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BIOCATALYSIS

# Enzymatic production and in situ separation of natural β-ionone from β-carotene

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**Abstract** A biotechnological process concept for generation and in situ separation of natural β-ionone from β-carotene is presented. The process employs carotenoid cleavage dioxygenases (CCDs), a plant-derived iron-containing nonheme enzyme family requiring only dissolved oxygen as cosubstrate and no additional cofactors. Organophilic pervaporation was found to be very well suited for continuous in situ separation of β-ionone. Its application led to a highly pure product despite the complexity of the reaction solution containing cell homogenates. Among three different pervaporation membrane types tested, a polyoctylmethylsiloxane active layer on a porous polyetherimide support led to the best results. A laboratory-scale demonstration plant was set up, and a highly pure aqueous–ethanolic solution of β-ionone was produced from β-carotene. The described process permits generation of high-value flavor and fragrance compounds bearing the desired label "natural" according to US and European food and safety regulations and demonstrates the potential of CCD enzymes for selective oxidative cleavage of carotenoids.

**Keywords** Dioxygenase · Organophilic pervaporation · β-Ionone · Flavor · Carotenoid

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## Introduction

The world market volume for flavors and fragrances is currently around US $20 billion, with an estimated 100 aroma chemicals being produced biotechnologically [2]. β-Ionone, a flavor and fragrance compound with fruity, violet-like characteristics, is used in particular in foodstuff and beverage industries. Due to increased consumer demand for "organic" or "bio" products [2], "natural" β-ionone has a market value approximately 10–100 times higher than the synthetic form. To date, two methods for production of natural β-ionone have been described: non-specific co-oxidative cleavage of carotenoids with lipoxygenases [23], and direct cleavage by a fungal peroxidase [24]. Both lead to the formation of a high share of byproducts. The enzyme *Arabidopsis thaliana* carotenoid cleavage dioxygenase 1 (AtCCD1) belongs to a family of iron-containing nonheme dioxygenases discovered in 2001 [20]. The CCD subgroup "1" cleave apocarotenoids and xanthophylls regiospecifically at the 9,10 and 9′,10′ double bonds, generating norisoprenoid C13 compounds with attractive organoleptic properties [22]. CCD enzymes require only dissolved oxygen as cosubstrate and no other cost-intensive cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH). Despite 10 years of intense research on this enzyme group and especially AtCCD1 [1, 6], the industrial potential of this enzyme group for synthesis of high-value plant secondary metabolites has not yet been exploited. Until recently, in vitro use of the most economical CCD1 substrate, β-carotene, was hindered by problems relating to delivery of this particularly hydrophobic substrate to the enzyme in the aqueous reaction medium that this enzyme requires. This challenge was overcome by using either nonionic micelles [13] or liposomes [12] as substrate delivery vesicles. Since the enzyme AtCCD1 was shown to be

product inhibited [18], continuous in situ product separation from the reaction solution is required to avoid negative influence on reaction kinetics.

Pervaporation is a method for selective separation of volatile substances from liquid mixtures using nonporous membranes. From a physical point of view, pervaporation is vacuum distillation across a nonporous polymer barrier [3]. Depending on the substance to be separated, the process is termed either organophilic or hydrophilic pervaporation. In technical use, it is especially well suited for separation of substances with molecular weights less than 200 Da at very low concentrations [15]. While hydrophilic pervaporation is frequently applied in a number of important unit operations such as dewatering of organic solvents or solvent separation [4], organophilic pervaporation is as yet not very widespread as a unit operation, and suitable organophilic pervaporation membranes are not very extensively described in the literature [10]. As a consequence, hydrophilic pervaporation units can be designed reliably using available physicochemical data, while organophilic pervaporation operations lack this background data and require more extensive testing of materials and conditions [15]. For this reason, data on membrane selection for organophilic pervaporation of $\beta$-ionone from aqueous solution are included in this report.

The goal of this work is to develop a selective biocatalytic process for production of natural $\beta$-ionone by oxidative cleavage of $\beta$-carotene using a cofactor-independent, regioselective enzyme. An aqueous–ethanolic solution of $\beta$-ionone was obtained using a laboratory-scale demonstration plant including in situ removal of the reaction product by organophilic pervaporation. Due to the substrate promiscuity of the biocatalyst, the process has a platform character and can also be used for production of other C13-norisoprenoids such as the fragrance compound 3-hydroxy-ionone by converting zeaxanthin instead of $\beta$-carotene.

## Materials and methods

### Chemicals

Unless mentioned otherwise, all chemicals were purchased from Carl-Roth (Karlsruhe, Germany) and, if not available there, from Sigma-Aldrich (Munich, Germany). Solvents were of analytical-grade purity and (in case of tetrahydrofuran) stabilized against peroxide formation by butylated hydroxytoluene (BHT).

### Technical equipment

A Bioengineering (Wald, Switzerland) KLF laboratory-scale fermenter with glass body and 2.4 l volume was used as enzyme reactor. The fermenter ports were fitted with a Bioengineering BioB 128/012 B1 EG2 impeller, two Bioengineering model 33519 heaters of 250 W maximum power each, a Bioengineering model Pt100 temperature sensor, and a Mettler-Toledo (Giessen, Germany) InPro 6800 32 cm oxygen electrode. The pervaporation module used was a prototype fabricated by Helmholtz Research Centre Geesthacht (HZG), Germany with usable membrane area of 0.099 $m^2$ [5]. The pressure gradient was generated using an RV3 vacuum pump (Edwards High Vacuum International, Sussex, UK). Organophilic pervaporation membranes were either provided by HZG (POMS/PEI) or purchased from GMT Membrantechnik GmbH, Rheinfelden, Germany (2420 G/V-1, 2421 G/V-2). Liquid-nitrogen-fillable glass cooling traps were custom made by KGW-Isotherm, Karlsruhe, Germany. To avoid losses of the product $\beta$-ionone into polymers in contact with the reaction solution, special hoses (PVC-FEP 6.4 × 9.5 mm; Reichelt, Heidelberg, Germany) were used for connection of the enzyme reactor to the pervaporation module. The hose segment within the peristaltic pump was of type EPDM/PP 8.0 × 11.1 mm (Reichelt, Heidelberg, Germany). Oxygen transfer into the enzyme reactor was performed with an Acurel no. 6114 (Membrana, Wuppertal, Germany) membrane hose loop of 10 cm length installed 1 cm above the enzyme reactor floor. Scanning electron microscopy was performed using an XL40 electron microscope with a CDU Leap EDAX detector (Philips, Hamburg, Germany).

### Microbial strains and plasmids, cultivation, and preparation of cell lysates

For heterologous expression, *Escherichia coli* strain BL21 (DE3) (Novagen, Darmstadt, Germany) and for plasmid amplification *E. coli* strain DH5α (Invitrogen, Karlsruhe, Germany) were used. Glutathione *S*-transferase (GST)-tagged AtCCD1 was cloned and expressed as described by Schilling [17]. Expression cells were harvested by centrifugation at 5,200*g* for 12 min at 4 °C and stored as pellets at −20 °C until use. Before use, pelleted cells were thawed at 20 °C and resuspended in reaction buffer for disruption. For enzyme reactor experiments, *E. coli* cells were disrupted by five passages in a high-pressure homogenizer (Avestin Emulsiflex C3, Ottawa, Canada) at 100,000 kPa with the heat exchanger at 4 °C. After the last passage, 0.3 % (w/v) Na-cholate was added to improve AtCCD1 solubilization and the solution was shaken and stored on ice for 10 min before use. For small-scale experiments (<20 ml total volume), *E. coli* cells were disrupted by sonication (W-250 D; Branson, Danbury, USA) with a 5-mm tip at 30 W for a total time of 120 s with intervals of 0.5 s sonication and 0.5 s breaks.

### Enzymatic reactions

In the enzyme reactor, carotenoid cleavage reactions were conducted at 30 °C (all components pretempered), 150 rpm impeller speed, 1 bar internal pressure, and compressed-air flow rate through the membrane hose of 20 $cm^3$ $min^{-1}$. The exhaust air cooler was operated at 4 °C. Unless mentioned otherwise, reaction solutions were composed of 320 ml enzyme-containing cell lysate and 80 ml micelle-based $\beta$-carotene solution. The progress of the reaction was determined by measuring the decrease in substrate concentration and the increase in the concentration of the product $\beta$-ionone. Controls for degradation of the substrate by native *E. coli* enzymes or by autoxidation were performed by conducting experiments with lysate of empty vector-expressing cells at the same total protein concentration. Negative controls were subtracted from experimental data to yield the results presented in the figures.

### Organophilic pervaporation

Apart from the membrane screening, all pervaporation experiments were performed with POMS/PEI membrane (HZG, Geesthacht, Germany). Permeate-side pressure was 0.2 mbar. Prior to experiments, preincubation of the membrane was performed for 60 min with 30 °C distilled water. Cooling traps were dipped into and filled with liquid nitrogen and were exchanged during the experiments by closing of the respective valves and re-establishment of the vacuum. The feed rate through the pervaporation module was 73 l/h in bioreactor experiments and 157 l/h in membrane screening experiments, ensuring turbulent flow conditions in both cases. The determination of permeate mass and the components and concentrations of permeate and retentate was performed as published [5] by periodical exchange of the freeze traps while the respective model or reaction solution underwent continuous pervaporation. For membrane screening, a model solution consisting of 9.6 mg/l $\beta$-ionone and 10 % (v/v) ethanol in 20 mM Tris pH 8.5 was used.

### Analytical methods

For enzyme reactor experiments, 1-ml samples of the reaction solution were taken via a syringe using a septum in the fermenter bottom. Sample preparation and extraction was performed in three steps. First, the 1-ml sample was mixed with 1 ml high-performance liquid chromatography (HPLC)-grade ethanol to stop the reaction and precipitate dissolved protein. After centrifugation, at 18,100*g* and 4 °C for 2 min, the supernatant was pipetted off and analyzed directly by HPLC. The pellet was extracted twice using 500 µl HPLC-grade acetone at 50 °C and 1,500 rpm for 5 min. Extracted pellets were recentrifuged at 18,100*g* and 4 °C for 2 min, and the acetone supernatants of each sample pooled for HPLC analysis.

Carotenoid HPLC analytics were performed using an Alltech Prevail C18 10 µm 250 × 4.6 mm column and a Shimadzu SPD-M10A diode array detector at 490 nm. Mobile phase A was 99:1 (v/v) methanol:water, and mobile phase B was tetrahydrofuran. The total flow rate was 1 ml/min with a pump program of 0–10.5 min 0 % B to 47.25 % B, 10.5–10.6 min 47.25 % B to 0 % B, and 10.6–21.5 min 0 % B. $\beta$-Carotene and $\beta$-ionone were quantified in a single measurement at 490 and 220 nm, respectively. All measurements were performed in triplicate and using external standards.

Determination of ethanol concentration in pervaporation permeate was performed by HPLC using the same column and detector as described above at 220 nm. An isocratic program was run with the mobile phase being 3 mmol/l $H_2SO_4$ at 0.6 ml/min.

Gas chromatography (GC)-flame ionization detector (FID) samples were prepared by mixing 900 µl pervaporation permeate sample with 100 µl internal standard solution (100 mg/l camphor in ethanol) and drying with $Na_2SO_4$. Analyses were performed using a 30 m 0.25 mm DB Wax ETR column (J&W Scientific, Folsom, USA) and a Shimadzu flame ionization detector.

GC–mass spectrometry (MS) samples were prepared by extraction with 1/5 of the sample volume in chloroform, centrifugation, and drying of the separated chloroform phase with $Na_2SO_4$. Analyses were performed at 3 µl injection volume using a 30 m 0.25 mm VB-5 column (ValcoBond, Schenkon, Switzerland) at split ratio of 22:1 and with helium as mobile phase. The temperature program consisted of an isocratic gradient from 80 to 240 °C at 8 °C/min and a subsequent isothermal phase of 5 min. The mass scan range was 40–400 *m*/*z*, and the injector and interface temperatures were 200 and 270 °C, respectively.

Scanning electron microscopy samples were prepared by vacuum gold sputtering and analyzed on aluminum sample holders. To obtain sharp breaking edges, membrane samples were dipped into liquid nitrogen and subsequently broken on a steel metal edge before sputtering.

## Results and discussion

### Selection and characterization of a suitable organophilic pervaporation membrane

The most suitable membrane type for separation of $\beta$-ionone from aqueous fermentation broth had not yet been determined. Physical parameters such as partial fluxes and selectivity had to be determined because sufficient literature data were not available. Three organophilic pervaporation

membrane types were screened for separation performance regarding the biotransformation product β-ionone. All three membrane types were composite membranes with a layout as shown in Supplementary Fig. 1, comprising a polymer fleece bearing the mechanical load, a porous polyetherimide (PEI) supporting layer, and a nonporous active layer consisting of either polydimethylsiloxane (PDMS) (2420G/V-1) or polyoctylmethylsiloxane (2421G/V-2 and POMS/PEI). Since the thickness of the active layer is a core characteristic of a pervaporation membrane and has significant influence on diffusion, total flux, and selectivity, the active layer thicknesses of the tested membranes were determined by scanning electron microscopy (SEM). Membranes fabricated by GMT had a 2.5 μm active layer, whereas those fabricated by HZG had a 12 μm active layer. Membrane characterization was performed using the same pervaporation module as in the demonstration plant. Model solutions were used to provide reproducible experimental conditions while at the same time mimicking the composition of the reaction solution as closely as possible. The concentration of β-ionone in the model solution was that which would prevail at 50 % conversion of a typical substrate concentration of 50 μM β-carotene.

Depending on the substrate type, the conversion of micelle-delivered carotenoids to C13-norisoprenoids by CCD enzymes can be accelerated by addition of water-soluble organic cosolvents [17]. However, cosolvents also reduce the separation efficiency of organophilic pervaporation processes due to coupling effects [9, 21]. To evaluate the balance between the economically required cosolvent-based activation of the reaction and the deterioration of separation selectivity, a typical cosolvent concentration of 10 % (v/v) was accounted for in the membrane characterization experiments. Figure 1 shows the data on the partial flux densities of water and β-ionone as well as the total flux density. At identical feed concentrations, membrane type POMS/PEI permitted a significantly higher partial flux density of the product β-ionone than the membrane types 2420G/V-1 and 2421G/V-2.

The suitability of a membrane for process use is judged by setting the partial flux density of the target substance in relation to the partial flux density of the solvent—in this case water—and the total flux density [15]. The achievable enrichment factor for β-ionone is largely dependent on the solvent flux, since the solvent flux density at low product concentrations is constant and independent of the concentration of all other components [7]. Figure 1b shows the partial flux densities of water and ethanol as well as the total flux. As expected, the flux density of the solvent water was independent of the product feed concentration. Furthermore, the data show increased flux densities during the first minutes of pervaporation. This effect is due to incorporation of water into the membrane during preincubation as described by Schäfer and coworkers [16]. When the pervaporation vacuum is built up, the water content of the membrane reduces to steady-state values, releasing unusually high volumes of permeate in the first minutes of operation. The mean total flux densities of the three membranes tested varied considerably: 2420G/V-1 and 2421G/V-2 showed comparatively high total flux densities of 1,063.4 and 728.5 g $m^{-2}$ $h^{-1}$, while the average total flux density using the POMS/PEI membrane was 95.5 g $m^{-2}$ $h^{-1}$.

The enrichment factor $\beta$ represents the increase in concentration of a component in the pervaporation permeate compared with the feed mixture. It is determined by the slope of the permeate versus retentate concentration of the product in a system without ongoing product formation. As shown in Fig. 1c, membrane type POMS/PEI permitted a significantly higher enrichment factor for β-ionone of 770 than the membrane types 2420G/V-1 ($\beta = 19$) and 2421G/V-2 ($\beta = 43$). While the enrichment factors for the product β-ionone differ significantly between the tested membrane types, the enrichment factors for the cosolvent ethanol were all at a similar order of magnitude and—at values between 4.9 and 6.4—very low for an organic cosolvent (Fig. 1d).

The investigation of membranes of different active layer material and thickness led to the following conclusions regarding a suitable pervaporation membrane for β-ionone: Active layer thickness was directly correlated with total flux density. Since the formation rate of β-ionone by AtCCD1 is comparatively low, a more than 10-μm-thick active layer is advantageous because solvent and cosolvent flux are greatly reduced while ionone flux is still well above the formation rate. Use of a silane substituted with C8 aliphatic chains (POMS) for the active layer rendered the active layer very hydrophobic and reduced the total flux density by 31 % compared with an active layer consisting of methyl-substituted silanes (PDMS) but at the same time increased the enrichment factor of the organic component 2.3-fold. Among the membranes tested, membrane type POMS/PEI by HZG met these requirements the best.

## Laboratory-scale demonstration plant

To provide a proof of concept for the process concept described, a laboratory-scale demonstration plant was set up. The layout of the demonstration plant was chosen similar to that of a published plant for biocatalytic production of 2-phenlyethanol by whole-cell biotransformation [5]. The piping and instrumentation diagram of the demonstration plant is depicted in Fig. 2. To minimize product inhibition, the reaction solution was continuously circulated through the pervaporation unit for constant in situ product removal. A set of valves permitted changing the cooling traps without depletion of the vacuum, hence shortening changeover times.

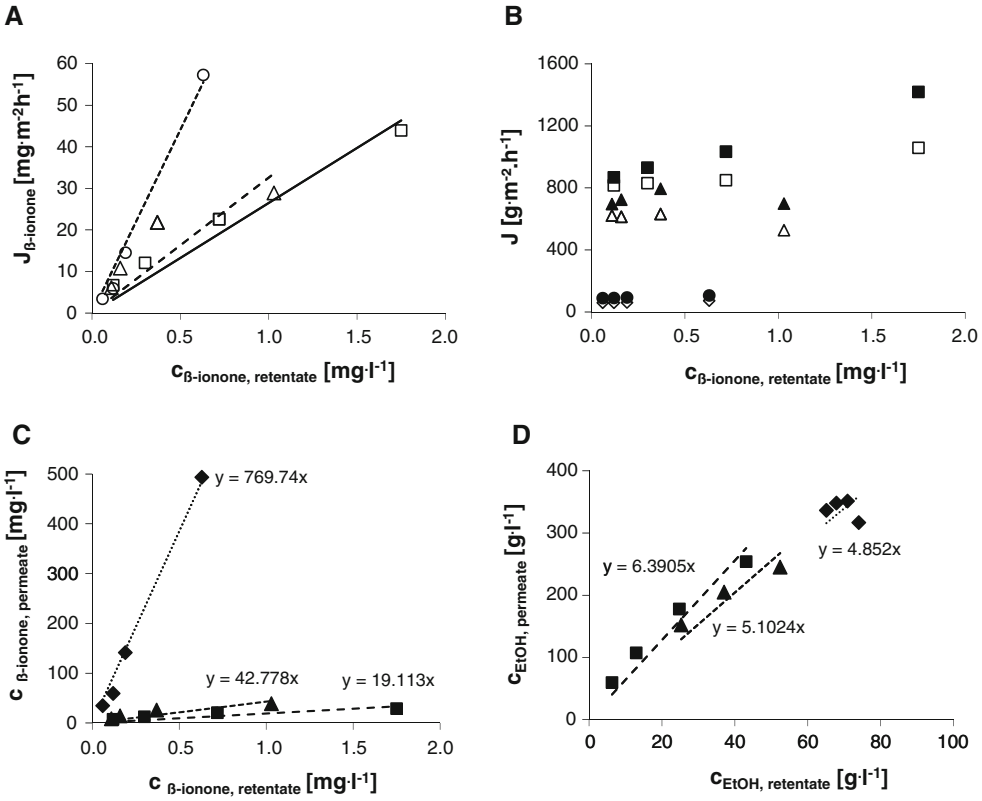


**Fig. 1** Partial flux density of *β*-ionone (**a**) and total flux density (**b**, *filled symbols*) or partial flux density of water (**b**, *empty symbols*). Concentrations of *β*-ionone (**c**) and ethanol (**d**) in permeate for different concentrations in retentate. Three pervaporation membrane types tested (2420G/V-1: *filled squares*, *open squares*; 2421G/V-2: *filled triangles*, *open triangles*; POMS/PEI: *filled circles*, *open circles*). Data obtained by periodical exchange of freeze traps while a model reaction solution was pervaporated continuously with the respective membrane

The reaction product *β*-ionone readily diffuses into many polymers, a property which is applied technically in the controlled release of this fragrance compound in laundry detergents or household cleansers [8, 11]. However, for biocatalytic production of *β*-ionone, this property represents a significant challenge. Since use of conventional hoses for pharmaceutical or chemical purposes led to significant product loss by diffusion into the polymer parts, screening of a large number of hose designs and materials needed to be conducted to determine the best suited set of materials for the process (data not shown). Product loss by incorporation could be minimized by avoidance of polymer parts in the demonstration plant and by use of double-walled hoses with a perfluoroethylenepropylene (FEP) core and a polyvinyl chloride (PVC) outer wall.

An AtCCD1 fusion with the solubility-enhancing tag glutathione *S*-transferase (GST) was used because Schilling and coworkers had reported a twofold increase in specific activity in the cell lysate when using this protein modification [17]. The cumulative amounts of substance of substrate and product show that the reaction could be conducted (Fig. 3a) and that the end point was not yet reached when the reaction was stopped after 11 h. More than 95 % of the product generated could be separated in situ from the reaction solution (Fig. 3b).

The symmetric 9,10/9′,10′ cleavage of *β*-carotene by AtCCD1 leads to two moles of *β*-ionone per mole of *β*-carotene. In Fig. 3, this ratio was not found as expected. In the demonstration plant, loss of *β*-ionone seemed to occur, which can probably be attributed to three factors: first, product loss through the aeration gas stream, which could be limited through use of exhaust coolers; second, loss by product diffusion into the separation membrane; and third, chemoenzymatic product degradation through components of the reaction solution. The pervaporation permeate was analyzed for side-products and impurities by HPLC and GC–MS. The GC–MS chromatogram in Fig. 4 shows that the process product was an almost pure aqueous solution of *β*-ionone. Organoleptically detrimental impurities such as indole from the lysed *E. coli* cells were not copervaporated. Apart from *β*-ionone, only two additional components were detected in the permeate extract: the isomerization product *α*-ionone, which is a desirable flavor and fragrance compound itself, and BHT, an easily avoidable stabilizer of the tetrahydrofuran used for substrate preparation.

## Oxygen supply by membrane tube insert

Cleavage of one mole of *β*-carotene by the dioxygenase AtCCD1 requires two moles of the cosubstrate oxygen [19]. To avoid oxygen limitation, a way of efficiently transferring oxygen into the reaction medium needed to be found. In bioreactors, oxygen transfer is typically accomplished by

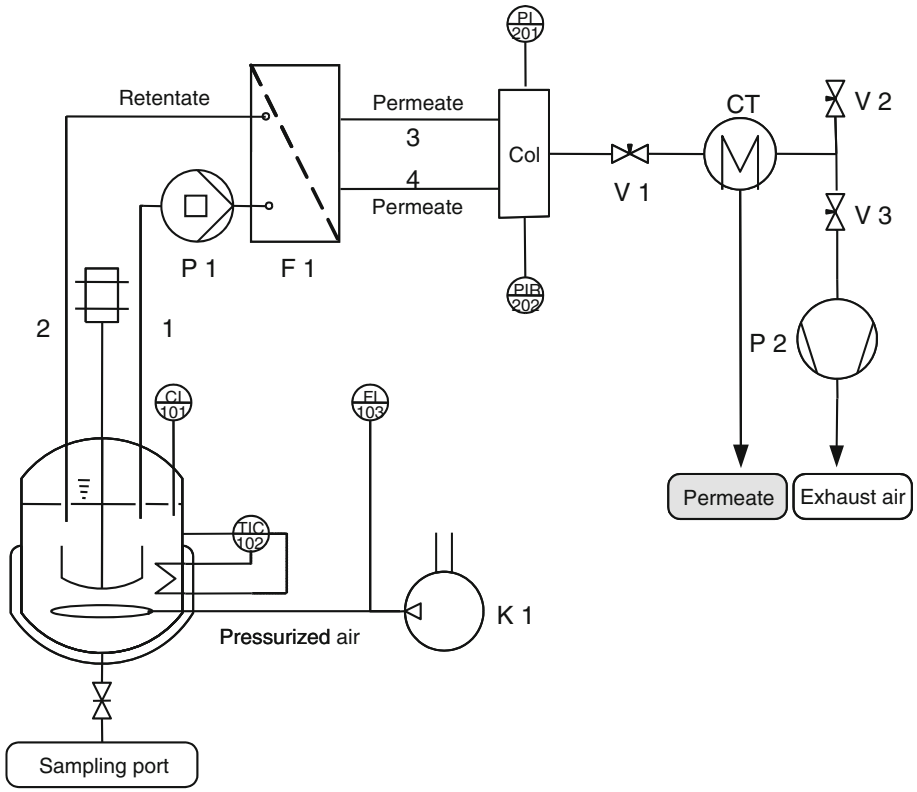


**Fig. 2** Piping and instrumentation diagram of the laboratory-scale demonstration plant. *P1* peristaltic pump, *P2* vacuum pump, *K1* air compressor, *Col* collector, *CT* cooling trap, *V1* rotary slide valve, *V2-3* needle valves, *CI 101* oxygen sensor, *TIC 102* temperature sensing and control unit, *FI 103* gas flow sensor, *PI 201* pressure sensor (high pressure range), *PIR202* pressure sensor (low pressure range), *1–2* double-walled PVC/FEP tubing, length 301 and 164 cm, respectively, *3–4* aluminum vacuum piping

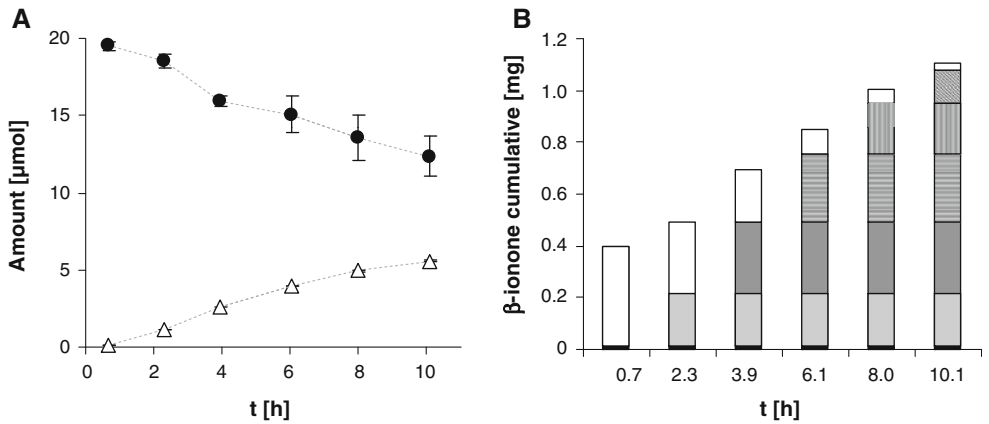


**Fig. 3** **a** Amount of substance of *β*-carotene (*filled circles*) and cumulative amount of substance of *β*-ionone (*filled triangles*) in conversion of micelle-delivered *β*-carotene by GST-AtCCD1 in a laboratory-scale demonstration plant with an active pervaporation unit. **b** Composition of cumulative amount of product over course of reaction: *white* reaction solution, *black* cooling trap (CT) 1, *light grey* CT 2, *dark grey* CT 3, *horizontally hatched* CT 4, *vertically hatched* CT 5, *diagonally hatched* CT 6

stirring and sometimes by additional sparging of compressed air or pure oxygen. In this investigation, the stirrer speed was limited to 150 rpm due to foaming caused by the surfactants required for substrate delivery and by the high concentration of protein in the reaction solution. Conventional ring spargers also led to extreme foaming at low gas flow rates and could not be used. To transfer sufficient amounts of oxygen into the reaction solution even at low flow rates, an aeration method with mean bubble diameter of less than 1 mm was required. This requirement could be met using a self-designed aeration insert with a gas-permeable membrane hose (Supplemental Fig. 2). The input of pressurized air into the medium through the membrane hose resulted in significant acceleration of the reaction by approximately 75 % (Fig. 5a). To avoid superposed effects on pervaporation and oxygen saturation, data were recorded without operating the pervaporation unit.

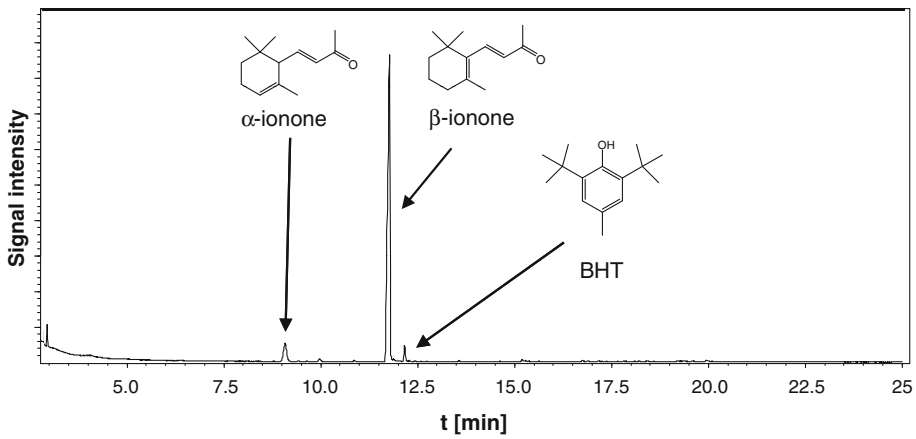


**Fig. 4** Conversion of $\beta$-carotene using GST-AtCCD1 in the demonstration plant. GC–MS total ion chromatogram of hexane extract of process product. Butylated hydroxytoluene (BHT) and $\beta$-ionone were identified using standards. α-Ionone was identified by comparison with a National Institute of Standards and Technology (NIST) MS fragmentation database

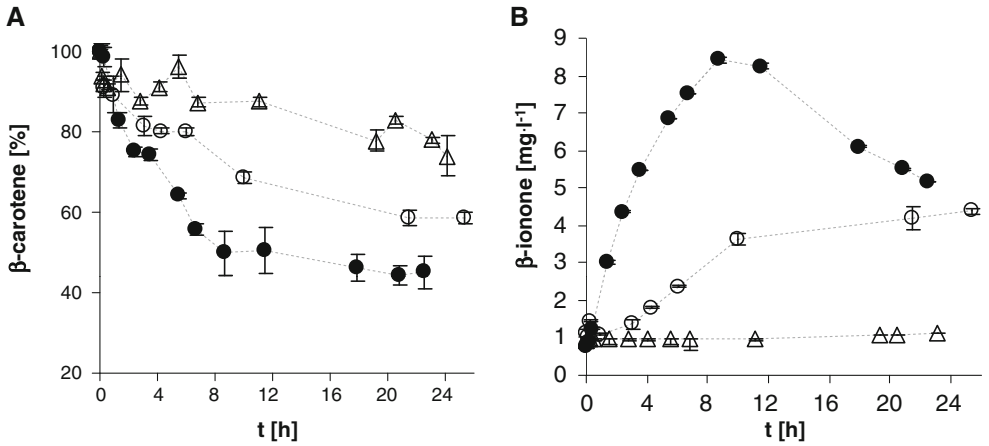


**Fig. 5** Effect of oxygen input on course of substrate (**a**) and product (**b**) concentration in reaction solution in conversion of micelle-delivered $\beta$-carotene using GST-AtCCD1 in a demonstration plant with the pervaporation unit inactive: without input of pressurized air via membrane hose (*open circles*), and with 20 $cm^3$ $min^{-1}$ input of pressurized air via membrane hose (*filled circles*). Control experiment with lysate of empty vector-transformed cells and 20 $cm^3$ $min^{-1}$ pressurized air flow (*open triangles*). Due to small differences in initial substrate concentration, results are displayed normalized in (**a**)

Besides a significantly increased reaction rate, the additional oxygen input also caused slightly accelerated carotenoid degradation, probably due to nonspecific autoxidation by dissolved reactive oxygen species. The acceleration of the enzymatic reaction by aeration is also represented by the faster increase in product concentration in the reaction solution (Fig. 5b). After 9 h reaction time, the concentration of $\beta$-ionone in the reaction solution began to decrease. The drop in product concentration was most likely due to a loss of the volatile product (vapor pressure of pure substance is 1.32 Pa at 298 K) through the aeration gas stream in the late phase of the reaction when the reaction velocity is lower than the stripping effect of the gas stream. The data in Fig. 5b are therefore overlaid with product loss by gas-phase stripping. This effect would however be significantly less pronounced had the pervaporation unit been active, resulting in a lower transient $\beta$-ionone concentration in the reaction solution. The oxygen saturation was measured during the reaction (data not shown), showing values between 80 and 90 %. Use of pressurized air is more economical, but it contains only 21 % oxygen. An even higher average oxygen saturation could be achieved by using pure oxygen for aeration. However, possible detrimental effects on substrate autoxidation remain to be investigated.

## Conclusions

A process concept for production and in situ separation of natural $\beta$-ionone from $\beta$-carotene using highly selective carotenoid cleavage dioxygenases (CCD) was described for the first time. In a laboratory-scale demonstration plant, micelle-mediated $\beta$-carotene was converted into an aqueous solution of $\beta$–ionone which contained as impurities only the isomerization product α-ionone and an experimentally

avoidable chemical stabilizer. Required downstream processing of the product is particularly low for a biocatalytic process product. Despite the high market prices achievable with natural flavor and fragrance compounds, the substrate conversion of 60 % requires further improvement by enhanced substrate delivery and enzyme stabilization. The CCD enzyme family has properties rendering it an ideal platform biocatalyst for oxidative cleavage: they accept a broad range of terpenoid C27 to C40 substrates and catalyze cleavage regioselectively at sites specific to the respective CCD subgroup [14]. In the future, we plan to design a modified demonstration plant adapted to more viscous media which will permit the combination of selective in situ product separation by organophilic pervaporation with the use of technical substrates such as plant or algal biomass with high carotenoid content. Some potential technical substrates such as carrot pomace from juice production are waste streams and would therefore be available at low cost and in sufficient amount, decreasing substrate expenses significantly. The rapid development in flavor and fragrance biotechnology has already led to the first biocatalytic production processes being more economical than chemical synthesis [2]. It is not unrealistic to assume that the biocatalytic process presented here can be developed into a platform technology for regioselective oxidative cleavage of carotenoids into volatile high-value products.

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