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Scavenging and antioxidant properties of compounds synthesized by carotenogenic yeasts stressed by heavy metals—EPR spin trapping study

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Abstract

Free radical scavenging and antioxidant activities of metabolites produced by carotenogenic yeasts of *Rhodotorula* sp. and *Sporobolomyces* sp. grown under heavy metal presence were studied using various EPR experiments. The thermally initiated decomposition of $K_2S_2O_8$ coupled with EPR spin trapping was shown to be the best choice to characterize antioxidant properties of yeast's samples. EPR spectroscopy revealed that yeast walls showed higher ability to scavenge free radicals than those from inside the cells. Since carotenogenic yeast differ to each other in resistance against the heavy metals due to their individual protective system, quenching properties and antioxidant activities of carotenogenic yeasts were modulated by Ni^{2+} or Zn^{2+} ions variously.

Keywords: Yeast; Carotenoids; EPR spectroscopy; Spin trapping; Antioxidants; Heavy metals

1. Introduction

Carotenoids are a widely distributed group of naturally occurring pigments that are essential to microbial, plant and animal life and exhibit the protective role against number of diseases [1–3]. They act as potent antioxidants and scavengers that negate the lethal effects of singlet oxygen and superoxide formed during oxygen stress or heavy metal presence [4,5]. Microbial cells have evolutionary developed efficient adaptation mechanisms for elimination of undesirable factors in order to survive in adverse environment [6–8]. Many yeasts have been described with an increased ability to produce carotenoids when they grown under unfavorable conditions [8–10]. Thus, activated biosynthesis of these compounds by carotenogenic yeasts exposed to heavy metal presence could be in part explained by their scavenger characters as a protection against the harmful effect of the environment.

One effective method for measurement of yeast's metabolites ability to quench free radicals is based on EPR spectroscopy applying spin trapping, which is a method of choice to characterize natural antioxidants as was previously shown in our laboratory [11–14], for the characterization of antioxidant activities of beer [11,12], wines [13] and teas [14]. The important role of several natural and synthetic carotenoids as scavengers of free radicals was already confirmed using EPR spin trapping technique [15–17]. It was shown that carotenoids can either increase or decrease the total yield of free radicals depend-

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ing on both the oxidation potential of the carotenoids and the nature of the radicals [16]. However, carotenogenic yeasts grown under exogenous stress produce various metabolites including both easily oxidizable materials as lipids, proteins, DNA or carbohydrates and antioxidants which are able to prevent their oxidation. The properties of antioxidants formed by yeasts can be substantially influenced by the environment surrounding the cells. Therefore, there is a necessity to develop the reliable methods to investigate sample total radical scavenging and antioxidant capacity without distinguishing the contributions from individual compounds. Simultaneously, the analysis of the yeast's metabolites composition and their comparison to the total antioxidant capacity of yeasts can help us to identify compounds that are the most efficient radical scavengers and antioxidants in the investigated samples. For that reason, information from different techniques is necessary to estimate radical scavenging and antioxidant capacities of yeast extracts more precisely.

In the presented study, the free radicals scavenging and antioxidant activities of natural antioxidants produced by carotenogenic yeasts of *Rhodotorula* sp. and *Sporobolomyces* sp. grown under heavy metal presence were studied applying different EPR assays in order to find the suitable procedure for investigation of the properties of metabolites localized either on the surface of cell walls or inside of the cells. Additionally, the role of Ni^{2+} and Zn^{2+} ions in reaction pathways resulting to reactive oxygen species (ROS) generation is discussed and the composition of yeast's carotenoids in connection to their antioxidant capacity is presented.

2. Experimental

All strains investigated in this study (*Sporobolomyces roseus* CCY 19-6-4, *Rhodotorula glutinis* CCY 20-2-26, *R. glutinis* CCY 20-2-31, *Rhodotorula rubra* CCY 20-7-28 and *Rhodotorula aurantiaca* CCY 20-9-7) are maintained in the Culture Collection of Yeasts (CCY; Institute of Chemistry, Slovak Academy of Sciences, Bratislava) on slant agar at 4 °C. Yeasts were cultivated on medium consisted of (g/l): glucose—20; yeast extract—4.0; $(NH_4)_2SO_4$—10; KH_2PO_4—1; $K_2HPO_4 \cdot 3H_2O$—0.2; NaCl—0.1; $CaCl_2$—0.1; $MgSO_4 \cdot 7H_2O$—0.5 and 1 ml solution of microelements [(mg/l): H_3BO_4—1.25; $CuSO_4 \cdot 5H_2O$—0.1; KI—0.25; $MnSO_4 \cdot 5H_2O$—1; $FeCl_3 \cdot 6\ H_2O$—0.5; $(NH_4)_2Mo_7O_{24} \cdot 4\ H_2O$—0.5 and $ZnSO_4 \cdot 7H_2O$—1]. All strains grew under a non-lethal and maximally tolerated concentration of either Ni^{2+} or Zn^{2+} ions (*Rhodotorula* sp. in the presence of 2.0 mM $NiCl_2$ or 2.0 mM $ZnCl_2$; *Sporobolomyces* sp. in the presence of 0.5 mM $NiCl_2$ or 2.0 mM $ZnCl_2$). The cultures were cultivated in 500 ml flasks containing 250 ml cultivation medium on a rotary shaker (150 rpm) at 28°C to early stationary grow phase. Isolated cells were suspended in distilled water and subjected to ultrasound treatment (Person-Ultragen UZD 300, Nitra, Slovakia) at 20 kHz, 21 °C and 110 W for 3×2 min under an inert nitrogen atmosphere. The cells were separated by centrifugation and the fibrilar part of cell wall was obtained from the supernatant by precipitation with 96% ethanol (1:2 v/v). This part of cells was marked as "walls". The sediment was frozen by liquid nitrogen. After defrosting, the cytosol material was isolated by ethanol precipitation from the supernatant and the sediment was marked as "cells". Both "walls" and "cells" were maintained in dimethylsulfoxide (DMSO) and stored at −20 °C until further experiments were performed. All cultivation experiments were carried out at triplicates and analyzed individually. It should be noted that no significant effect of sonication on carotenes was observed under above described conditions.

Carotenoids were isolated from "walls" and "cells" by 96% ethanol and analyzed by RP-HPLC using column Biospher (4.6×150 mm) filled with reverse phase PSI 200 C18 [9]. Carotene pigments were eluted by methanol as mobile phase (1.1 ml/min, 45 °C) and identified by spectrophotometric detector at 450 nm. Phytoene, as a precursor of carotene pigments, was analyzed with methanol/acetonitrile (5:95, 1.1 ml/min, 45 °C) and identified by spectrophotometric detector at 285 nm.

Zinc and nickel ions content in "walls" and "cells" was analyzed by a method of Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) in axial configuration. The microwave module disintegrated the lyophilized samples and the zinc and nickel ions were determined by flame atomic absorption spectrometer with background correction by a deuterium lamp. The acetylene-air flame was used in the ratio of 1:1.2 with the fuel flow of 1.2 l min^{-1} and both metals were measured at 232.0 nm.

Ultraviolet–visible (UV–Vis) spectra were measured by UV–Vis spectrometer PC 2000 (Sentronic, Germany) accompanied with DH 2000 light source (deuterium and halogen lamps). A Bruker EMX EPR spectrometer (Germany) along with a Bruker ER4111 VT variable-temperature control unit was used in EPR experiments.

The total antioxidant capacity (TAC) was measured by standard procedure using the ferric reducing ability of plasma (FRAP) reagent [18]. FRAP reagent (25 ml 0.3 M acetate buffer, 2.5 ml 10 mM 2,4,6-tripyridyl-*S*-triazine, and 2.5 ml mM $FeCl_3$) was warmed to 37 °C and absorbance of blank was taken at 593 nm. After adding of the sample (30 μl) to the FRAP solution (900 μl) ferrictripyridyltriazine (Fe^{III}-TPTZ) complex was reduced to the ferrous (Fe^{II}) form by antioxidants present in sample and an intense blue color with an absorption maximum at 593 nm was developed. Absorbance was recorded at 15 s intervals. The reaction was monitored up to 10 min. The absorbance at 10 min was selected for calculation of FRAP values. These were obtained by comparing the absorbance change at 593 nm in test reaction mixtures, including samples, with solutions containing one antioxidant in purified form (using calibration curve of Trolox) [18].

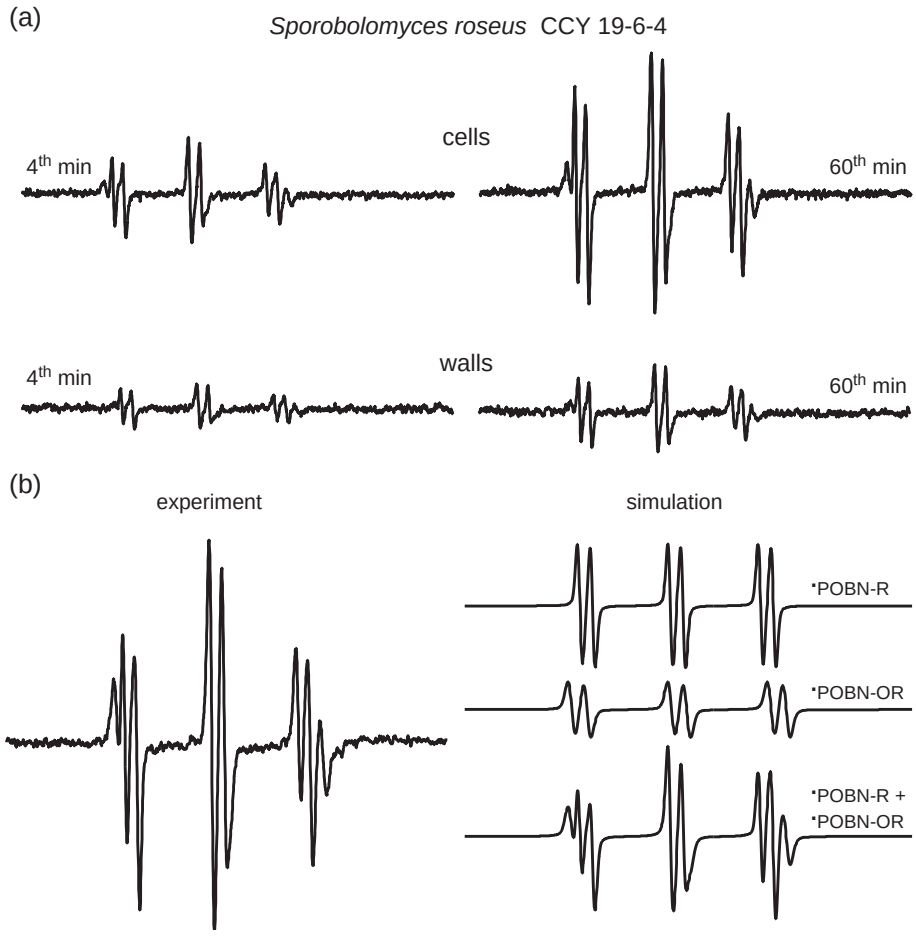

Fig. 1. (a) The first and the last EPR spectra from the series of 20 spectra taken for DMSO supernatants of yeast's cell walls ("walls") cell rests ("cells") extracted from yeast *Sporobolomyces roseus* CCY 19-6-4 grown in the absence of heavy metal ions measured in H_2O_2/NaOH/DMSO solution in the presence of POBN spin trapping agent. (b) Experimental and simulated EPR spectra of POBN spin adducts measured in H_2O_2/NaOH/DMSO solution in the presence of POBN spin trapping agent.

5,5-Dimethylpyrroline-*N*-oxide (DMPO) from Aldrich was redistilled before application. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) from Fluka, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) from Sigma and $K_2S_2O_8$ from Merck were used as received. All solutions were prepared using redistilled water and spectroscopic grade

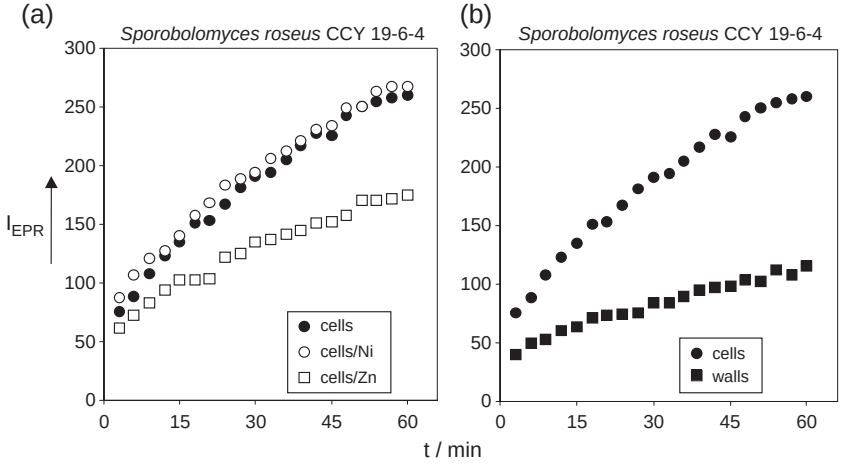

Fig. 2. Time evolution of EPR intensity (double integral) observed during a 60 min period at room temperature for different DMSO supernatants applying H_2O_2/NaOH/DMSO system in the presence of POBN spin trapping agent. (a) The samples "cells" extracted from yeast *Sporobolomyces roseus* CCY 19-6-4 grown in the absence of heavy metal ions (full circles) and in the presence of nickel (empty circles) or zinc (empty squares). (b) The samples "cells" (full circles) and "walls" (full squares) extracted from yeast *S. roseus* CCY 19-6-4 grown in the absence of heavy metal ions.

dimethylsulfoxide (DMSO) from Lachema (Czech Republic).

3. Results and discussion

The radical scavenging ability of DMSO supernatants of yeast's "walls" and "cells" extracted from two yeast samples (*S. roseus* CCY 19-6-4, *R. aurantiaca* CCY 20-9-7) was tested applying the system H_2O_2/NaOH/DMSO [19] which produces superoxide anion, hydroxyl radical and methyl radical simultaneously. POBN was used as the spin trap. In the experiment the air saturated mixture of 50 μl of 25 mM NaOH in water, 50 μl DMSO and 50 μl POBN in DMSO (0.035 g/1 ml) was mixed with 100 μl of sample (yeast's extract), followed by the addition of 50 μl 30% H_2O_2 in water. In reference sample DMSO/H_2O (1:1) was added to the system instead of yeast's supernatant solution. The solution was immediately transferred to the quartz EPR flat cell. The first measurement in EPR cavity started exactly in the 4th minute after addition of hydrogen peroxide into the system. To obtain a time evolution of radical spin adducts, 20 EPR spectra of each sample were repeatedly recorded at regular 3 min intervals (each spectrum represents the accumulation of three spectra). Fig. 1a shows the first (4th min) and the last (60th min) EPR spectra from the series of 20 spectra taken for the samples of "walls" and "cells" extracted from yeast *S. roseus* CCY 19-6-4 grown in the absence of heavy metal ions. The obtained EPR spectra of $^{\bullet}$POBN-adducts can be well fitted with two individual EPR signals (Fig. 1b) with the following splitting constants: a_N=1.41 mT, a_H=0.20 mT (carbon centered radicals; 70%) and a_N=1.56 mT, a_H=0.24 mT (oxygen centered radicals; 30%). In contrast to the yeast supernatants, the oxygen centered adducts fully dominated in reference sample (DMSO) and EPR intensity was much higher (more than 5 times) than in the yeasts supernatants. The detailed studies concerning this point using more suitable system including thermal decomposition of $K_2S_2O_8$ are discussed below. The abilities of the yeast's samples to decrease spin adduct concentration was tracked during 60 min after addition of hydrogen peroxide to the system under conditions analogous to those for the reference sample (DMSO). A time evolution of relative EPR intensities recorded for different representative samples is summarized in Fig. 2. Generally, all yeast's samples were considerably stronger scavengers compared to the reference sample. This behavior can be explained by the reaction mechanisms which include the suppression of generation of ROS and scavenging of ROS by antioxidants present in yeast's samples. In the case of e.g. carotenoids (CAR) usually three mechanisms of radical scavenging are proposed [15] as (i) radical addition to the conjugated system of double bonds (CAR+ROO$^{\bullet}$ → ROOCAR$^{\bullet}$), (ii) hydrogen abstraction from the carotenoid and (iii) the electron transfer reactions. All these processes prevent free radicals to be scavenged by spin traps. Generally, the decrease of EPR intensity is a result of competitive reactions of antioxidants present in yeast's supernatants and DMPO spin trap with generated reactive

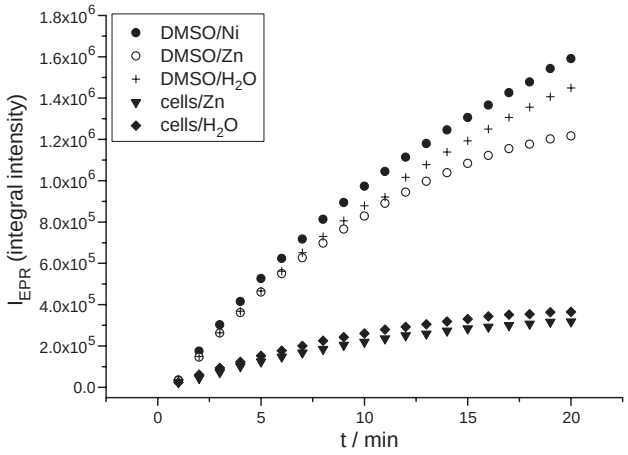

Fig. 4. Time course of EPR integral intensities of DMPO adducts for the reference and yeast samples: 175 μl DMSO/25 μl H_2O (crosses), 175 μl DMSO/25 μl $NiCl_2$(aq) (full circles), 175 μl DMSO/25 μl $ZnCl_2$ (aq) (empty circles), 175 μl of cell's extracts from *Sporobolomyces roseus* CCY 19-6-4 grown under absence of exogenous stress with the addition of 25 μl distilled water (full diamonds) or 25 μl $ZnCl_2$(aq) (full triangles) recorded during first 20 min of the thermal decomposition of $K_2S_2O_8$.

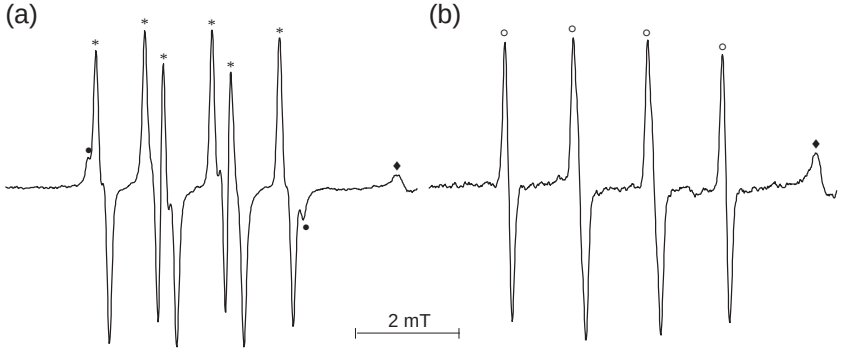

Fig. 3. Representative EPR spectra of spin adducts (super · DMPO-O_2^-, super · DMPO-OCH_3, super · DMPO-OR) recorded during the thermally initiated decomposition of $K_2S_2O_8$(aq.) at 333 K coupled with spin trapping (DMPO) in the presence of (a) reference sample DMSO and (b) DMSO supernatant from *Rhodotorula rubra* CCY 20-7-28 ("cells").

free radicals. Additionally, as seen from Fig. 2, EPR spectroscopy revealed that antioxidants present in yeast's walls showed higher ability to scavenge free radicals than those from inside the cells. More effective radical scavenging capacity was found in yeast samples grown under presence of Zn^{2+} ions than with Ni^{2+} ions. This tendency was observed for both samples from *S. roseus* CCY 19-6-4 (Fig. 2) and *R. aurantiaca* CCY 20-9-7 (not shown). This phenomenon could be probably explained by both the presence of Zn^{2+} ions in "walls" and "cells" (as discussed in detail below) and by processes where Zn^{2+} ions during cultivation might induce more efficient scavenging and antioxidant capacities of yeasts metabolites compare to the cultivation in the absence of exogenous metal. For example, this could occur by the enhanced production of Zn proteins (e.g. Zn-superoxide-dismutase) with possible antioxidant function.

In the next step the thermal decomposition of $K_2S_2O_8$ in H_2O/DMSO solutions at 333 K was used as a powerful source of both oxygen- and carbon-centered reactive radicals [20]. DMPO was used as the spin trap, which can trap not only carbon-centered but also very effectively oxygen-centered radicals. The advantage of this technique in comparison to the system H_2O_2/NaOH/DMSO described above is the absence of time stress in the preparation phase as the reaction is initiated precisely by setting the temperature for $K_2S_2O_8$ decomposition in the EPR cavity at the moment when all other setup parameters were fine-tuned. Additionally, we used a special EPR standard in the form of thin laminated [21] paper slip containing traces of homogeneously distributed DPPH, which was fixed on the outer wall of the flat part of the EPR cell. This enabled us to compare quantitatively all experimental results from EPR experiments similarly to the procedures with the Mn-

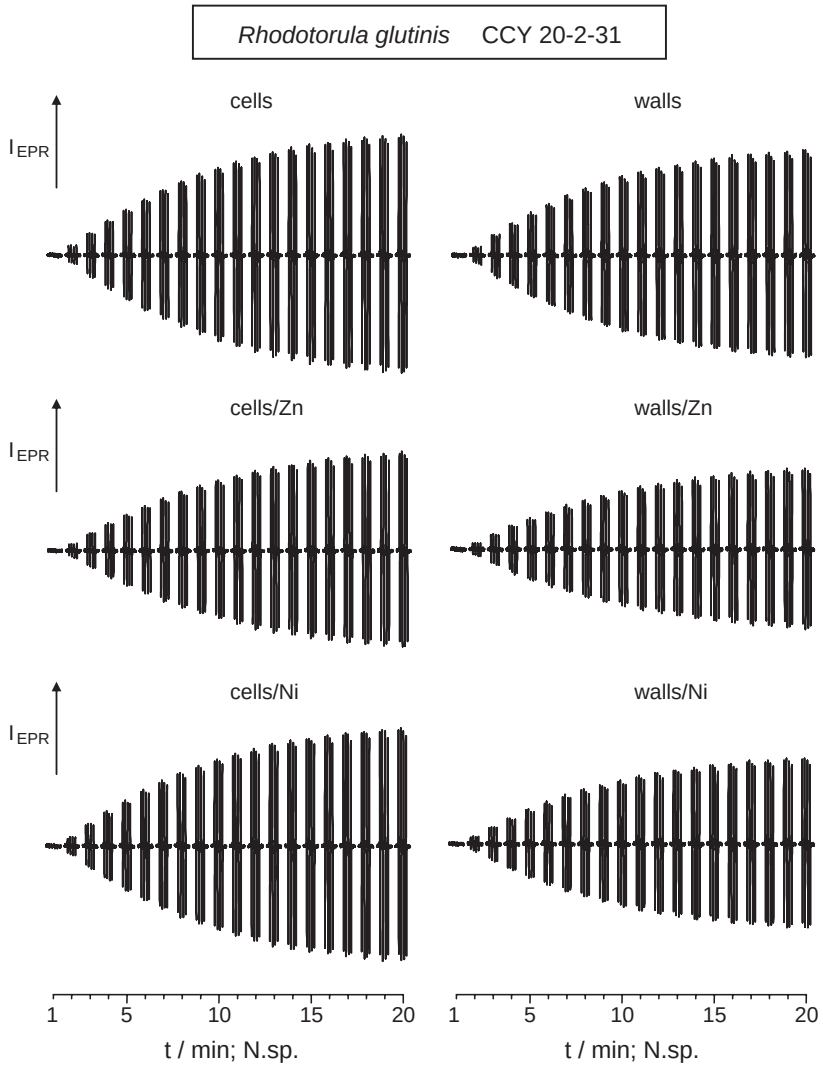

Fig. 5. Time course of 20 individual EPR spectra obtained for samples of DMSO supernatants ("cells" and "walls") from carotenogenic yeasts *Rhodotorula glutinis* CCY 20-2-31 grown under absence or presence of $NiCl_2$ or $ZnCl_2$. All sets of 20 EPR spectra of DMPO adducts monitored during the thermal (333 K) decomposition of $K_2S_2O_8$ in the presence of DMSO supernatants were taken for 20 min at the same experimental conditions as for reference sample (DMSO instead of DMSO supernatant). *X*-axis depicts both the time of experiment (*t*) and the number of individual spectrum (N.sp.).

standards used in quantitative EPR [21]. The use of the DPPH line (marked with diamond in Fig. 3a) balances the changes in the Q-factor and, therefore, it was possible to perform the correct determination of the spin adduct concentration. The characteristic EPR spectra of reference (200 μl DMSO/H_2O (4:1) solution) and yeast DMSO supernatant (200 μl) samples taken in 25 μl DMPO in DMSO (0.2 M), 25 μl 0.01 M $K_2S_2O_8$ (aq.) solution at 333 K are shown in Fig. 3. Fig. 3a shows experimental EPR spectrum obtained in reference sample (marked with asterisk in Fig. 3a), which can be simulated with hyperfine splittings a_N=1.31 mT, a_H=0.94 mT, a_H=0.13 mT. This spectrum well correlates with the oxygen centered adduct, namely $^{\bullet}$DMPO-O_2^- superoxide anion radical adduct [14]. Additionally, an another EPR signal of low intensity characterized with hyperfine splittings a_N=1.44 mT, a_H=1.05 mT, a_H=0.13 mT is evident in the spectra (marked with solid circles in Fig. 3a) and is characteristic to oxygen centered spin adduct $^{\bullet}$DMPO-OR [13]. In very recent study using HPLC-EPR, ESI-MS and MS/MS techniques [22] this radical was unambiguously attributed to the DMPO/methoxyl radical adduct. The domination of $^{\bullet}$DMPO-O_2^- and $^{\bullet}$DMPO-OOH (formed by the protonation of superoxide anion radical) adducts in the reference sample can be successfully explained taking into account rapid consecutive reactions of $SO_4^{-\bullet}$ radical leading to the production of reactive $^{\bullet}$OH radicals [20]. In the presence of oxygen and water superoxide anion and $^{\bullet}$OOH radicals are formed due to the consecutive chemical reactions of $^{\bullet}$OH radicals with neighborhood. DMPO/methoxyl radical adduct is formed due to the reaction of methyl radicals produced from DMSO reacting with $^{\bullet}$OH radicals with oxygen under formation of less stable methylperoxyl radicals ($(CH_3)_2SO+^{\bullet}OH \rightarrow {}^{\bullet}CH_3+CH_3SO_2H$; $^{\bullet}CH_3+O_2 \rightarrow {}^{\bullet}OOCH_3$). These are not detectable at room temperature as due to the consecutive reactions the methoxyl radicals are formed from methylperoxyl radicals [22].

On the other hand in the case of yeast supernatants the EPR spectra with hyperfine splittings a_N=1.45 mT and a_H=1.32 mT fully dominate (Fig. 3b, empty circles) and correspond to DMPO/alkoxyl radical adducts [22]. The total concentration of spin adducts is generally much lower compared to the reference sample (DMSO instead of DMSO supernatant) as illustrated in Fig. 4. Alkoxyl radicals are formed as a result of the reactions of primary formed carbon centered radicals $^{\bullet}$R originating from yeast supernatants with the oxygen present in the system under formation of peroxyl and alkoxyl radicals which can cause considerable biological damage ($^{\bullet}R+O_2 \rightarrow {}^{\bullet}OOR \rightarrow$consecutive reactions$\rightarrow {}^{\bullet}OR$) [22]. No EPR signals matching $^{\bullet}$DMPO-OH and $^{\bullet}$DMPO-O_2^- adducts formation were detected indicating that mostly the small reactive radicals are effectively scavenged with the antioxidants present in the yeast's samples. The rest of not scavenged reactive radicals forms the less reactive long chain oxygen- and carbon-centered radicals, which are clearly detectable by EPR as already mentioned above. Their amount can be used as a good measure of the radical scavenging capacity of the samples under study.

Fig. 5 shows complete series of EPR measurements for samples of supernatants from carotenogenic yeasts of *R. glutinis* CCY 20-2-31 grown under presence of $NiCl_2$ and $ZnCl_2$, respectively. All sets of 20 EPR spectra of DMPO adducts monitored during the thermal (333 K) decomposition of $K_2S_2O_8$ in the presence of DMSO supernatants were taken under the same experimental conditions as described above for reference sample (DMSO). Such time development of EPR spectra is characteristic for all investigated samples. Fig. 6 shows a time course of EPR integral intensities (evaluated from the integration of sets of 20 individual EPR spectra for each measurement, see Fig. 5) representatively for the samples of *R. glutinis* CCY 20-2-31 (both "walls" and "cells", Fig. 6a) and *R. glutinis* CCY 20-2-26 (Fig. 6b) recorded during first 20 min. Similarly as observed in the EPR experiments with H_2O_2/NaOH/DMSO system these results can be explained in terms of different scavenging ability of the samples which increases in the order: reference (DMSO)<cells(Ni)<cells<cells(Zn)<walls<

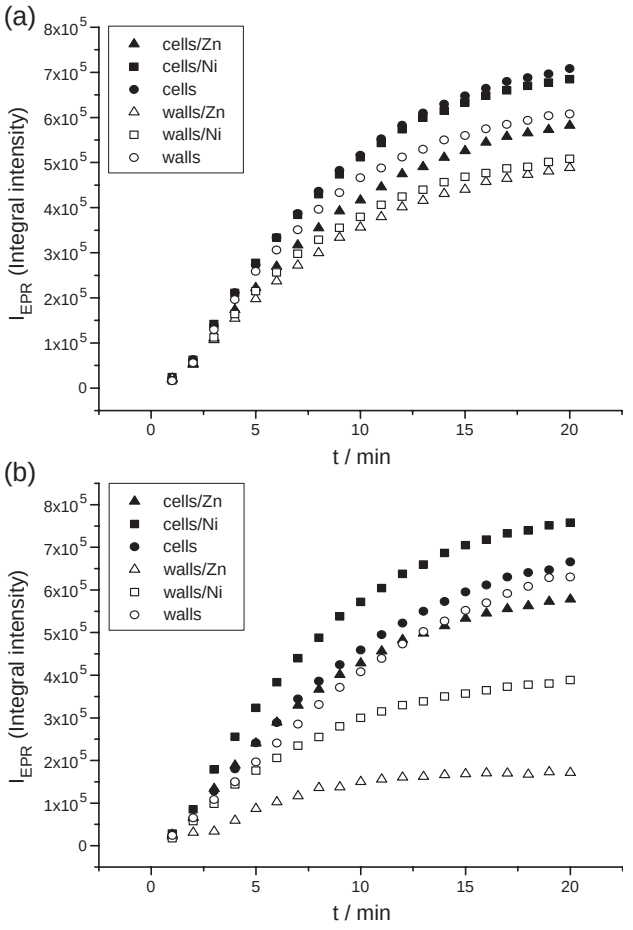

Fig. 6. Time course of EPR integral intensities of DMPO adducts for the samples (a) *Rhodotorula glutinis* CCY 20-2-31 and (b) *R. glutinis* CCY 20-2-26 recorded during first 20 min of the thermal decomposition of $K_2S_2O_8$ in the presence of DMSO supernatants.

walls(Ni)<walls(Zn). As can be again seen from experiments using $K_2S_2O_8$ system, the best radical scavenging capacity was observed for "walls" of yeasts grown under presence of Zn^{2+} ions. On the other hand, low capacity to scavenge radicals was found for cytosol fractions ("cells") from yeasts cultivated under presence of Ni^{2+} ions.

To explain this behavior, several EPR experiments and quantitative/qualitative analyses using HPLC and ICP-OES were performed. It is clear from evaluated EPR spin trapping experiments that extracts from zinc stressed yeasts show the best radical scavenging capacity. Therefore we studied the influence of Zn^{2+} and Ni^{2+} ions presence on the time course of EPR signal on the reference sample. Fig. 4 presents the comparison of the time evolution of the integral intensity of EPR signals for reference sample in the absence or in the presence of metal ions. In these experiments to the standard reaction mixture containing 175 μl DMSO instead of yeast supernatant was added either 25 ul distilled water (labeled as DMSO/H_2O) or aqueous solution of 10^{-3} M MCl_2 (labeled as DMSO/Zn if M was Zn and DMSO/Ni if M was Ni). It is evident that the presence of Zn^{2+} ions decreases the quantity of radicals produced in the reaction system. On the other hand the presence of Ni^{2+} ions increases the total amount of free radicals under analogous experimental conditions. The same effect was also observed for the yeast samples as illustrated for *S. roseus* CCY 19-6-4 grown under absence of exogenous stress with the addition of 25 μl distilled water (full diamonds, labeled as cells/H_2O) or 25 μl $ZnCl_2$(aq) (full triangles, labeled as cells/Zn) recorded during first 20 min of the thermal decomposition of $K_2S_2O_8$. This is in accordance with the recent studies [23,24], which described that zinc ions play critical roles in the defense system of cells against reactive oxygen species (ROS), and thus represents an excellent protective agent against the oxidation of several vital cell components, such as membrane lipids and proteins, chlorophyll, SH-containing enzymes and DNA. On the other hand the presence of Ni^{2+} ions in the system can lead to the enhanced production of ROS as metal-induced generation of reactive oxygen species (Fenton reactions) may play an important role in this process [25,26].

Table 1 illustratively shows the carotenoid composition of "walls" and "cells" from several yeasts incubated with Zn^{2+} and Ni^{2+} ions. While *R. glutinis* CCY 20-2-26 and *R. glutinis* CCY 20-2-31 can be characterized by high content of β-carotene, *R. rubra* CCY 20-7-28 displays increased content of both β-carotene and phytoene. The yeasts treatment with metals caused substantial change in the carotenoid composition of the yeast. For instance, we observed increase of luteine and rapid decrease of phytoene in "walls" of *R. glutinis* CCY 20-2-31 cultivated under Zn^{2+} ions in comparison to unstressed culture. On the other hand, level of luteine was very low in *R. glutinis* CCY 20-2-26 and increase amount of α-carotene was found in both "cells" and "walls" of the strains incubated with metal ions. However, changes in carotenoids composition do not correlate exactly with EPR studies described above. This illustrates the complexity of the investigated systems and the contribution of further compounds to the total antioxidant capacity except of carotenoides. Also, as described above, the influence of metal ions cannot be neglected because after cultivation they remain adsorbed in both "cells" and "walls" (analyses of detailed metal composition in yeasts structures will be published in forthcoming paper).

Finally, we compared the radical scavenging capacity evaluated by EPR spin trapping technique with the total

Table 1
The carotenoid composition of selected yeasts

Strain	Conditions	Carotenoids [%]					
		Luteine	Torulene	Lycopene	α-Carotene	β-Carotene	Phytoene
R. glutinis CCY 20-2-26	cells	1.9	4.9	0.1	5.0	83.5	4.7
	cells+Ni	1.0	3.4	4.2	16.6	68.5	6.2
	cells+Zn	0.2	23.0	0.1	9.0	54.3	13.4
	walls	0.2	0.7	1.2	3.0	94.3	0.6
	walls+Ni	3.3	9.5	0.8	15.2	62.8	8.5
	walls+Zn	0.1	3.3	3.3	21.6	69.0	2.7
R. glutinis CCY 20-2-31	cells	4.4	0.5	1.9	7.0	64.2	22.0
	cells+Ni	0.2	4.5	0.1	5.4	80.7	9.1
	cells+Zn	2.5	0.5	1.4	6.5	75.8	13.4
	walls	4.0	3.0	1.1	2.2	54.6	35.1
	walls+Ni	6.2	2.5	1.2	4.8	67.6	17.7
	walls+Zn	30.5	14.3	0.2	1.6	49.2	4.2
R. rubra CCY 20-7-28	cells	8.3	0.6	1.4	5.4	34.4	50.0
	cells+Ni	13.6	3.9	0.2	1.8	37.0	43.4
	cells+Zn	6.3	0.4	1.4	5.5	38.6	47.8
	walls	28.6	1.5	3.7	6.2	37.9	22.1
	walls+Ni	0.4	0.1	0.1	0.2	15.6	83.6
	walls+Zn	13.5	0.7	0.9	4.9	74.2	5.8

Values are presented as the means of triplicates that varies between 4% and 8%.

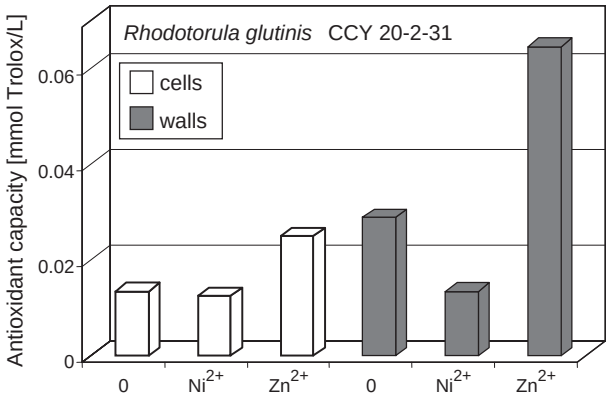

Fig. 7. Total antioxidant capacity (TAC) evaluated by the standard procedure based on the FRAP reagent of the DMSO supernatants from *Rhodotorula glutinis* CCY 20-2-31 grown on different environmental stress (both "walls" and "cells").

antioxidant capacity (TAC) of the investigated samples evaluated by the standard procedure based on the FRAP reagent (see Experimental Section). In Fig. 7 the TAC values of the samples *R. glutinis* CCY 20-2-31 as assayed by FRAP method are summarized. It is evident that Fe^{3+} reducing capacity of the samples very well correlates with the radical scavenging capacity of all samples except of the sample of walls obtained from the yeast grown in the absence of stress. Such behavior was also observed for the samples *R. glutinis* CCY 20-2-26 and *R. rubra* CCY 20-7-28 investigated in these series of experiments. This confirms the reliability of new techniques used in this study, which are suitable for the determination of radical scavenging and antioxidant properties of complex biochemical systems. Full details on the carotenogenic yeast composition cultivated under different exogenous stress are the subject of our further extensive studies and will be published in due course.

4. Conclusions

Applying different EPR experiments, the suitable procedure to investigate the radical scavenging and antioxidant properties of pigments localized on the surface of cell walls and inside of the cell bodies of yeasts grown on exogenous stress was found. The obtained results can be explained in terms of different scavenging ability of the samples which increases in the order: cells(Ni)< cells<cells(Zn)<walls(Ni)<walls<walls(Zn). Zn^{2+} ions induced changes in yeast leading to more efficient scavenging and antioxidant capacities compared with Ni^{2+} ions, and antioxidants present in yeast's walls showed higher ability to scavenge free radicals than those from inside the cells. For comparison the total antioxidant capacity was additionally investigated using spectrophotometric methods including a FRAP-based method, which confirmed the conclusions based on EPR spin trapping experiments.

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