1984; Drawert *et al.*, 1986) reporting the production of volatile aldehydes and alcohols by apple peripheral tissues from *in situ* or added linoleic acid or linolenic acid. Amongst the apple pomace enzymes involved in aroma production, lipoxygenase (EC 1-13-11-12) catalysis of fatty acid peroxidation leads to the formation of 9- or 13-hydroperoxides (Kim & Grosch, 1979), whereas hydroperoxide lyase ensures the formation of volatile compounds by cleavage of the hydroperoxides (Schreier & Lorenz, 1982; Hatanaka *et al.*, 1986; Bensoussan *et al.*, 1993).

The present work advances some factors likely either to improve the production of volatile aldehyde for industrial purposes or to orientate the enzymatic reactions preferentially towards the formation of a particular aroma compound.

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MATERIALS AND METHODS

Materials

The enzyme source was a commercial 'Golden Delicious' apple, pomace was reproduced on a laboratory scale by crushing apples in an electric mixer and pressing them with a small press. The dry weight was approximatively 24% and the ratio of sugar to dry weight 29%. The pomace was stored in buffer in the presence of preservatives at −18°C (Almosnino & Belin, 1991). This wet pomace was micronized with a Polytron PT 45-80 (Kinematica, Littau-Luzern, Switzerland) at the beginning of bioconversion. Substrates were technical linoleic acid (Prolabo, Paris, France) containing 75% linoleic acid, 12% oleic acid, 2% linolenic acid and 1% saturated fatty acid, and carthame oil (Bjorg, Saint-Genis-Laval, France) containing 76% linoleic acid hydrolyzed by a non-specific *Aspergillus niger* SP 398 lipase (Novo, Bagsvaerd, Denmark). Tween 80 at 0.25 ppm (Prolabo, Paris, France) was used as emulsifier (De Pooter *et al.*, 1983). Two preservatives were added: SO_2 in the form of Na_2SO_3 at 60 ppm (to inhibit the polyphenoloxidase) and ascorbic acid at 500 ppm (to prevent the browning by reduction of quinones).

Conducting the bioconversion

The liquid medium had a total reaction volume of 150 ml of Mac Ilvaine buffer including preservatives and

consisted of 6.6% (w/v) of apple pomace, 0.1% of technical linoleic acid and Tween 80 (37.5 μl). The bioconversion was carried out at 18°C for 30 h in 500 ml flasks with constant agitation on a rotary shaker (100 rpm).

The concentrated media, in a 2-litre buffered reaction mixture, contained (w/v) 33 or 50% apple pomace, 0.5 or 2% linoleic acid (given as technical linoleic acid or hydrolyzed carthame oil), Tween 80 and preservatives. The bioconversion was run into 5- or 10-litre Trimix reactors (Rayneri, Montreuil, France) equipped with three coaxial movings parts: the speed of the scrapers was constant at 30 rpm, the speed of the central countershaft was maintained at 100 rpm and the speed of the central tri-blade set at 300 rpm. The temperature of the process, if not indicated, was 25°C. The bioconversion was run for various periods from 24 to 48 h.

Oxygen was brought to the reaction medium as pure gas or as compressed air by direct injection into the medium or by addition into the head space of the reactor either continuously during the bioconversion, or sequentially at $t = 0$, 4, 6, 8, 24 and 30 h with a flow rate of 5–8 litres/min for 15 min. Initial pH conditions were generally established with Mac Ilvaine buffer (citric acid–phosphate) 0.1 M, pH 5.8, according to Drawert *et al.* (1986). Nevertheless, other buffers were utilized during some bioconversions: Sorensen buffer (citrate–NaOH) at various ionic strengths and pH and, in alkaline conditions, Clark and Lubs buffer (borate–NaOH) 0.1 M, pH 9.0.

Extraction and analysis of aroma compounds

Ten grammes of the reaction medium were added to 50 ml distilled water containing 1 ml solution of internal standard (heptanoic acid methyl ester at 100 ppm; Berger & Drawert, 1984). The volatile compounds were extracted from the mixture by steam stripping in a Buchi apparatus and recovered in 200 ml of distillate. The aroma compounds were then recovered by liquid–liquid extraction in 20 ml of hexane and concentrated to 1 ml under nitrogen flux. The samples (1 μl) were analysed by gas chromatography. A Packard chromatograph CP 9000 (Chrompack, Middelburg, The Netherlands) was used, equipped with a capillary column (50 m×0.32 mm. I.D) model CPWAX 58 CB, an 'on column' injector and a flame ionization detector. The injector temperature was 60°C, that of the detector 310°C. The temperature was programmed from 60 to 120°C at 3°C/min and above 120°C at 5°C/min. The carrier gas was nitrogen.

Bioconversion follow-up

The biochemical phenomena occuring during bioconversion were examined by considering the hexanal production, which reflects the expression of a double enzyme system, lipoxygenase and hydroperoxide lyase, and the 2,4-decadienal production, which reflects lipoxygenase expression and non-enzymatic decomposition of the hydroperoxides by β-scission (Gardner, 1985, 1989). All results represent the average of three replicates with 8% uncertainty.

RESULTS AND DISCUSSION

Effects of enzyme source concentration on aldehyde production

The bioconversion was conducted in three media A, B and C differing in their apple pomace concentration: 6.6 (A), 33 (B) and 50% (C), but with the same unchanged enzyme source–fatty acid substrate ratio (66.6).

In comparison to the low pomace concentration (medium A), the bioconversion conducted with medium B gave a clear increase in aldehyde production in 6 h, i.e. 7.5 times more hexanal and 5.4 times more 2,4-decadienal (Fig. 1). Productivities for hexanal and 2,4-decadienal in the B (33%) medium were 7.6 and 54.5 mg/kg apple pomace per h, respectively, during 6 h bioconversion. In spite of the same enzyme/substrate ratio, these results can be explained by the fact that 33% pomace concentration promoted better contact between apple pomace and linoleic acid, and more efficient expression of the enzymatic pool. With the most concentrated pomace medium (C), productivities during 6 h for hexanal and 2,4-decadienal (respectively 3.6 and

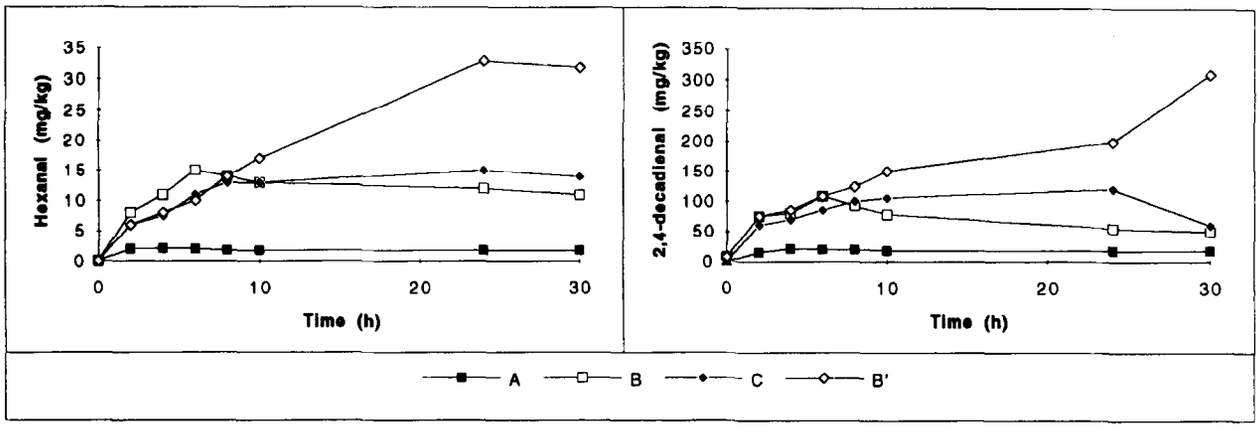

Fig. 1. Effect of apple pomace concentration on aldehyde production.

Table 1. Influence of oxygen supply on aldehyde production (mg/kg)

Time (h)	Hexanal		2,4-Decadienal	
	Control	O_2 supply	Control	O_2 supply
0	0	0	8	12
4	11	10	80	60
6	15	14	108	100
8	14	16	93	108
10	13	17	78	110
24	11	20	55	120

Results represent the average of three replicates (8% uncertainity).

Table 2. Hexanal and 2,4-decadienal productivities (mg/kg/h) vs different kinds of oxygen supply

O_2 supply	Hexanal		2,4-Decadienal	
	0–24 h	24–48 h	0–24 h	24–48 h
O_2: sequential	7	20	30	50
O_2: continuous	8	7.5	51	38
Air: sequential	1.5	1.2	15.7	5.4
Air: continuous	4.4	4.5	40	32.5

Results represent the average of three replicates (8% uncertainity).

28.3 mg/kg/h) are low and inferior than those of medium A.

Influence of oxygen supply

Interest of an exogenous oxygen supply

The productivities of hexanal and 2,4-decadienal in medium B without oxygen supply and with the addition of oxygen in the headspace of the reactor were similar during the first hours of bioconversion (Table 1). However, between 8 and 24 h, the yields decreased in the absence of oxygen supply, whereas the addition of oxygen permitted the tendency observed in the first hours of bioconversion to continue.

As co-substrate of the bioconversion, oxygen supports the lipoxygenase activity and prevents a decrease in aldehyde concentration in the reaction medium.

Comparison of different kinds of oxygen supply

In accordance with the observations of Dolev *et al.* (1967) indicating that oxygen incorporated in hydroperoxides came from oxygen dissolved in the aqueous phase, the oxygen supply was added directly to the medium at the base of the reactor (Table 2). Productivities issued from bioconversion processes involving compressed air were significantly lower than those involving pure oxygen whether supplied by continuous or sequential methodology. The productivity of 2,4-decadienal after 48 h with continuous compressed air supply was quite similar to that obtained with pure oxygen.

Hexanal productivity in the presence of pure oxygen was higher than in the presence of compressed air.

Chan *et al.* (1976) observed that the absence of oxygen in the reaction medium allowed the conversion of 13-hydroperoxides into 9-hydroperoxides by isomerization leading to high concentrations of 2,4-decadienal. Grosch (1982) also noticed that the production of hexanal from a mixture of 13- and 9-hydroperoxides was dominant in the presence of oxygen.

Nevertheless, in the same conditions, as hexanal is more volatile than 2,4-decadienal the low productivities observed for hexanal may be due to losses during the aeration procedure, as shown by complementary experiments in model conditions. Apple pomace inhibited by heating and supplied by aldehyde at $t=0$ (50 and 300 mg/kg of apple pomace, respectively, for hexanal and 2,4-decadienal) under continuous oxygen supply exhibited a loss of more than 80% of the initial hexanal content after 8 h, whereas the 2,4-decadienal content was slightly increased (+6%) (Table 3).

Influence of concentration and nature of the lipidic substrate

In comparison to medium B (0.5% linoleic acid), bioconversion in medium B′ (2% linoleic acid) produced 2.7 times more hexanal (33 mg/kg) and 3.2 times more 2,4-decadienal (200 mg/kg).

Table 3. Residual aldehyde content (mg/kg) under continuous O_2 flux (5 litres/min) during 24 h

Time (h)	Hexanal	2,4-Decadienal
0	50	300
2	50	301
4	39	311
8	8	320
24	1	335

Results represent the average of three replicates (8% uncertainity).

Table 4. Influence of the nature of the lipidic substrate on aldehyde production (mg/kg) in B′ conditions

Time (h)	Hexanal		2,4-Decadienal	
	Technical linoleic acid	Carthame oil + lipase	Technical linoleic acid	Carthame oil + lipase
0	0	0	15	15
4	8	12	90	100
8	10	18	130	160
24	25	40	255	290
30	2	55	310	360

Results represent the average of three replicates (8% uncertainity).

For hexanal, these high values were obtained after 24 h and the corresponding productivities were 1.5 with B and 4.1 mg/kg/h with B′ (Fig. 1).

Productivities of 2,4-decadienal over 24 h, seemed better in medium B′ (25 mg/kg/h) than in medium B (7 mg/kg/h). But in comparison to the substrate concentration, the aldehyde yield appeared to be reduced in medium B′: the concentration of linoleic acid was increased 4 times, whereas and the production of aldehyde was only doubled.

To evaluate the effects of the state of the lipidic substrate in concentrated medium, similar to medium B′, carthame oil hydrolyzed by an *Aspergillus niger* lipase was used in comparison with technical linoleic acid in a 10-litre Trimix reactor. The quantity of oil added to the reaction medium was based on the free fatty acid concentration given by the final rate of hydrolysis of the oil (64%), which would be similar to the control medium containing technical linoleic acid (2%). Table 4 shows that aldehyde production from hydrolysed carthame oil was higher than from the technical linoleic acid bioconversion. The productivity of hexanal, in the first 8 h, was significantly different in the presence of oil and lipase (6.8 mg/kg/h) to the control (3.7 mg/kg/h). The utilization of oil with lipase enables hexanal production to be improved. The hexanal/2,4-decadienal ratio was 0.16 with oil and 0.10 with technical linoleic acid at the end (30 h) of the bioconversion

The use of carthame oil and lipase seems to promote aldehyde production because the progressive liberation of fatty acids in the reaction medium is more favourable to enzyme activity. Similary, Egmond *et al.* (1976), and Nicolas & Drapron (1981) observed a strong inhibition of lipoxygenase activity when the substrate concentration was high. According to Gardner (1985), high quantities of hydroperoxides generated by lipoxygenase activity on linoleic acid can inhibit hydroperoxide lyase activity involved in the formation of hexanal leading to condensation reactions giving aldehydes with high molecular weights. Among the products derived from lipolysis of carthame oil, the monoglycerides act as emulsifiers (Tahoun & Ali, 1986), such as Tween 80, and facilitate the contact between fatty acid (hydrophobic phase) and enzymes (hydrophilic phase).

Effects of temperature and pH conditions

The temperatures

Figure 2 shows aldehyde production during bioconversion at 15, 18 and 25°C in concentrated medium with carthame oil and lipase. The best hexanal productivity after 30 h was obtained in the bioconversion carried out at 25°C (5.0 mg/kg/h). Productivities were 1.4 or 2.7 times lower at the other temperatures, i.e. 3.5 (18°C) and 1.8 mg/kg/h (15°C). Similar productivities were obtained for 2,4-decadienal at 25 and 18°C, i.e. 28.2 and 25.2 mg/kg/h, respectively, after 30 h.

These results showing an increase in aldehyde production during bioconversion, more apparent with

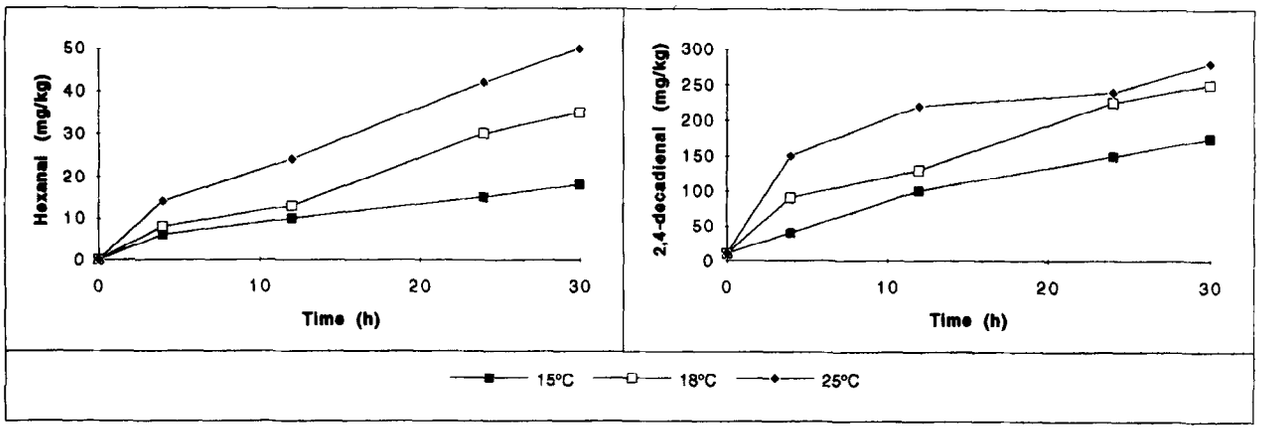

Fig. 2. Effects of temperature on hexanal and 2,4-decadienal production.

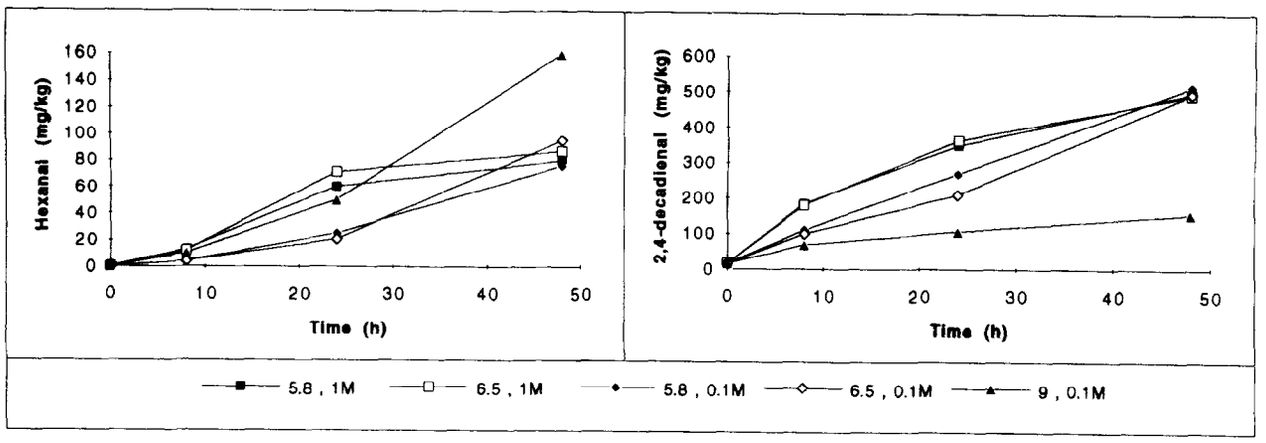

Fig. 3. Effects of ionic strength and pH of the buffer on aldehyde production.

hexanal than with 2,4-decadienal, indicate that the higher temperature (25°C) promotes both lipoxygenase activity and the hydroperoxide lyase activity involved in hexanal formation. As is apparent from the literature data, temperature effects on aldehyde production remain debatable. While Kim & Grosch (1979) and Schreier & Lorenz (1982) used apple enzymes at 25°C, Drawert *et al.* (1986) showed that the best yields of hexanal were observed at 10°C.

The pH

The effects of the pH associated with the ionic strength of the buffer on aldehyde production are shown in Fig. 3. During the first half period of the bioconversion (0–24 h), in acidic conditions, the aldehyde productivities were were 2–3 times greater with high molarity buffers.

Similar results were observed for 2,4-decadienal productivities, but with a lower ratio (1.4–1.8). Between 24 and 48 h, the production of aldehydes in the 1 M buffered media decreased and the concentrations of hexanal and 2,4-decadienal were very similar at the end of bioconversion (48 h).

Lipoxygenase and hydroperoxide lyase appeared to be unaffected by the acidic conditions of the reactional medium. The presence and concentration of citric acid in the 1 M buffer may prevent enzymatic browning particularly at the beginning of the bioconversion.

The yields of the two aldehydes evolved differently in alkaline conditions. At the end of the bioconversion, hexanal production was 2.1 times higher and 2,4-decadienal production 3.3 times lower than in the control medium (0.1 M, pH = 5.8). In these bioconversions performed in alkaline conditions, the hexanal/2,4-decadienal ratio was greatly modified after 48 h and hexanal production (160 mg/kg) appeared to be higher than 2,4-decadienal production (155 mg/kg).

These results do not concord with those of Drawert *et al.* (1986) which indicated maximum hexanal production at pH 4 and a regular decrease in production over the range pH 5–8.

Nevertheless, our results show the importance of the pH conditions on orientation of the enzymatic reaction leading to hexanal or 2,4-decadienal. The pH variation in the reactional medium involving apple pomace mainly affects lipoxygenase activity. Alkaline conditions (pH = 9) allow better expression of this enzyme with formation of 13-hydroperoxides to the detriment of 9-hydroperoxides. These results are supported by the following data related to other enzyme sources. Soybean lipoxygenase L1 produce a majority (95%) of 13-hydroperoxides (Iacazio *et al.*, 1990) at pH 11, while in acidic conditions the proportion of 9-hydroperoxides increases and can be up to 50% of the total hydroperoxides produced (Christopher *et al.*, 1972). A similar observation is reported with corn germ lipoxygenase, (Veldink *et al.*, 1972) for which the 9-/13-hydroperoxides ratio may be reversed depending on pH conditions.

Several parameters have influenced at different levels, the production of aroma by bioconversion of linoleic acid with apple pomace. Our results show that aldehyde production by the apple pomace enzyme system can be oriented in favour of hexanal or 2,4-decadienal by monitoring the pH conditions of the reaction medium. Furthermore, we also showed that simultaneous hydrolysis of oil by a lipase and biotransformation of the free fatty acids obtained by the system lipoxygenase hydroperoxide–lyase considerably improved aldehyde production.

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