Journal of Photochemistry and Photobiology A: Chemistry 223 (2011) 14–24

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology A: Chemistry

journal homepage: www.elsevier.com/locate/jphotochem

The spectral and catalytic studies of chlorophylls and pheophytins in mimetic biotransformation of α-pinene

M. Trytek[a,*], E. Janik[b], W. Maksymiec[b], J. Fiedurek[a], A. Lipke[c], M. Majdan[c]

[a] Department of Industrial Microbiology, Institute of Microbiology and Biotechnology, Maria Curie-Skłodowska University, Akademicka St. 19, 20-033 Lublin, Poland
[b] Department of Plant Physiology, Institute of Biology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[c] Faculty of Chemistry, Maria Curie-Skłodowska University, M. Curie-Skłodowskiej Sq. 2, 20-031 Lublin, Poland

ARTICLE INFO

Article history:
Received 15 February 2011
Received in revised form 11 July 2011
Accepted 19 July 2011
Available online 29 July 2011

Keywords:
Biomimetic catalysis
Biotransformation
Pinene
Chlorophyll
Pheophytin
Photooxidation
UV–vis spectroscopy

ABSTRACT

Natural porphyrin pigments isolated from spinach in the form of a crude extract, chlorophyll a, chlorophyll b, pheophytin a and pheophytin b were studied as potential catalysts for the biomimetic transformation of α-pinene to trans-pinocarveol, pinocarvone and myrtenal. The reaction of monoterpene oxidation was performed in an organic solution under visible light irradiation. Significant changes in the distribution of the products were achieved using β-pinene as the substrate. The pheophytins showed the highest catalytic efficiency among the photocatalysts examined. The photographic visualization of α-pinene oxidation course at the beginning of the reaction, after 4 and 24 h exposure to light showed a distinct shift in the colour excited catalysts. This may indicate a change in their structure leading to their degradation. The absorbance and fluorescence spectra revealed that pheophytins are significantly stable photocatalysts in comparison to both chlorophylls. Pheophytin b displayed the highest stability in the biotransformation, whereas the highest rate of photodecomposition was proved for chlorophyll b under the reaction condition. Differences were noticed in the behaviour of the chlorophylls in the photodegradation process. From the changes in the intensity of the singlet oxygen band on infrared fluorescence spectra of pigments upon pinene addition, and from the reactivity experiments with radicals scavengers, it can be deduced that the biotransformation of pinene by chlorophylls and pheophytins proceeds by common oxygenation mechanism involving singlet oxygen and free radicals.

1. Introduction

Monoterpene hydrocarbons such as α-pinene, β-pinene and p-cymene are inexpensively available in large quantities. Oxygenated monoterpenes (terpenoids) are valuable compounds used in flavour and fragrance industries. Biotransformation of monoterpene hydrocarbons to oxygenated ones is of interest because it allows production of stereoselectively pure compounds under mild conditions and also the products may be considered as natural products [1]. Pinene exists in two forms, α-pinene and β-pinene. Both forms are present in many essential oils but are mostly obtained from turpentine (obtained by the dry distillation of wood or other dry plant material).

Microbial transformation of pinenes has been investigated since the early 1960s [2,3]. However, the traditional biocatalysts have several limitations in application to oxidative biotransformations, including stability and activities, especially to those substrates and products that are toxic and require being performed in reaction media other than water. Bioconversion yields of pinenes have usually been in the milligrams per liter range and thus rather low compared to other terpene biooxidations, possibly due to the more complex bicyclic structure of the pinenes [4].

For improvement of the activity and selectivity in the biocatalytic oxidation of monoterpenes, the active-site of heme monooxygenases has been engineered by designed mutagenesis [5,6]. An alternative approach is replacement of expensive naturally occurring biocatalysts or the reproduction of their catalytic properties by designing and preparation of active site analogues of heme monooxygenases. It is known that a crucial role in most transition metal-enzymes is played by the porphyrin prosthetic groups (e.g., protoporphyrin IX). Therefore, substantial efforts have been devoted to making use of metalloporphyrins in biomimetic catalysis to engineer an artificial catalytic system resembling the function and activity of monooxygenases like cytochromes P-450, peroxidases, and catalase to catalyze important and difficult transformations such as regioselective hydroxylation of unactivated C–H bonds [7–11]. The presence of the porphyrins as the active site of chlorophylls and pheophytins makes them enticing targets for biocatalysis.

Several interesting examples of the activity of porphyrins and metalloporphyrins as biomimetic catalysts in oxidation of monoterpenes have been reported [12–15]. However, to our

* Corresponding author. Tel.: +48 81 537 5958; fax: +48 81 537 5959.
E-mail address: mtrytek1@o2.pl (M. Trytek).

1010-6030/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotochem.2011.07.012

knowledge, only one paper deals with the utilization for this purpose of natural chlorophyll in the form of a crude leaf extract. The main disadvantage of this process is the requirements for the use of hazardous reactants and UV-light, and the final product is in the form of trans-pinocarvyl hydroperoxide, while pinocarveol rather than the hydroperoxide is the desired product [16].

Our work is aimed at investigation of plant pigments (chlorophyll a, chlorophyll b, pheophytin a and pheophytin b, and in the form of a crude extract), all isolated from spinach, as novel catalysts for biotransformation of α-pinene to pinocarveol, pinocarvone and myrtenal under mild conditions.

2. Materials and methods

2.1. Chemicals

(1S)-(−)-α-Pinene (98%), (1S)-(−)-β-pinene (99%), (−)-trans-pinocarveol (96%), (−)-myrtenol (98.5%), and petroleum ether were purchased from Sigma–Aldrich, USA. 2,6-Di-tert-butylphenol (BHT), synthetic vitamin E (Trolox), and tert-butyl hydroperoxide (tert-BuOOH) were stored at 5 °C. All reagents and solvents were of analytical grade.

2.2. Chlorophyll a and chlorophyll b isolation

Fresh spinach leaves were homogenized with acetone (1:4 (w:v)) and filtered through Miracloth. Next, the acetone extract was mixed with distilled water (5:1 (v:v)) and petroleum ether (2:1 (v:v)). The ether layer was concentrated by evaporation under a nitrogen stream and chromatographed on HPTLC Kieselgel 60 plates (Merck, Germany) using the mobile phase of petroleum ether:isopropanol:water (100:10:0.25 (v:v:v)). Chlorophylls dissolved in 100% acetone were identified on the basis of spectral properties.

The content of chlorophyll in the acetone extracts of fresh spinach leaves was determined using a spectrophotometric method [17]. The ratio of chlorophyll a to b and pheophytin a to b was 1.8 and 3.5, respectively.

2.3. Conversion of chlorophylls to pheophytins

The acetone extract was obtained as described above. Next, the extract was evaporated to a small volume and supplemented with hexane to 50 ml and shaken for 1 min with an equal volume of 9.2% HCl. The hexane layer was washed with 50 ml water three times. After evaporation of hexane from the sample, the extract was supplemented with petroleum ether to 3 ml and chromatographed using the HPTLC method. Pheophytin a and pheophytin b dissolved in 80% acetone were identified on the basis of absorption spectra and calculated according to Yang et al. [18].

The purity of chlorophylls and pheophytins was controlled by means of absorbance spectroscopy according to the literature data [19,20], which allowed exclusion of pigment protonation or metalation. The pigment concentrations used to record the spectra were 8.3×10^{-3} g/l, 4.8×10^{-3} g/l, 7.3×10^{-3} g/l and 3.8×10^{-3} g/l for chlorophyll a, chlorophyll b, pheophytin a and pheophytin b, respectively.

2.4. Spectral measurements

UV–vis measurements were carried out using a Shimadzu UV-160 A (Japan) spectrophotometer with 1 cm Hellma quartz cells. Spectra were recorded between 300 and 700 nm at a temperature of 21 ± 1 °C.

The emission and excitation spectra of chlorophylls and pheophytins in chloroform solutions were recorded on a FP-6300 spectrofluorometer (JASCO) in the 400–750 nm region at room temperature (21± °C). The spectra of solutions with reagent concentration of 10^{-5} mol × dm^{-3} were measured using a 1 cm quartz cell. The slit of the spectrofluorometer was adjusted automatically. The absorption, emission and excitation spectra were recorded digitally and the SigmaPlot (Jandel Corp.) program was used in manipulation and plotting the data. No filters were used, as the FP-6300 apparatus had sufficiently high resolutions and the xenon lamp "lines" did not interfere with our spectra.

The singlet oxygen luminescence spectrum was registered by using Photon Technology International Inc. spectrofluorometer equipped with an infrared module (NIR PMT Module with InP/InGaAs photocatode material, Hamamatsu Photonics K.K.) operating at spectral response range: 950–1700 nm.

The products of biotransformation exogenously added to the spectral controls, were prepared from the post-reaction solution by the isolation of one fraction on a short silica gel column eluting with a methyl chloride/hexane mixture. The products, trans-pinocarveol, pinocarvone and myrtenal/myrtenol were added as a stock in chloroform to the chloroform containing the pigments (2 mM) to a final concentration of 0.5, 1 and 0.3 g/L, respectively.

All pigments for spectroscopic measurements were previously dissolved to the final concentration of 5×10^{-4} M.

2.5. Photooxidation procedure

Biotransformation experiments were carried out under controlled temperature (20 °C), using an incubator consisting of four fluorescent visible lamps (Osram Lumilux, Cool White) regularly placed above the reaction vessel. The catalytic oxidation reaction was performed at atmospheric pressure, under oxygen dissolved in the system in closed 12-ml glass vials. 150 μmol of α-pinene was dissolved in 3 cm^3 of chloroform or 1,4-dioxane containing an isolated crude extract, chlorophyll or pheophytin (in their respective concentration), and irradiated for 24 h under estimated intensity of light ranging from 120 to 150 μmol/m^2 s.

After the specified time of photooxidation, 250 μl of the reaction medium were taken, diluted rapidly with a 5-fold volume of an internal standard solution and used for GC-FID and GC–MS analyses conducted according to the method reported previously [14]. Quantification of volatile compounds was done by comparison with the added standard. 0.05% (v/v) n-decane (for substrate) and linalool (for oxidation products) in hexane were used as an internal standard for gas chromatography. The substances were identified by comparison of their mass spectra and retention indexes (RI) with those of authentic sample spectra in a standard library database system (NIST 2004 and MassFinder ver. 3).

Reference chemical blank experiments were performed in the same way, but in darkness, to exclude the possibility of any non-photochemical transformation reactions during the specified period of time.

Control experiments were carried out in the presence of 1, 2 and 0.3 equivalent of tert-BuOOH, BHT and Trolox, respectively.

Biotransformations were performed in two replicate samples and the analyses were carried out in duplicate. The error associated with the GC quantification of the samples was ±5% and is quoted for a confidence interval of 95%. The data presented are reported as the average values.

3. Results and discussion

The hydrocarbon α-pinene is an attractive precursor substrate for bioconversion into valuable natural flavour and fragrance compounds (such as verbenone, pinocarvone and myrtenol), because of its widespread occurrence and low price. Pinenes (α- and

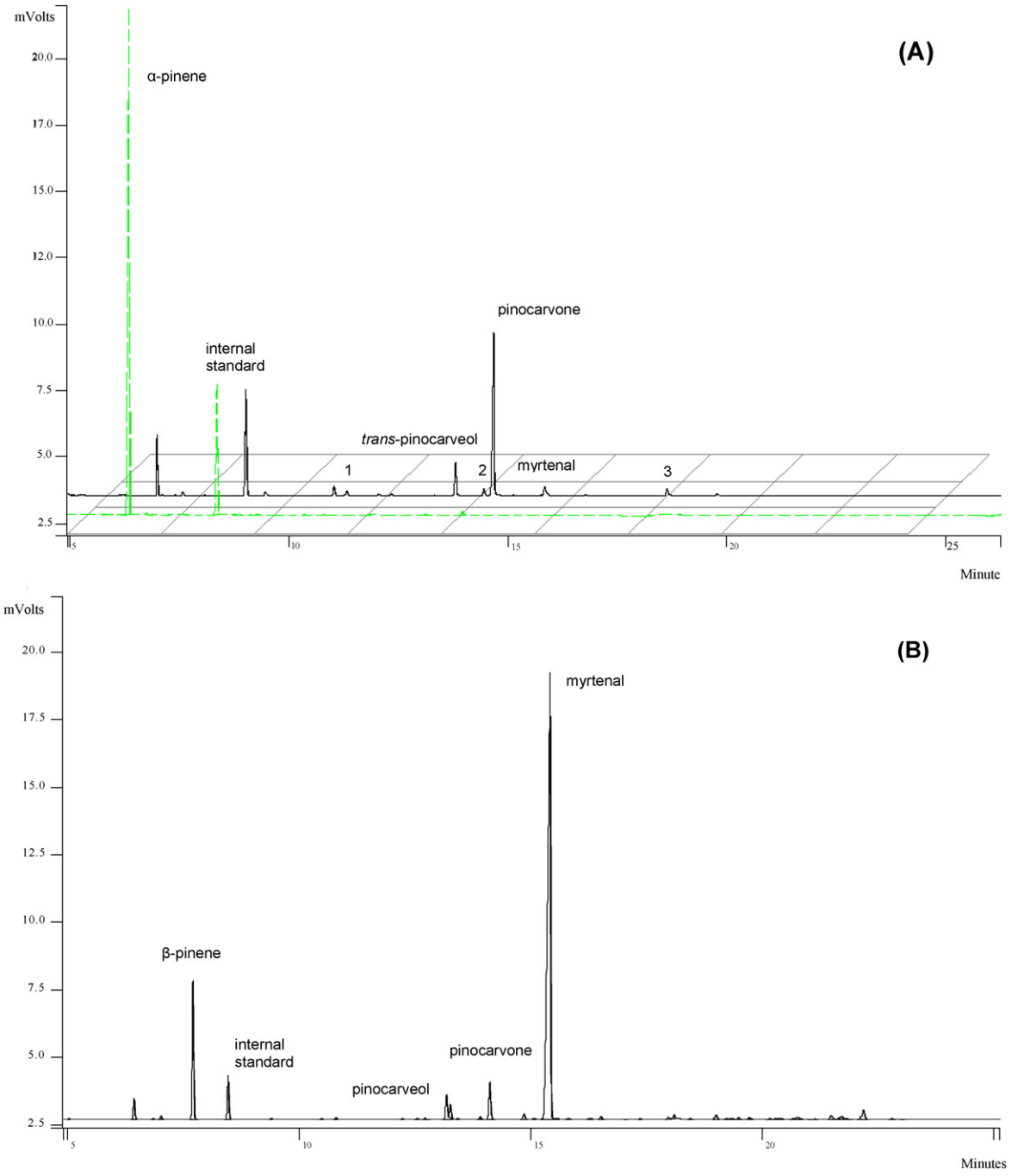

Fig. 1. The example model chromatograms of the products of (A) α-pinene and (B) β-pinene photocatalytical biotransformation with chlorophylls and pheophytins: upon exposure to light. (Solid line); without light (dashed line); 1, 2 and 3 – unidentified products.

β-) are major components of turpentine, a by-product of the pulp making industry. One of the approaches in controlled and selective biotransformation of monoterpenes (under mild conditions) is the use of porphyrins as biomimetic catalysts. It would help overcome the problem of sensitivity of traditional biocatalysts to toxic terpenes [8,14,15]. Therefore, elaboration of porphyrin macrocycle-containing catalysts, e.g., chlorophylls, imitating enzymes is desirable.

During our initial studies, we examined the ability of natural tetrapyrrole pigments in the form of a crude extract, chlorophyll a, chlorophyll b, pheophytin a and pheophytin b – all isolated from spinach – to biotransform α-pinene. The analysis with the use of GC and GC/MS techniques showed that α-pinene formed three main products: trans-pinocarveol, pinocarvone and myrtenal. Myrtenol and all three unidentified products noticeable on GC traces (Fig. 1A) were obtained only in trace amounts. By contrast, the biotransformation of another isomer β-pinene favors myrtenal as the major product, whereas myrtenol, trans-pinocarveol and pinocarvone were obtained in substantially lower amounts (Fig. 1B), owing to the shift of the double bond in the pinene molecule. Addition of tert-BuOOH to the photocatalytic systems with pheophytins and chlorophylls had little, if any, effect on the rate or course of the reaction. Similarly, the reaction performed in the darkness in the presence of tert-BuOOH as an oxidant, yielded no products.

Among the chlorophyll preparations examined for α-pinene biotransformation, the highest accumulation of oxidative

Table 1
The photocatalytic properties of chlorophyll-derived pigments involved in α-pinene biotransformation.

Photocatalyst	Trans-pinocarveol		Pinocarvone		Myrtenal		Remaining substrate
	C [g/l]	Turnover number[g]	*C* [g/l]	Turnover number	*C* [g/l]	Turnover number	*C* [g/l]
Crude extract[a,g]	0.94	(70)	2.55	(190)	1.0	(74)	0.01
Crude extact[b,g]	0.67	(94)	2.32	(330)	0.17	(24)	2.07
Chlorophyll a[c]	0.49	24.2 (1648)	1.96	96.3 (6550)	0.17	8.5 (578)	7.13
Chlorophyll b[d]	0.30	24.8 (1664)	0.99	82.4 (5520)	0.23	18.7 (1254)	6.24
Pheophytin a[e]	0.51	36.7 (2563)	1.25	89.7 (6260)	0.59	42.6 (2970)	0.02
Pheophytin b[f]	0.58	105.4 (7240)	1.39	253 (17,394)	0.62	113.5 (7798)	0.004
Chlorophyll a[h]	0.40	78.1	1.04	207.1	0.27	53.2	2.47
Chlorophyll a + BHT	0.26	62.6	0.45	107.2	0.02	5.4	4.52
Pheophytin a[h]	0.57	113.0	1.51	302.0	0.40	79.2	1.6
Pheophytin a + Trolox	0.71	139.8	1.14	228.3	0.23	47.6	2.1
Pheophytin a + BHT	0.21	40.9	0.33	66.8	0.008	1.7	7.95

[a] Crude extract from spinach, weight of the catalyst used – 13.5 mg, solvent – chloroform.
[b] Crude extract from spinach, weight of the catalyst used – 7.1 mg, solvent – 1,4-dioxane.
[c] Chlorophyll a, weight of the catalyst used – 0.3 mg, solvent – chloroform.
[d] Chlorophyll b, weight of the catalyst used – 0.18 mg, solvent – chloroform.
[e] Pheophytin a, weight of the catalyst used – 0.2 mg, solvent – chloroform.
[f] Pheophytin b, weight of the catalyst used – 0.08 mg, solvent – chloroform.
[g] Turnover number: the moles of products per mole of pigments used in biotransformation; in the parenthesis: effectiveness was calculated as the concentration of the product per gram of the catalyst used in the reaction.
[h] Amount of the catalyst used – 0.1 μmoles, biotransformation time – 20 h, solvent – chloroform.

derivatives were obtained using extract containing a mixture of dyes (extract of spinach) as a photocatalyst, due to its larger number of molecules used in the reaction compared to the purified dyes (Table 1). A distinctly different ratio of myrtenal/pinocarvone in two solvents: chloroform and 1,4-dioxane was observed using spinach extract, but the reason for this phenomenon is hard to explain. However, the highest catalytic efficiency expressed as turnover number (here defined as the moles of products per mole of pigments used in biotransformation) was displayed by pheophytin b. No oxidative products were detected in the absence of light. This confirmed the photochemical character of the biotransformation reactions involving the chlorophyll-derived catalysts in the organic media. Similar products composition of pinenes was found under identical conditions with chlorophylls as well as pheophytins indicating a common oxidation mechanism which is largely independent of the used pigments. These products are expected to result from the singlet oxygen ($^1O_2$) ene addition to terpenoic olefins, although in the case of both pinenes, non-ene products were also formed. As has been postulated, the common mechanism of the photooxidation is rather complex, and determined by the concurrent involvement of the reactive oxygen species: singlet oxygen, superoxide radical anions ($O^{2\cdot-}$), and hydroxyl radicals [21,22]. To gain a better insight into the nature of photocatalytic biotransformation, the infrared fluorescence spectra of the pigments in chloroform with and without pinene addition were registered. The ability to the $^1O_2$ generation by pheophytin and chlorophyll in chloroform was directly demonstrated by spectroscopic monitoring of luminescence at 1274 nm (Fig. 2). Moreover, we noted that upon pinene presence in the photocatalytic system, the intensity of fluorescence band of $^1O_2$ significantly decreased in relation to the pheophytin a in chloroform without pinene, showing unambiguous evidence of singlet oxygen participating in the biotransformation process. On the other hand, addition of the free-radical inhibitor BHT (with 2 equiv related to pinene) retarded the photooxygenation seriously (Table 1), clearly indicating that a free radical oxidative process was also involved in the biotransformation reaction. The typical radical-initiated autooxidation should be ruled out because a broad oxygenated terpenes mixture is obtained via this route, e.g., pinene epoxides, diols, verbenol, verbenon, myrtenol, myrtenal, pinocarveol, and hydroperoxides [23,24]. Based on the above data we concluded that the photocatalytic biotransformation may involve two different reaction pathways: (i) direct oxygenation

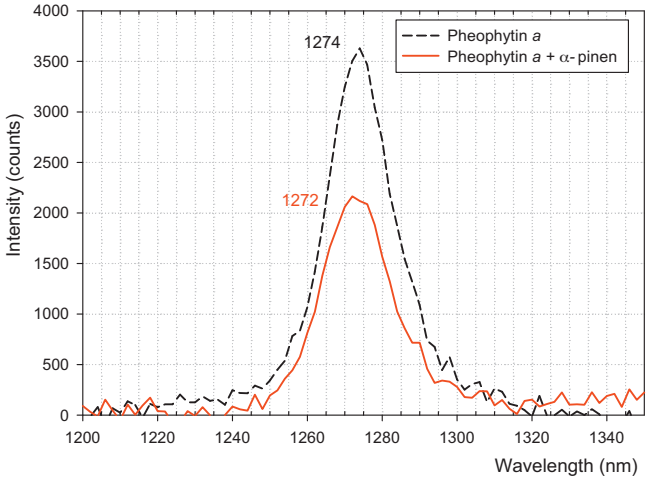

Fig. 2. Effect of α-pinene on the emission spectrum of $^1O_2$ generated in chloroform containing pheophytin a by light excitation at 410 nm.

of pinene with singlet oxygen generated by photocatalysts and (ii) abstraction of an electron from pinene by excited pigments followed by the generation and subsequent recombination of pinenyl and oxygen radical intermediates. It remains unclear how the pinocarvone is formed. So far it has been assumed that the ketone originates from either rapid decomposition of corresponding hydroperoxide or the abstraction of an allylic H atom from alkoxyl radical by O_2 [24,25]. Presumably, in our system, some free radicals participate in pinocarvone formation, since a substantial decrease in its fraction was noticed after quenching the radicals by BHT and Trolox (Table 1).

The summary of the biotransformation kinetics of pinene is provided in Fig. 3. The pinene biotransformation reaction seems to be of the first order type. The dependencies: $\log(c/c_0)$ (where c and c_0 are the pinene concentrations at time t and 0) have the character of a straight line with r^2 determination coefficients 0.98–0.99. One can conclude that the pinene biotransformation consists of the parallel reactions: pinene → trans-pinocarveol; pinene → pinocarvone; pinene → myrtenal.

Additional support for this idea, resulting directly from the theory of parallel reactions [26], is the presence of straight line correlations: $c_{\text{trans-pinocarveol}}$ vs $c_{\text{pinocarvone}}$ for all the studied

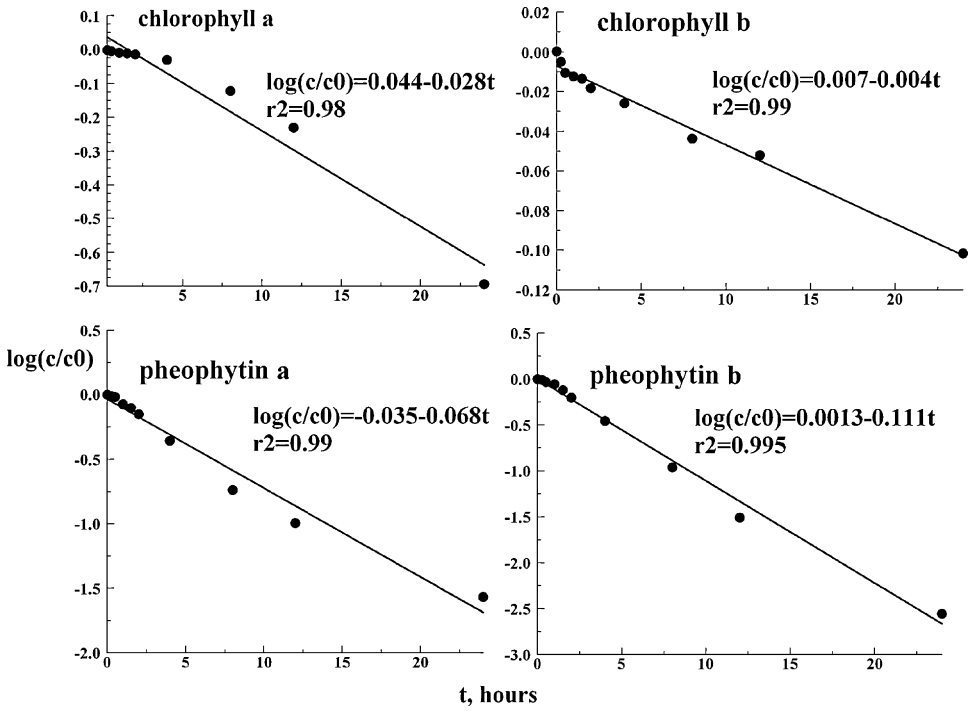

Fig. 3. The logarithmic change of the pinene concentration with time during the biotransformation of pinene by chlorophyll a; chlorophyll b; pheophytin a; pheophytin b.

systems, i.e., for chlorophylls and pheophytins. The pinene → myrtenal reaction case is somewhat complex. The lack of straight line correlations: $c_{\text{pinocarvone}}$ vs c_{myrtenal}, $c_{\text{trans-pinocarveol}}$ vs c_{myrtenal} together with the different course of pinene biotransformation in the case of elimination of radicals from the reaction medium, implies an important role of a radical intermediate, comparable with singlet oxygen, in pinene oxidation, especially, into the myrtenal. Indeed, as in the presence of BHT or Trolox in the reaction mixture, the myrtenal/pinocarvone concentration ratio has dropped from 0.26 as much as to approximately 0.02 ± 0.005, the distribution of the observed products upon addition of the radical scavengers provides further evidence for the reaction mechanism based on two (probably, cooperative) pathways.

We supported the suggestion of parallel reactions in an independent reactivity experiment, since neither pinocarvone nor myrtenal or other derivatives were detected under typical reaction conditions using trans-pinocarveol or myrtenol as the substrates. Interestingly, only one, unidentified compound arises in myrtenol photooxidation.

It is easy to notice from the kinetic data (Table 2) that for the systems with pheophytins almost 100% of pinene was consumed, whereas in the system with chlorophylls approximately 20% or 80% of it remained unchanged at the end of the experiment, which directly results from the higher catalytic activity of pheophytins in the biotransformation process. Compared to α-pinene, the biotransformation of β-pinene, for instance, by pheophytin a, proceeded with higher efficiency and selectivity, yielding 2.56 g/L myrtenal, 0.176 g/L pinocarvone and 0.098 g/L pinocarveol (with the total turnover number of 630.6 after 18 h), whereas in the case of α-pinene, 1.51, 0.57 and 0.4 g/L pinocarvone, pinocarveol and myrtenal, respectively, were obtained (with the total turnover number of 494 after 20 h). Generally the biotransformation of pinene is more pronounced for the systems with pheophytins, as the evidently higher rate constants, i.e., higher slopes of $\log(c/c_0)$ values, for this case can be noticed. A similar conclusion can be drawn on the basis of the total turnover number being the higher for the pheophytins than for chlorophylls after 24 h-photooxidation (Table 2). These differences in activity can be explained in terms of the lack of Mg in the core of the pheophytins structure resulting in the presence of free electrons, which can be excited to higher electronic states and participate in singlet oxygen formation during intersystem crossing.

In the case of pinocarveol and pinocarvone, for all pigments, the highest photooxidation rate was observed during the first 8–10 h of the reaction (Fig. 4). After that time, the reaction is evidently retarded, most likely due to accumulation of the products or/and the photodegradation of pigments. Appearance of myrtenal production with time, despite parallel destruction of the pigments, probably results from extension of the action of free radicals on pinene in the photocatalytic system.

The photographic visualization of the α-pinene oxidation course by using chlorophyll-derived pigments at the beginning of the reaction, after 4 and 24 h exposure to light is shown in Figs. 5–7. A distinct shift in the colour of the catalysts used may indicate a change in their structure leading to their degradation (last photo). Chlorophyll and a number of related compounds such as chlorophyllides, chlorophyllins, pheophytins, chlorins and bacteriochlorophylls have been examined as photosensitizers for many types of biological systems, including biomolecules, cells and multicellular organisms. Some of these compounds are now being examined as sensitizers for photochemotherapy [27]. However, chlorophylls obtained by extraction with an organic solvent from living leaves have seldom been used as photocatalysts because they are quite unstable under illumination. A photostable PEG-chlorophyllin (polyethylene glycol derivatives) conjugate, that has the photochemical function of hydrogen gas evolution and carbon dioxide fixation under visible light, was reported. PEG-chlorophyllin acquired a photostable nature and a high photosensitizing ability to produce superoxide anions by illumination with visible light, as compared to chlorophyllin. The

Table 2
Kinetic data of the α-pinene biotransformation using chlorophylls and pheophytins during 24-h irradiation with visible light.

Time of biotransformation (h)	Photocatalyst[a]	Total turnover number[b]	Pinene consumption (%)
8	Chlorophyll a	141.6	24.5
24		350.7	79.8
8	Chlorophyll b	118.2	9.6
24		130.2	20.9
8	Pheophytin a	348.3	81.8
24		495.3	97.1
8	Pheophytin b	384.2	89
24		394.5	99.7

[a] The concentration of the pigments used in the photocatalytic biotransformation was 3.3×10^{-5} M; pinene concentration: 35 mM.
[b] Sum of the turnover number for the main α-pinene oxidation products.

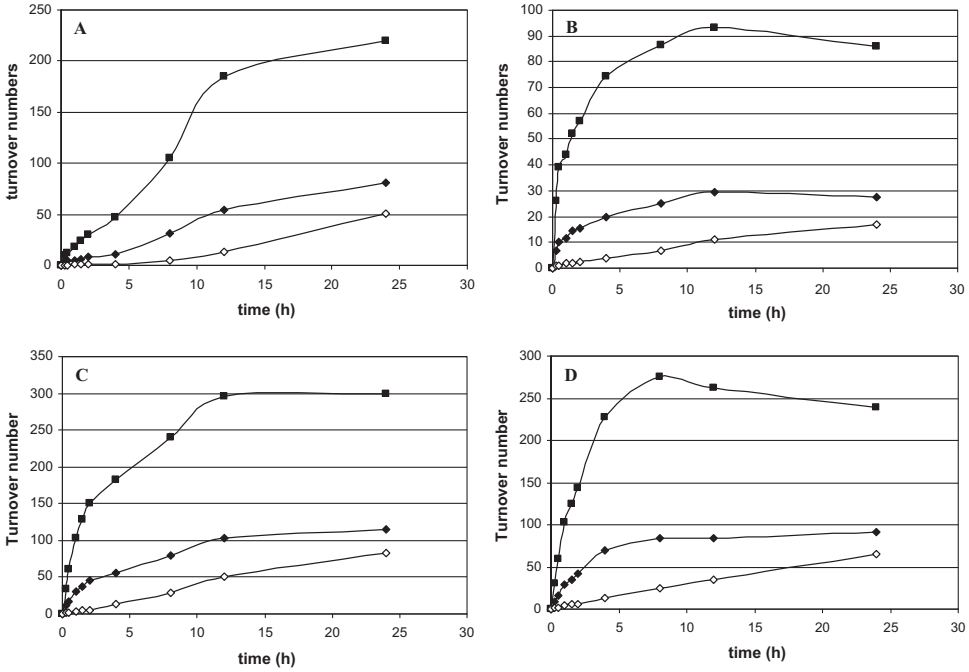

Fig. 4. Turnover numbers of porphyrin-containing pigments as a function of irradiation time of 3.3×10^{-5} M solutions of (A) chlorophyll a; (B) chlorophyll b; (C) pheophytin a; (D) pheophytin b in the process of pinene biotransformation; (black squares) pinocarvone; (black diamonds) trans-pinocarveol and (white diamonds) myrtenal.

photosensitizing ability may be closely associated with stabilization of chlorophyllin conjugated with PEG, although its mechanism is yet unclear [28,29].

In further studies, the stability and physicochemical properties of the catalysts during the biotransformation of pinene was investigated by absorption and emission spectroscopy. For a more detailed study of the low photostability of the natural chlorophyll a and chlorophyll b under photocatalytic reaction, their absorption and emission spectra in chloroform in the absence of pinene were additionally registered during 24-h visible light irradiation.

Chlorophylls and pheophytins are porphyrin pigments having characteristic absorption bands in "blue" (Soret-band) and "red" (*Q*-band) spectral regions. Two main maxima: B_x and B_y occur in the Soret band. Analogously, the *Q* band is characterized by two maxima named Q_x and Q_y. The position of these bands depends on the pigment solvent. According to Wellburn [30], the main absorption maxima of chlorophyll a and b in chloroform are located at 431, 665 and 460, 647 nm, respectively. Figs. 5A and 6A show the absorbance spectra of the chlorophyll a- and b-pinene system in chloroform at the beginning of the experiment and during illumination with visible light. A gradual decrease in the intensity of Soret and *Q* bands of both chlorophylls in chloroform was observed in the presence of α-pinene during illumination. Similar spectral changes were examined in the case of illumination of chlorophylls in chloroform without α-pinene (Figs. 5B and 6B). This effect could be caused by the negative influence of the light on the chlorophyll molecule, known as chlorophyll photobleaching, involving irreversible degradation of the pigments. It was reported previously that the excited chlorophyll molecule can be oxidized by light-induced, very reactive oxygen species, such as free radicals and/or singlet oxygen [31]. As can be seen in Fig. 5A, the chlorophyll a breakdown is associated with the reduction of light absorption in the B_x band vs to the B_y band. The "red"-shifted Q_y electronic region was observed concurrently. In our work, the photocatalyst degradation was stronger in the case of chlorophyll b and it was followed by formation of spectral forms absorbing in the 480–600 nm region (Fig. 6). It is worth noting that, in the chlorophyll b–pinene solution, its characteristic change in colour from green to red took place about 2 h earlier, compared to the system without pinene. This indicates that the presence of an electron donor (as most likely pinene is in the reaction with the electrophilic singlet oxygen) may accelerate the initial step of the chlorophyll b degradation.

The *B*/*Q* ratio (where *B* and *Q* are defined as the absorbance intensity of the Soret and *Q* region, respectively) increased during illumination for both chlorophylls (data not shown). This phenomenon suggests changes in the molecule structure connected with a faster decrease in absorbance in the "red" region-band under light irradiation. The light-induced breakdown of chlorophylls is

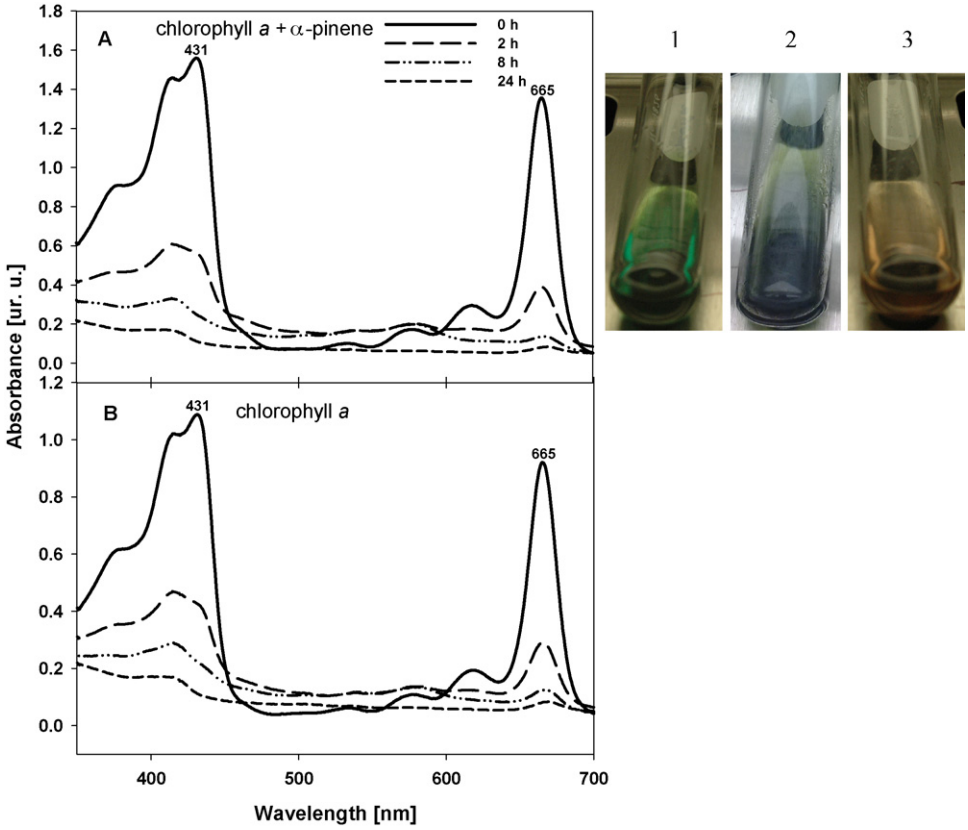

Fig. 5. UV–vis absorption spectra of the chlorophyll a – B, and the chlorophyll a–α-pinene system in chloroform during light-promoted biotransformation of α-pinene – A. Beside: the photographic visualization of pigment involved in α-pinene biotransformation (1) at the beginning of the reaction; (2) after 4 h of the reaction and (3) after 24 h of the reaction.

in all probability based on the destruction of the porphyrin ring in position C5 [32] and/or double bond of the phytyl chain of the pigment molecule, which finally leads to formation of numerous transient products. Previously, such an effect in a chlorophyll solution was observed by Jen and MacKiney [33], Hynninen [34] and Rontani et al. [35]. In our system, the more rapid photodestruction of chlorophyll b than chlorophyll a probably results from the presence of the –CHO group in the former pigment. On the basis of the absorbance spectra of pheophytin a and b, we can conclude that, under the biotransformation reaction condition, these metal free pigments are more stable forms of porphyrin photocatalysts in comparison to the chlorophylls (Fig. 7). The complete decomposition of pheophytins occurred not earlier than after 24 h of illumination. Also, they showed neither changes in the absorption spectrum shape during illumination, which suggests lack of a transient form of this pigment molecule during degradation, nor any signs of the metallation process of the central nitrogens or protonation of the macrocycle with formation of dication. In the event when the metal ion is complexed by a pyrrole ring or dications of pheophytins are formed, the position and shape of *Q* bands differs from that of free based pheophytins [36–41]. This makes them a new potential photocatalysts not only for the biotransformation of α-pinene but also for other electron rich hydrophobic compounds.

Chlorophylls and pheophytins are able to fluorescence in visible light. The fluorescence emission spectrum of chlorophyll a and b in chloroform has a maximum located at 672 and 651 nm, respectively (Fig. 8). In our experiment, a clear decrease in the intensity of fluorescence emission of chlorophyll a and b was observed along with the time of illumination, being more pronounced for chlorophyll b. This effect is connected with the shift of the chlorophylls emission bands and occurrence of an extra emission band in the 420–600 nm spectral region. The "short"-wavelength bands confirm pigment molecule photobleaching and appearance of chlorophyll degradation products [42]. In the chlorophyll a–pinene system with prolonged time of illumination, the "short"-wavelength band was "blue"-shifted, which suggests that different products of chlorophyll degradation are formed during the biotransformation reaction. Interestingly, the location of the "short"-wavelength band corresponding to degradation products was not changed in the case of chlorophyll b during illumination. However, in contrast to chlorophyll a the "red"-shift of the "long"-wavelength band (to 658 nm) was observed after 8 and 24 h illumination (Fig. 9). Therefore, we suggest that the photodecomposition process of these chlorophyll forms proceeds in two different ways.

The fluorescence emission intensity detected also indicates stronger chlorophyll b photodestruction under the biotransformation reaction, in comparison to chlorophyll a. The ratio of fluorescence intensity in the "red"-region to fluorescence intensity in the "blue"-region after 2 h illumination was 5.4 and 0.2 in the case of chlorophyll a and b (with pinene), respectively. As can be seen from the presented spectra, chlorophylls seem to be more stable in the system, which does not contain α-pinene. The above ratio calculated for chlorophylls in the system without α-pinene was higher than in the system containing α-pinene. The considerably more intensive fluorescence at the short-wavelength bands of the chlorophyll–pinene systems, as compared to the chlorophylls without pinene, can be explained by stopping the total decomposition of the chlorophylls probably through stabilization of the transient compounds by the terpenoid products of the photocatalysis. This is especially evident for chlorophyll a, which also demonstrated less decreased band intensity in the long-wavelength at

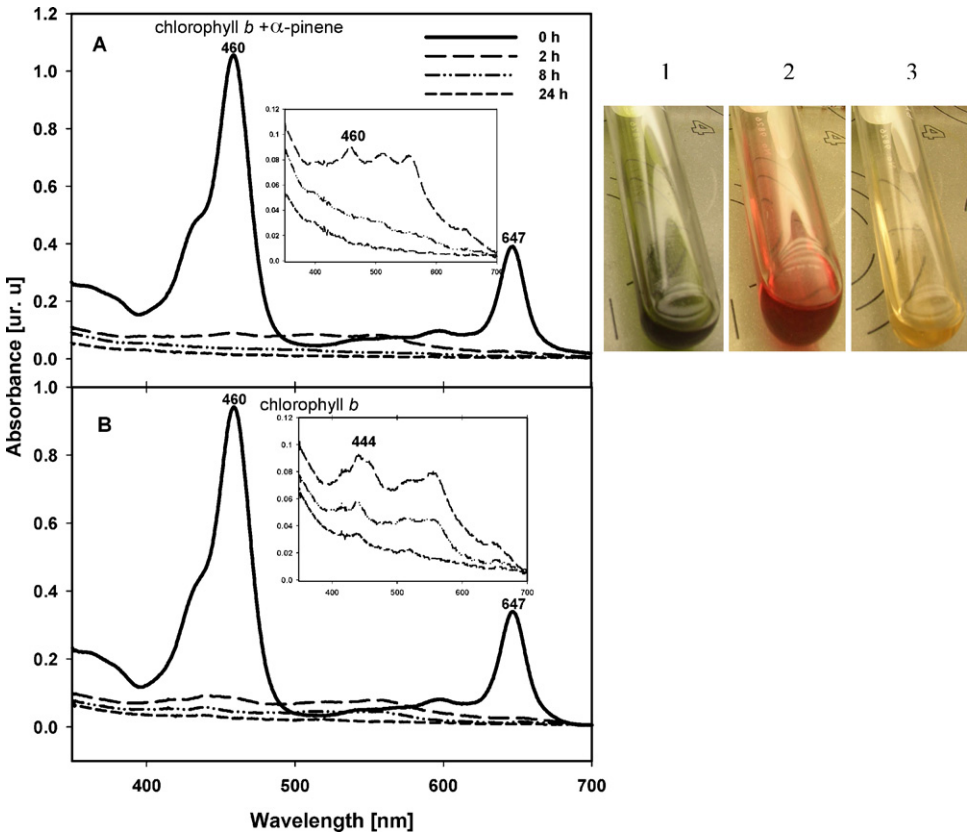

Fig. 6. UV–vis absorption spectra of the chlorophyll b – B, and the chlorophyll b–α-pinene system in chloroform during light-promoted biotransformation of α-pinene – A (inset – enlarged spectra). Beside: the photographic visualization of pigment involved in α-pinene biotransformation (1) at the beginning of the reaction; (2) after 4 h of the reaction and (3) after 24 h of the reaction.

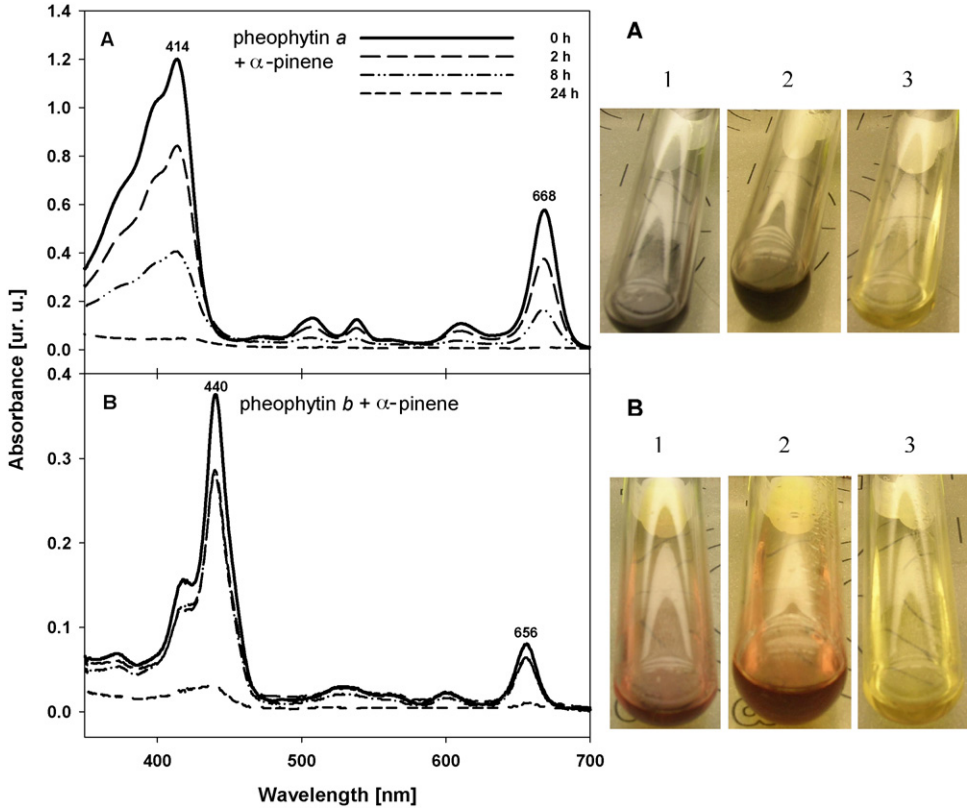

Fig. 7. UV–vis absorption spectra of the pheophytin a–α-pinene – A, and the pheophytin b–α-pinene – B system in chloroform during light-promoted biotransformation of α-pinene. Beside: The photographic visualization of pigments involved in α-pinene biotransformation (1) at the beginning of the reaction; (2) after 4 h of the reaction and (3) after 24 h of the reaction.

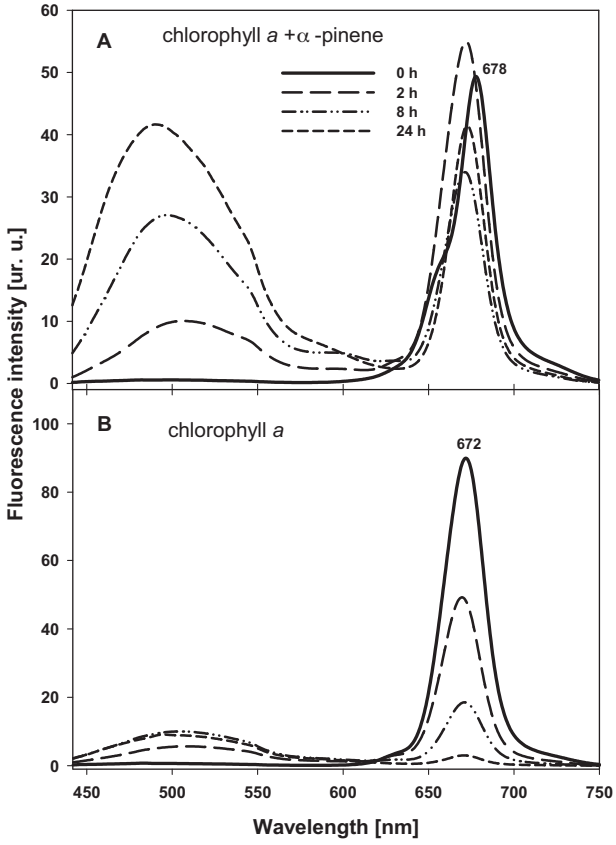

Fig. 8. Emission spectra of the chlorophyll a – B, and the chlorophyll a–α-pinene system in chloroform during light-promoted biotransformation of α-pinene – A. The excitation wavelength was at 460 nm.

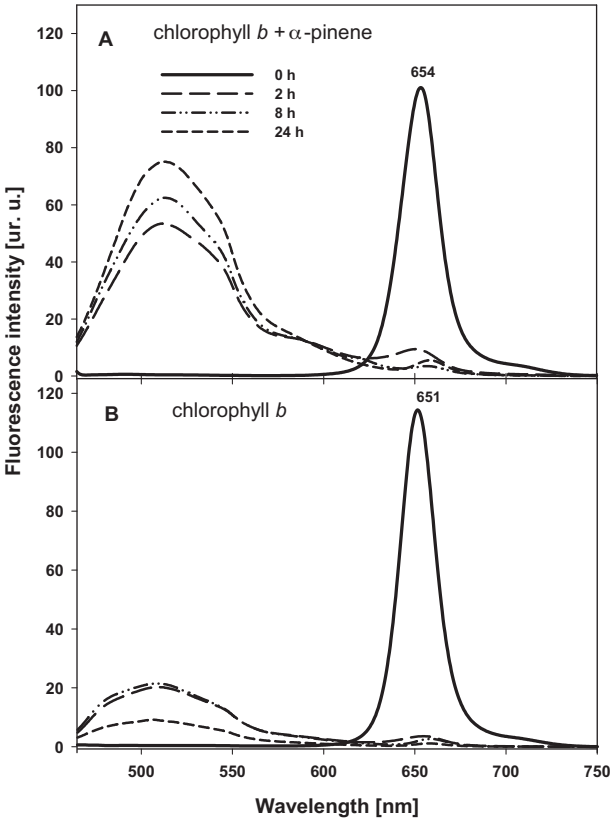

Fig. 9. Emission spectra of the chlorophyll b – B, and the chlorophyll b–α-pinene system in chloroform during light-promoted biotransformation of α-pinene – A. The excitation wavelength was at 460 nm.

the presence of monoterpenes. The thesis that products of the biotransformation reaction may stabilize the transient products of chlorophyll degradation under the irradiation is particularly affirmed by the excitation spectrum of chlorophyll b measured after 2 h of illumination in the presence of exogenously added products (trans-pinocarveol, pinocarvone and myrtenal). This spectrum has the same shape as that of chlorophyll b one measured without products in the dark (Fig. 10). Similarly, the protective role of the products was confirmed by the emission spectra of fluorescence of chlorophyll b in their presence (Fig. 11). The ratio of fluorescence intensity in the "red"-region to fluorescence intensity in the "blue"-region after 2 h illumination was 17-fold higher in the case of exogenous addition of the products than directly under the α-pinene biotransformation.

In such non-polar solvents as chloroform, chlorophyll at a concentration range of 2–3 mM appears merely in dimeric forms [43]. Probably due to the decreasing polarity, the α-pinene in the system with chlorophylls promotes enhancement of the pigment agglomeration. This was confirmed, firstly, by the red-shifted emission maximum at the beginning of the photoreaction (from 672 to 678 nm for chlorophyll a, and from 651 to 654 nm for chlorophyll b) and, secondly, by subsequent changes in the position of their bands (Figs. 8 and 9) during light excitation associated with biotransformation progress, in turn leading to a decrease in pinene concentration. The above results are confirmed also by the fluorescence excitation spectra measured for the chlorophyll b in chloroform or chlorophyll b with α-pinene in the presence of exogenously added oxidative derivatives in the dark (Fig. 12). In the Soret region, chlorophyll b has a band centered at 460 nm. Upon α-pinene addition, excitonic splitting of this Soret band into

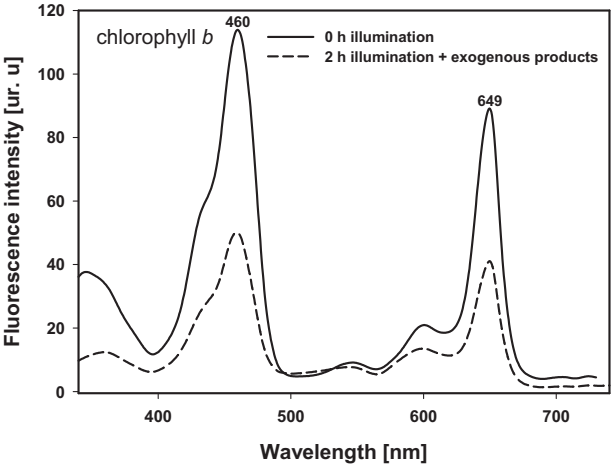

Fig. 10. Excitation spectra of the chlorophyll b (solid line) in chloroform and the chlorophyll b in chloroform with biotransformation reaction products (dashed line). The emission wavelength was at 650 nm.

two bands located at 435 and 443 nm, respectively, is observed indicating appearance of structurally aggregated pigment forms.

In contrast to chlorophylls, the fluorescence emission spectra of pheophytin a and b (Fig. 13) proved high stability of these pigments during the biotransformation reaction. A clear decrease in fluorescence intensity was observed only after 24 h illumination followed by slight "blue"-shift of the main fluorescence maximum. Interestingly, the destruction of pheophytin a after 24 h was accompanied by formation of an additional spectral form with band emission at 635 nm. The excitation, emission and absorbance spectra of pheo-

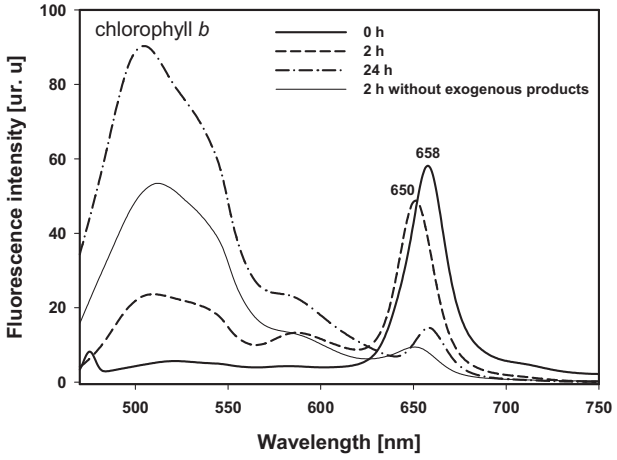

Fig. 11. Emission spectra of the chlorophyll b–α-pinene in the chloroform system with exogenously added products after 0, 2 and 24 h of illumination and the chlorophyll b–pinene system in chloroform light-promoted biotransformation of α-pinene after 2 h of illumination (thin line). The excitation wavelength was at 475 nm.

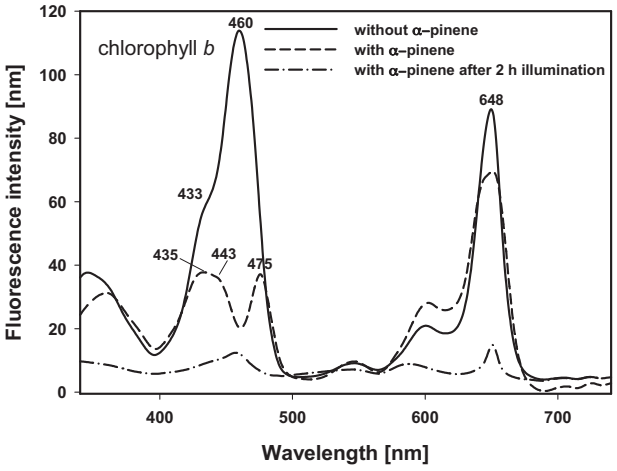

Fig. 12. Excitation spectra of the chlorophyll b (solid line) in chloroform and the chlorophyll b–α-pinene system in chloroform with exogenously added products (dashed line). The emission wavelength was at 650 nm.

phytins did not show a relationship between the destruction of pigments and presence of exogenously added products of the biotransformation reaction (data not shown).

There are many interesting products resulting from oxyfunctionalization of pinenes, due to many diverse degradation pathways. However, the drawbacks of the classical biotransformation systems, such as excess volatility of terpenes, insolubility in aqueous solutions, and their toxicity towards microorganisms result in low bioconversion yields, which have usually been in milligrams per liter range [4,44]. Two noteworthy exceptions were the α-pinene biotransformation with Hormonema sp. yielding 0.3 g/L verbenone and 0.4 g/L verbenol after 96 h [45], and production of pinene oxide, verbenol and myrtenol with total products of over 1 g/L using recombinant *Escherichia coli* in an aqueous-organic two-phase system [46]. In this context, the total product concentrations of over 2.5 g/L after 24 h of biotransformation presented in this work mark a very promising step towards further optimization of the process.

4. Conclusions

The significance of the data presented here lies in the fact that chlorophyll-derived pigments were the first time used as

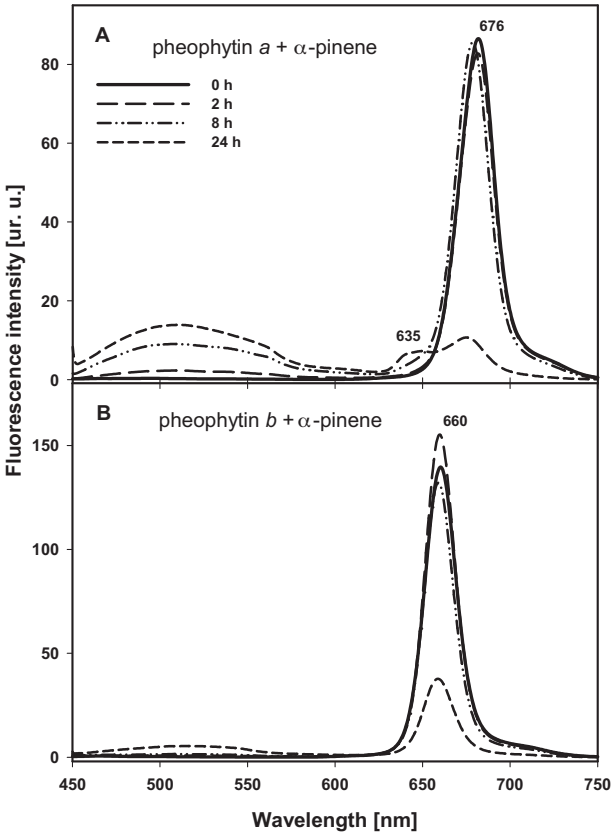

Fig. 13. Emission spectra of the pheophytin a–α-pinene – A, and the pheophytin b–α-pinene – B system in chloroform during light-promoted biotransformation of α-pinene. The excitation wavelengths were at 415 and 440 nm in the case of pheophytin a and b, respectively.

biomimetic catalysts for the photooxidation of monoterpene into products that attract great interest in the fragrance and flavour industry. Pheophytins have proved to be the most effective among the photocatalysts examined. A pheophytin-based photocatalytic system would be an appropriate, mild and clean method not only of transformation of α-pinene, but also other precursors widely distributed in nature. The spectral investigations have provided considerable information on the stability of the porphyrin pigments during the biotransformation of α-pinene. Based on the analysis of the absorbance and fluorescence emission spectra, we can state that under 24-h of light-promoted biotransformation, pheophytin a and b, the metal free pigments, are more stable photocatalysts, in comparison with chlorophylls.

Kinetic study confirms a weakening in the catalytic activity together with an increase in the photodecomposition rate of the pigments, however with the exception of myrtenal formation. According to our suggestion, this is due to the parallel type of mechanism of photocatalytic biotransformation, which could be exploited in the planning of the improvement of the reaction selectivity, i.e., changing the proportion of the radical to singlet oxygen pathway. For future work, the optimal pigment concentration should also be taken into consideration, since an increased concentration of the pigments results in a decrease in their activity. As follows from the above-presented results, the course of photodecomposition of chlorophyll a and chlorophyll b in the biotransformation process is evidently different. One of the reasonable solutions aimed at increasing the stability might be the use of heterogeneous catalysts through the immobilization of the pheophytins or chlorophylls in photostable and light-transparent solid supports (e.g., sol–gels materials), which would also be advan-

tageous with respect to easier recovery and recycling of these biocatalysts. In the case of chlorophyll b, binding of the –CHO group should be carried out, for example by employing it in the immobilization process. Further improvement of the catalytic activity of chlorophylls may be achieved by increasing the photostability by chemical modification of these catalysts with synthetic polymers such as polyethylene glycol derivatives or others.

References

[1] C.C. DeCarvalho, M.M. De Fonseca, Biotransformation of terpenes, Biotechnol. Adv. 24 (2006) 134–142.
[2] O.P. Shukla, P.K. Bhattacharyya, Microbiological transformations of terpenes: Part – XI pathways of degradation of α- & β-pinenes in a soil pseudomonad (PL-strain), Indian J. Biochem. 5 (1968) 92–101.
[3] P.W. Trudgill, Microbial metabolism and transformation of selected monoterpenes, in: C. Ratledge (Ed.), Biochemistry of Microbial Degradation, Kluwer, London, 1994, pp. 33–61.
[4] J. Schrader, Microbial flavour production, in: R.G. Berger (Ed.), Flavours and Fragrances Chemistry, Bioprocessing and Sustainability, Springer, Berlin/Heidelberg, 2007, pp. 509–573.
[5] S.G. Bell, R.J. Sowden, L.L. Wong, Engineering the heme monooxygenase cytochrome P450(cam) for monoterpene oxidation, Chem. Commun. 7 (2001) 635–636.
[6] S.G. Bell, X. Chen, R.J. Sowden, F. Xu, J.N. Williams, L.L. Wong, Z. Rao, Molecular recognition in (+)α-pinene oxidation by cytochrome P450, J. Am. Chem. Soc. 125 (2003) 705–714.
[7] D. Mansuy, Biomimetic catalysts for selective oxidation in organic chemistry, Pure Appl. Chem. 62 (1990) 741–746.
[8] B. Meunier, Metalloporphyrins as versatile catalysts for oxidation reactions and oxidative DNA cleavage, Chem. Rev. 92 (1992) 1411–1456.
[9] J. Groves, Reactivity and mechanism of metalloporphyrin-catalyzed oxidation, J. Porph. Phthal. 4 (2000) 350–352.
[10] T.J. McMurry, J.T. Groves, Metalloporphyrin models for cytochrome P-450, in: P.R. Ortiz de Montellano (Ed.), Cytochrome P450, Structure, Mechanism and Biochemistry, Plenum Press, New York/London, 1986, pp. 1–28.
[11] L. Que, W. Tolman, Biologically inspired oxidation catalysis, Nature 455 (2008) 333–340.
[12] V. Maraval, J. Ancel, B. Meunier, Manganese(III) porphyrin catalysts for the oxidation of terpene derivatives: a comparative study, J. Catal. 206 (2002) 349–357.
[13] C.C. Guo, W.J. Yang, Y.L. Mao, Selectivity aerobic oxidation of C=C and allylic C–H bonds in α-pinene over simple metaloporphyrins, J. Mol. Catal. A 226 (2005) 279–284.
[14] M. Trytek, J. Fiedurek, K. Polska, S. Radzki, Photoexcited porphyrin system as a biomimetic catalyst for D-limonene biotransformation, Catal. Lett. 105 (2005) 119–126.
[15] M. Trytek, J. Fiedurek, S. Radzki, A novel porphyrin-based photocatalytic system for terpenoids production from (R)-(+)-limonene, Biotechnol. Prog. 23 (2007) 131–137.
[16] R. Kenney, G. Fisher, Preparation of trans-pinocarveol and myrtenol, Ind. Eng. Chem. Prod. Res. Dev. 12 (1973) 317–319.
[17] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Methods Enzymol. 148 (1987) 349–382.
[18] C.M. Yang, K.W. Chang, M.H. Yin, H.M. Huang, Methods for the determination of chlorophylls and their derivatives, Taiwania 43 (1998) 116–122.
[19] L. Shuang, D. Feng-Qin, Y. Chun-Hong, T. Chong-Qin, K. Ting-Yun, Reconstitution of photosystem II reaction center with Cu-chlorophyll a, J. Integr. Plant Biol. 48 (2006) 1330–1337.
[20] P.H. Hynninen, Protonation–deprotonation equilibria in tetrapyrroles. Part 1. Protonation titrations of 13-(demethoxycarbonyl)pheophytin a in methanolic hydrochloric acid by electronic absorption spectra, J. Chem. Soc. Perkin Trans. 2 (1999) 669–678.
[21] J. Chacon, J. McLearie, R. Sinclair, Singlet oxygen yields and radical contributions in the dye-sensitised photo-oxidation in methanol of esters of polyunsaturated fatty acids (oleic, linoleic, linolenic and arachidonic), Photochem. Photobiol. 47 (1988) 647–656.
[22] I. Ahmad, Q. Fasihullah, A. Noor, I. Ansari, Photolysis of riboflavin in aqueous solution: a kinetic study, Q. Ali, Int. J. Pharm. 280 (2004) 199–208.
[23] L. Weber, R. Hommel, J. Behling, G. Haufe, H. Hennig, Photocatalytic oxygenation of hydrocarbons with (tetraarylporphyrinato)iron(III) complexes and molecular oxygen. Comparison with microsomal cytochrome P-450 mediated oxygenation reactions, J. Am. Chem. Soc. 116 (1994) 2400–2408.
[24] U. Neuenschwander, F. Guignard, I. Hermans, Mechanism of the aerobic oxidation of alfa-pinene, Chem. Sust. Chem. 3 (2010) 75–84.
[25] W. Schrader, J. Geiger, D. Klockow, E.H. Korte, Degradation of alpha-pinene on Tenax during sample storage: effects of daylight radiation and temperature, Environ. Sci. Technol. 35 (2001) 2717–2720.
[26] K.A. Connors, Chemical Kinetics: The Study of Reaction Rates in Solution, VCH Publishers, Inc., 1991, pp. 62–66.
[27] J.D. Spikes, J.C. Bommer, Chlorophyll and related pigments as photosensitizers in biology and medicine, in: H. Scheer (Ed.), Chlorophylls, CRC Press, London, 1991, pp. 1181–1204.
[28] T. Itoh, A. Ishii, Y. Kodera, M. Hiroto, A. Matsushima, H. Nishimura, Y. Inada, Chlorophyllin coupled with polyethylene glycol: a potent photosensitizer, Res. Chem. Intermed. 22 (1996) 129–136.
[29] T. Itoh, H. Asada, K. Tobioka, Y. Kodera, A. Matsushima, M. Hiroto, H. Nishimura, T. Tsuzuki, T. Kamachi, I. Okura, Y. Nada, Hydrogen gas evolution and carbon dioxide fixation with visible light by chlorophyllin coupled with polyethylene glycol, Bioconjug. Chem. 11 (2000) 8–13.
[30] A.R. Wellburn, The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution, J. Plant Physiol. 144 (1994) 307–313.
[31] A.N. Melkozernov, R.E. Blankenship, Photosynthetic functions of chlorophylls, in: B. Grimm, R.J. Porra, W. Rüdier, H. Scheer (Eds.), Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Function and Application, Springer, Amsterdam, 2006, pp. 397–412.
[32] B. Kräutler, B. Jaun, P. Matile, K. Bortlik, M. Schellenberg, On the enigma of chlorophyll degradation: the constitution of a secoporphinoid catabolite, Angew. Chem. Int. Ed. Engl. 30 (1991) 1315–1318.
[33] J.J. Jen, G. MacKiney, On the photodecomposition of chlorophyll in vitro. I. Reaction rates, Photochem. Photobiol. 11 (1970) 297–302.
[34] P.H. Hynninen, Modifications, in: H. Scheer (Ed.), Chlorophylls, CRC Press, Boca Raton, FL, 1991, pp. 145–209.
[35] J.F. Rontani, G. Baillet, C. Aubert, Production of acyclic isoprenoids compounds during the photodegradation of chlorophyll in seawater, J. Photochem. Photobiol. A 59 (1991) 369–377.
[36] W. Oettmeier, T.R. Janson, M.C. Thurnauer, L.L. Shipman, J.J. Katz, Spectroscopic characterization of the pheophytin a dication, J. Phys. Chem. 61 (1977) 339–342.
[37] P.H. Hynninen, Protonation–deprotonation equilibria in tetrapyrroles. Part 1. Protonation titrations of 132-(demethoxycarbonyl)pheophytin a in methanolic hydrochloric acid by electronic absorption spectroscopy, J. Chem. Soc. Perkin Trans. 2 (1991) 669–678.
[38] H. Scheer, J.J. Katz, Peripheral metal complexes: chlorophyll isomers with Magnesium bound to the ring E β-ketoester system, J. Am. Chem. Soc. 100 (1978) 561–571.
[39] F. Hong, Z. Wei, G. Zhao, Mechanism of lanthanum effect on the chlorophyll of spinach, Sci. China Ser. C: Life Sci. 45 (2002) 166–176.
[40] J. Petrović, G. Nikolić, D. Marković, In vitro complexes of copper and zinc with chlorophyll, J. Serb. Chem. Soc. 71 (2006) 501–512.
[41] J. Zvezdanović, D. Marković, G. Nikolić, Different possibilities for the formation of complexes of copper and zinc with chlorophyll inside photosynthetic organelles: chloroplasts and thylakoids, J. Serb. Chem. Soc. 72 (2007) 1053–1062.
[42] J. Zvezdanović, T. Cvetić, S. Veljović-Jovanović, D. Marković, Chlorophyll bleaching by UV-irradiation in vitro and in situ: absorption and fluorescence studies, Radiat. Phys. Chem. 78 (2009) 25–32.
[43] R.J. Abraham, D.A. Goff, K.M. Smith, N. M. R. spectra of porphyrins. Part 35. An examination of the proposed models of the chlorophyll a dimer, J. Chem. Soc. Perkin Trans. 1 (1998) 2443–2451.
[44] U. Krings, R.G. Berger, Biotechnological production of flavours and fragrances, Appl. Microbiol. Biotechnol. 49 (1998) 1–8.
[45] M.S. van Dyk, E. van Rensburg, N. Moleleki, Hydroxylation of (+)limonene, (−)α-pinene and (−)β-pinene by a Hormonema sp., Biotechnol. Lett. 20 (1998) 431–436.
[46] H. Schewe, D. Holtmann, J. Schrader, P450(BM-3)-catalyzed whole-cell biotransformation of alpha-pinene with recombinant *Escherichia coli* in an aqueous-organic two-phase system, Appl. Microbiol. Biotechnol. 83 (2009) 849–857.