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THE ANTIOXIDANT POTENTIAL OF CAROTENOID EXTRACT FROM *PHAFFIA RHODOZYMA*[*]

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Background. Carotenoids are components playing an important role in biological systems, starting with light protection, immunoenhancement, protection against carcinogens and finishing with antioxidant activity. Food additives market is based mainly on synthetic additives; however, higher consumer awareness has resulted in an increased use of natural substances. One of the potentially antioxidant compounds could be a lipid soluble carotenoid – astaxanthin (xanthophyll), found in the microbial world. The aim of this study was to evaluate the antioxidant potential of carotenoid extract from Phaffia rhodozyma extract.
Material and methods. Carotenoids extracted from *Phaffia rhodozyma* and the astaxanthin standard was selected for the investigations. Antioxidant potential was evaluated by radical scavenging activity ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals) and in lipid oxidative stability measurements (Rancimat, Oxidograph and Schaal oven tests).
Results. It was found that the examined extracts presented a significantly higher ability to scavenge the $DPPH^{\bullet}$ radical in comparison to the $ABTS^{\bullet+}$ radical. Evaluations of linoleic acid emulsion oxidative stability showed a higher antioxidant effect of the *Phaffia rhodozyma* extract than that of astaxanthin during 19 h of incubation. That potential however, was not detected in linoleic acid emulsion incubated for 96 h, where both additives accelerated oxidation process. In bulk sunflower oil a protective effect of *Phaffia rhodozyma* extract was observed. In both Rancimat and Oxidograph tests antioxidant activity measured using the induction period was evaluated. However, results of the Schaal oven test indicated that a 144 h incubation of sunflower oil offered a significantly better protection of the lipid against oxidation when the *Phaffia rhodozyma* extract was added.
Conclusions. On the basis of recorded results it was found that the *Phaffia rhodozyma* carotenoid extract showed moderate antioxidant properties, depending on the environmental conditions of methods used.

Key words: *Phaffia rhodozyma*, carotenoids, astaxanthin, lipids oxidation, radical scavenging activity

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INTRODUCTION

Carotenoids are components that play an important role in biological systems, starting with light protection, immunoenhancement, protection against carcinogens and finishing with antioxidant activity [Kurihara et al. 2002, Dufosse et al. 2005, Sikora et al. 2009]. There are many carotenoids widely applied in feed, pharmaceutical, food and cosmetics industry [Fraser and Bramley 2004]. The world market of food additives is based mainly on synthetic additives; however, higher consumer awareness has resulted in an increased use of natural substances [Pokorny 2007]. One of the new potentially antioxidant compounds could be a lipid soluble carotenoid – astaxanthin (xanthophyll) [Jackson et al. 2008]. Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione), a reddish pink-colored carotenoid, is an indispensable component of feeds that are used in commercial salmon, rainbow trout and shrimp cultures [Gil-Hwan and Eui-Sung 2003, Guerin et al. 2003].

Almost 95% of astaxanthin preparations available on the market contain a synthetic pigment [Lorenz and Cysewski 2000], which is much more expensive and less stable than the natural one. One of the methods to reduce costs is the application of microorganisms in natural astaxanthin synthesis. A primary commercial source of astaxanthin could be *Phaffia rhodozyma* (syn. *Xanthophyllomyces dendrorhous*) yeast, since astaxanthin makes up as much as 83-87% of total carotenoids produced [Guerin et al. 2003, Calo et al. 1995, Flen et al. 1999]. Another advantage of *Phaffia rhodozyma* use is its capacity to assimilate various carbon sources, cheapness and easy culture [Palagyi et al. 2001]. A major disadvantage is the low level of synthesized pigment (0.2-0.4 g/kg dry matter of yeast) [Guerin et al. 2003, Lorenz and Cysewski 2000, Visser et al. 2003], but the following procedures could minimize that problem: manipulations with media pH, temperature, culture media composition, etc. [Palagyi et al. 2001, Flores-Cotera et al. 2001, Ramirez et al. 2001, Wang et al. 2006, Stachowiak and Czarnecki 2006].

To date antioxidant potential of carotenoid extracts is a new and insufficiently investigated chapter of potential wider use in food industry. There is scarce information in the available literature on the role of the antioxidant potential of *Phaffia rhodozyma* in lipid systems. The possible utilization of astaxanthin and carotenoid compounds in food industry could improve quality and safety of food products. One of the applications of carotenoid compounds could be stabilization of lipids and lipid-containing products.

Lipids undergo oxidation, both during production stages and storage, causing a sequence of unfavourable changes, deterioration of product sensory attributes (rancidity, changes of texture and colour) and reduced nutritive value [Gray 1978, Frankel et al. 1998, Klensporf-Pawlik and Jeleń 2009]. Enrichment of lipids with alternative natural substances could have an advantageous effect on its oxidative stability. Nowadays second very important aspect is their additional introduction to human body, which would positively influence human health and reduce morbidity caused by degenerative diseases. From both the technological and nutritional point of view it is highly desirable to control the oxidation process by an addition of antioxidants.

Depending on the research system used no multifunctional antioxidant has yet been found. The antioxidant activity depends on the system used, the source of components (especially in case of natural substances), their concentration and affinity to the specific phase [Gramza and Korczak 2005]. Research showed that today's consumer prefers natural additives rather than synthetic ones because of the potential toxicity and car-

cinogenicity of the latter [Barlow 1990, Prior and Cao 2000]. The modern world needs to look for new sources of substances, which could influence lipids and food stability, be harmless for consumers and exhibit high antioxidant activity when added to food.

Present investigations aimed at finding a new source of antioxidant compounds to be used in stabilization of lipids. In view of remarkable carotenoids activity in different biological systems [Hussein et al. 2006], the present study examined the antioxidant potential of carotenoid extract from *Phaffia rhodozyma* and the astaxanthin standard.

MATERIAL AND METHODS

Chemicals. All chemicals used were of analytical grade: ethanol, methanol, ddH_2O, chloroform, hexane, acetic acid, HCl, KJ, hydrolysable starch, $Na_2S_2O_3$, isooctane, KOH, $K_2S_2O_8$, glucose, the hexane fraction, acetonitrile, ethanolamine, ethyl acetate (POCH, Poland), K_2HPO_4, KH_2PO_4, $Na_2HPO_4 \cdot 12\ H_2O$, $FeSO_4 \cdot 7\ H_2O$, NH_4SCN, $Fe(NH_4)(SO_4)_2 \cdot 12\ H_2O$ [Merck], p-anisidine, tiobarbituric acid, Tween 20 (Sigma), trichloroacetic acid (Ubichem), linoleic acid (NuChek Prep), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), Trolox, 2,2-diphenyl-1-picrylhydrazyl, astaxanthin (Sigma-Aldrich), yeast malt (YM), malt extract, agar, NaCl (BTL Poland), bactopeptone (Dickinson and Co.), DMSO (Riedel-de Haen).

Yeast and culture conditions. *Phaffia rhodozyma* CBS 5626 (now *Xanthophylomyces dendrorhous*) from the DSMZ collection was used in the study. This strain was cultured on slants of yeast malt (YM) at 4°C and transferred monthly. The components of the proliferation medium (YM) were 10 g glucose, 5 g bactopeptone, 3 g yeast extract, 3 g malt extract and 20 g agar (for slants) in 1 L distilled water [Calo et al. 1995]. Cultivation: The yeast culture was rinsed from slants with ten mL of medium. Four mL of this suspension were transferred into 250 mL Erlenmayer flasks containing 76 mL of autoclaved medium. Proliferation experiments were carried out for 4 days at 22°C in orbital shakers (agitation speed, 200 rpm) at a constant illumination of 600 lx.

Carotenoid extraction from yeast cells of *Phaffia rhodozyma*. Yeast culture was centrifuged and harvested cells were washed with distilled water and dissolved in DMSO [Sedmak et al. 1990]. Hexane fraction pure and 20% NaCl solution were serially added with vortexing between additions. After extraction the upper hexane layer containing carotenoids was collected for quantitative and qualitative pigment analyses and antioxidant determination. Extracts were stored in air-tight and light protected vials at (–40°C) until further use. Carotenoid extract dry matter was analysed with the use of two-stage dryer method. A known yeast extract volume was repeatedly dried in a weighing bottle (2 h at 55°C, and then at 105°C until constant mass was received). Results are expressed as μg/mL of the extract and g/kg of dry weight of culture.

Total carotenoid contents and profile determination. Biomass was measured by DCW (dry cell weight). The total carotenoid yield was quantified by an HPLC (Waters) model Alliance 2695 with PAD detection. Operating conditions: a X-Terra C_{18}RP column, Waters (3.9 × 150 mm, particle size 5 μm); oven temperature 30°C; gradient elution (flow rate = 0.5 mL·min^{-1}; mobile phase: 95% solvent A (acetonitrile with 0.05% [v/v] ethanolamine addition) and 5% solvent B (methanol: ethyl acetate 11:9 (% v/v) for 19 min, change up to 60% solvent A and 40% solvent B in 20 min. The last concentration remained constant for an additional 10 min. All carotenoids were quantified

at λ = 480 nm, by manual integration using astaxanthin as an external standard. Astaxanthin was identified by their retention times and by comparing VIS-UV spectra with those of pure compounds.

Antiradical activity. Carotenoid extract was first concentrated (Buchi, Rotavapor) and the solvent from the hexane-kerosene fraction was replaced with methanol. In the obtained extracts the yields of dry matter and total carotenoids were also controlled. Radical scavenging activity of carotenoid extracts from *Phaffia rhodozyma* was measured with the use of $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ methods, based on a decrease in absorbance of examined solutions, which is a result of radical scavenging activity of the additive.

$DPPH^{\bullet}$ radical scavenging method. The effect of *Phaffia rhodozyma* extracts was estimated according to the procedure described by Sanchez-Moreno et al. [1998]. An aliquot of methanol (0.1 ml), a solution containing the extracts, was added to 3.9 ml of $DPPH^{\bullet}$ 0.025 $g \cdot l^{-1}$ in methanol prepared daily. A decrease in absorbance of samples was measured at λ = 515 nm (Jena Optik). The faster the decrease in absorption, the stronger the antioxidant, possessing a higher ability of hydrogen donation. The range of extract concentrations and measurement frequencies was established experimentally. The antiradical value represents the percentage of radical scavenging ability according to the following formula:

$$\% \text{ scavenging effect} = [(Ab_k - Ab_b) / Ab_k] \cdot 100$$

where:

Ab_b – sample absorbance,
Ab_k – control sample absorbance.

$ABTS^{+\bullet}$ radical scavenging method. Radical scavenging activity of extracts was measured according to Re et al. [1999]. The $ABTS^{+\bullet}$ solutions with a stable absorbance at λ = 734 nm were used for the determination. Antiradical activity of examined samples was presented as the percentage of radical scavenging and calculated using the same equation as in the $DPPH^{\bullet}$ method.

Antioxidant activity. Antioxidant activity of carotenoid extracts was measured in emulsified and bulk lipid systems: linoleic acid emulsion and bulk sunflower oil.

Linoleic acid emulsion. The linoleic acid emulsion (NuChek Prep. USA) was used to evaluate the antioxidant potential of *Phaffia rhodozyma* carotenoid extract. Emulsions [10 mM] were freshly prepared according to the method by Lingnert et al. [1979] with the use of phosphate buffer (pH 7.0) and Tween 20 (Sigma). On the basis of earlier analyses the activity of additives was tested at 0.02, 0.05 and 0.1%, counted over the lipid content, while the control sample was lipid with no additives. Extracts were dissolved in methanol and added to the emulsion, mixed and stored. Oxidative stability of the fatty acid emulsion was evaluated using two methods. The first one was a popular method for fast measurement of antioxidant potential of additives after 19h sample incubation at 37°C. The method is based on the evaluation of antioxidant efficiency (AE) with changes in contents of linoleic acid conjugated dienes [Lingnert et al. 1979]. Antioxidant efficiency is the ratio of the difference between an increase in conjugated diene content in an emulsion sample with no antioxidants added and an increase in conjugated diene content in a sample with additives, to an increase in conjugated diene content in an emulsion sample with no antioxidants. Results expressed as AE > 0 represent antioxidant properties, AE < 0 represent prooxidative properties of the additive.

The other method consisted in assessment of linoleic acid emulsion oxidation stage with periodical measurements of peroxides [Kawashima et al. 1981], thiobarbituric acid reactive substances (TBARS) [Buege and Aust 1978] and conjugated linoleic acid (CLA) diene contents [Lingnert et al. 1979]. Lipid emulsions with additives were incubated at 37°C for 96 h, until the completion of the induction period. For the purpose of the study it was decided to apply extract concentration of 0.1% counted in relation to linoleic acid content.

Sunflower oil. The oxidative stability of bulk oil – sunflower oil, with an addition of carotenoids was determined by three methods: Rancimat, Oxidograph and Schaal oven tests. Based on earlier results additive concentrations of 0.02, 0.05 and 0.1% in relation to lipid content were used (Rancimat and Oxidograph test), for further analysis using the Shaal oven test additive concentrations were limited to 0.1%.

The first method was the Rancimat test (Metrohm, Switzerland) [Laubli and Bruttel 1986]. The amount of 2.5 g lipid was weighed into a reaction vessel, placed in a heating block kept at 110°C, with airflow of 20 L/h. During the oxidation process volatile compounds are released and collected in the receiving flask filled with ddH_2O, and then their conductivity is measured and recorded. Results of the experiments are automatically evaluated by the Rancimat software. The completion of the induction period (Ip) is indicated with a sudden conductivity increase caused by the dissociation of volatile carboxylic acids. On the basis of obtained Ip readings, antioxidant efficiency of additives was established. The protection factor (PF) was determined as the ratio of the induction period of a sample with antioxidants to the induction period of the control.

Protection factor (Pf) = Induction period of sample / Induction period of control

The second test used was the Oxidograph test (Microlab Aarhus, Denmark) [Larsen 1989, Gramza et al. 2007]. The amount of 5 g of lipid was weighed into a reaction vessel, placed in a heating block kept at 110°C. The method is based on the measurement of pressure reduction in a reaction vessel with a heated (110°C) lipid sample. The induction period was evaluated graphically. The protection factor was evaluated similarly as in the Rancimat test.

The third test used was the Schaal oven test, where lipid samples with additives were incubated at 60°C in darkness for six days. The incubation period was limited by peroxide content (lipid samples were incubated until the level of 50 meq O_2/kg was recorded) [Pardun and Kroll 1970]. The rate of lipid oxidative stability was recorded periodically, every 24 h of samples incubation. The chemical analysis of bulk sunflower oil oxidation included the determination of peroxide (PV) [PN ISO 3960: 2005], anisidine (AV) and total oxidation values (TOTOX) [PN EN ISO 6885: 2001]. Similarly to Rancimat and Oxidograph tests, the protection factor (PF) of additives was determined.

Statistical analysis. The results were obtained from a minimum of three independent experiments and averaged. Data were analysed by the analysis of variance ($p \leq 0.05$) to estimate the differences between values of tested compounds. The correlations between variables were analysed by simple and multiple regressions. Results were processed by the computer program Statistica™ 8.0 (StatSoft).

RESULTS AND DISCUSSION

Total carotenoid content

The two-step drying method made it possible to determine dry matter of *Phaffia rhodozyma* yeast CBS 5626 at 5.1 g/L. The analysis of total carotenoid content in the produced extract showed 1.32 mg in terms of a culture unit (1 L) and 0.26 g in terms of yeast dry matter (1 kg). Astaxanthin was the main carotenoid produced by the studied yeast and accounted for almost 80% total carotenoids (Fig. 1). Obtained pigment yields were typical of *Phaffia rhodozyma* wild strains [Flen et al. 1999, Palagyi et al. 2001, An et al. 1989, Echavarri-Erasun and Johnson 2004, Stachowiak and Czarnecki 2007]. The primary carotenoid concentration was too low for antioxidant activity measurements, so *Phaffia rhodozyma* carotenoid extracts were concentrated up to 21.67 μg/mL and further used.

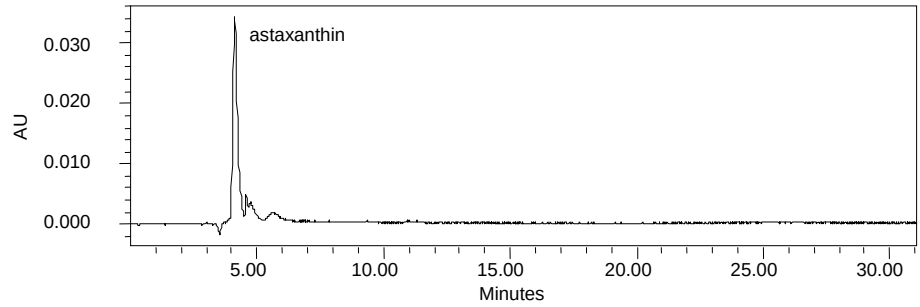

Fig. 1. HPLC chromatogram of carotenoid extract from *Phaffia rhodozyma* CBS 5626

Antiradical activity

Investigations of radical scavenging activity of *Phaffia rhodozyma* yeast extract were conducted for two different radicals: $DPPH^{\bullet}$ and $ABTS^{+\bullet}$. To determine antiradical activity the original yeast extracts solvent – hexane was replaced with methanol. Total carotenoid content was evaluated to be 5.08 μg/mL.

The $DPPH^{\bullet}$ radical scavenging activity was presented as the percentage of radical scavenging (%). Results showed that there were no differences in percentage of radicals scavenged in examined extract concentrations (Table 1). The highest percentage of scavenged radicals was recorded for an addition of 0.05% carotenoid extract (94.58%), while the other extracts at different concentrations were slightly weaker scavengers.

The $ABTS^{+\bullet}$ radical scavenging activity was presented similarly as in the $DPPH^{\bullet}$ method, as the radical scavenging activity (%). Results showed that all tested concentrations of the *Phaffia rhodozyma* extract scavenged the $ABTS^{+\bullet}$ radical at a similar percentage of 48% (Table 1).

Results of antiradical activity measurements showed a high potential of the *Phaffia rhodozyma* extract. A comparison of two methods results showed a higher radical scavenging activity of examined additives in the presence of the $DPPH^{\bullet}$ radical than in case of $ABTS^{+\bullet}$. Because antiradical activity measurement methods depending on the manner

Table 1. Antiradical potential of *Phaffia rhodozyma* carotenoids extract

Additive	Concentration %	DPPH$^{\bullet}$	ABTS$^{+\bullet}$
		radical scavenging %	
Phaffia rhodozyma extract	0.02	93.33 ^{ab}	48.42 ^{a}
	0.05	94.58 ^{a}	48.70 ^{a}
	0.10	90.44 ^{b}	48.89 ^{a}

a, b – mean values with different letters differ statistically in the column ($p < 0.05$).

of radical generation and its type, it suggests that the carotenoid extract could have a higher affinity to the lipophilic fraction rather than hydrophilic [Gramza-Michałowska 2007]. Also the antiradical potential measurements, suitable for simple non-complex products, could possibly fail in the presence of complex substances that are composed of a number of intensifying or masking substances [Huang et al. 2005]. No correlation between carotenoid content and antiradical activity was observed.

Miller et al. [1996] examined antiradical activity of carotenoids in the presence of ABTS$^{\bullet+}$ and found that the highest activity was exhibited by lycopene than β-carotene, while astaxanthin showed a much weaker activity. Results suggested that relative abilities of carotenoids to scavenge the ABTS$^{\bullet+}$ radical cation are influenced by the functional groups with increasing polarities and the number of conjugated double bonds. Liu and Osawa [2007] examined the antiradical activity of astaxanthin and its isomers in the DPPH radical system. It was found that astaxanthin, generally known as all-*trans* astaxanthin, was a less potent antioxidant than its isomers, especially 9-*cis* astaxanthin, which activity was 4-fold higher than that of all-*trans* astaxanthin. Similar tendencies were shown in the lipid peroxidation system and ROS generation in human neuroblastoma cells. Different results were presented by Kobayashi and Sakamoto [1999], who examined singlet oxygen quenching activities of carotenoids, free astaxanthin and its esters. Results showed that astaxanthin esters function as powerful antioxidant agents under both hydrophobic and hydrophilic conditions. Naguib [2000] examined the antioxidant activity of astaxanthin and related carotenoids in fluorometric assay involving peroxyl radical generation. It was found that astaxanthin exhibited a relatively higher antioxidant activity as compared to selected carotenoids tested. According to studies by Di Mascio et al. [1990] and Baron et al. [2005], astaxanthin and canthaxanthin were the best singlet oxygen quenchers, followed by Trolox and α-tocopherol. Mortensen et al. [1997] examined antiradical activity of astaxanthin and β-carotene by pulse radiolysis. It was observed that the rate and mechanism of scavenging are much more dependent on the nature of the oxidizing radical species than the carotenoid structure. In another review it was stated that there is still limited evidence on carotenoid antioxidant activity to support its high effect *in vivo* through their antioxidant potential [1997].

Antioxidant activity

In order to evaluate the antioxidant potential of the obtained carotenoid extract its activity in lipid systems was measured. Linoleic acid emulsion and sunflower oil were selected for the analyses.

Emulsified lipid system – linoleic acid emulsion

The antioxidant activity of carotenoid extracts was evaluated with the use of the linoleic acid emulsion system. Firstly the Lingnert method [1979] was used to find additives with the highest activity concentration. Conjugated diene content was measured in the beginning of the experiment and after 19 hours of storage at 37°C. On the basis of recorded results the antioxidant efficiency (AE) was determined.

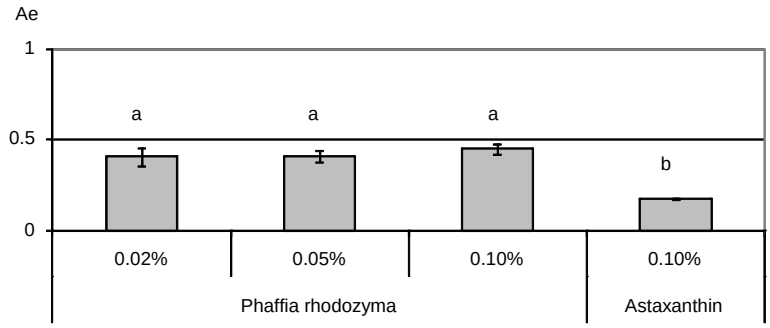

Fig. 2. Antioxidant efficiency (AE) of linoleic acid emulsion with carotenoid extract and astaxanthin incubated for 19 h: a, b – mean values with different letters differ statistically in the row ($p < 0.05$)

It was found that all additives inhibited the oxidation process in the linoleic acid emulsion (Fig. 2). The highest AE value, amounting to 0.45, was found in the carotenoid extract at a 0.1% concentration. The value for other concentrations was slightly lower, but no statistically significant differences were found. A significantly weaker protection was observed in case of the linoleic acid emulsion with an addition of astaxanthin. It was stated that antioxidant efficiency (AE) for the linoleic acid emulsion with the carotenoid extract from *Phaffia rhodozyma* was almost three times higher than that of astaxanthin at the examined concentration.

The next step included linoleic acid emulsion storage for 96 hours and the evaluation of primary and secondary oxidation products (Fig. 3). During the 96-hour incubation of linoleic acid emulsion with carotenoid extract three autoxidation process stages were distinguished: initiation, propagation and the termination period. Evaluation of peroxide content in the examined emulsion samples showed higher levels than in the control sample during the 96-hour incubation. The highest peroxide content was recorded for astaxanthin (0.1%) and it was lower in the *Phaffia rhodozyma* extract (0.1%) emulsion samples. Peroxide content was consistently increasing until the 48th hour of incubation to increase rapidly at the 72nd hour, to decrease slightly again at the 96th hour. The experiment was planned to last until the induction period is observed. When analysing peroxide content it was suggested that an addition of the carotenoid extract and astaxanthin enhanced the oxidation process. The level of TBARS was consistently increasing until the 48th hour of incubation to increase rapidly at the 72nd hour. Results showed that until the second day of incubation all additives had a negative effect on the oxidative stability of the emulsion, which was reversed starting from the third day. The lowest

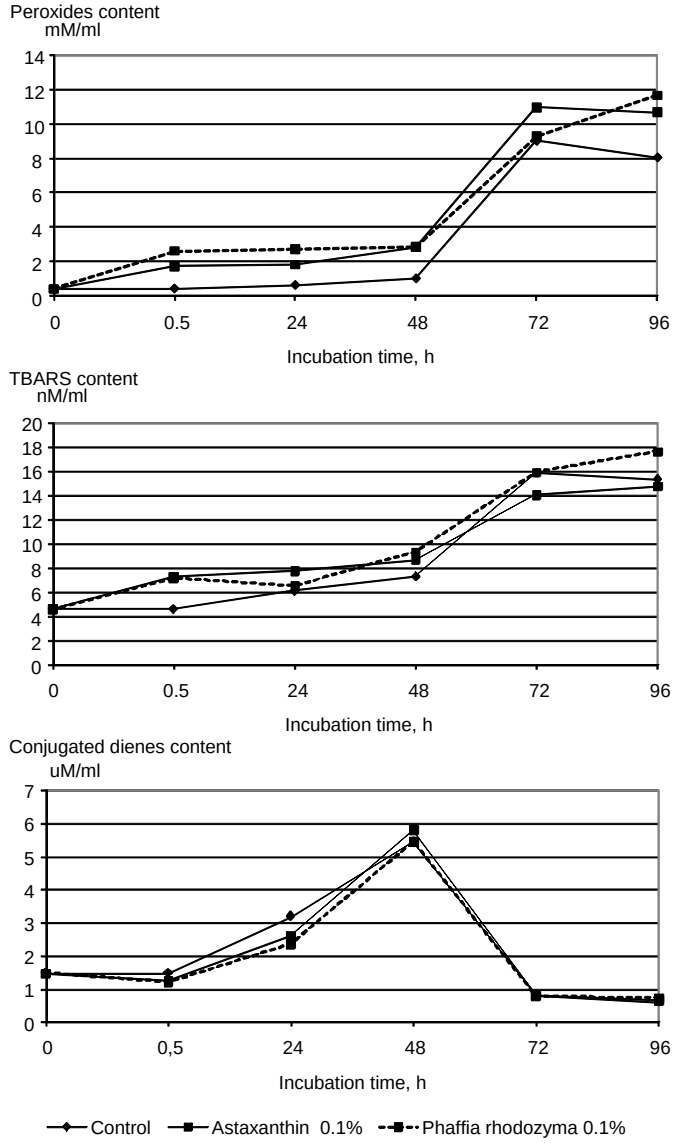

Fig. 3. Oxidative stability of linoleic acid emulsion incubated for 96 h with an addition of *Phaffia rhodozyma* extract and astaxanthin

increase in the level of secondary oxidation products was recorded in the emulsion with an addition of the *Phaffia rhodozyma* extract and astaxanthin. Linoleic acid conjugated diene content increased rapidly until the 48th incubation hour, to decrease suddenly at the 72nd hour and stability. Longer storage resulted in the conjugated diene content of linoleic acid comparable to that of the control sample.

It was observed that in all experiments *Phaffia rhodozyma* extracts exhibited better antioxidant potential in emulsified linoleic acid. No significant antioxidant effect of *Phaffia rhodozyma* carotenoid extracts and the astaxanthin standard on primary products of oxidation process was observed in the experiment. Hydroperoxides produced during the lipid oxidation process are very unstable and undergo different transformations. One of its disintegration products is malondialdehyde. Analyses of secondary oxidation product contents showed their higher content in all samples containing additives as compared to the control. Other samples showed no antioxidant effect under analysed conditions. A further increase in malondialdehyde content in linoleic acid emulsion is evidence of a continuing oxidation process.

There is scarce information in the literature on the role of the antioxidant potential of *Phaffia rhodozyma* astaxanthin in lipid systems. Results reported in a study by Terao [1989] showed that astaxanthin was more effective than β-carotene in retarding hydroperoxide formation on azo-initiated lipid peroxidation in homogeneous methyl linoleate/AMVN systems. It was found that astaxanthin, which contains oxo groups at the 4 and 4' positions in the beta-ionone ring, retarded hydroperoxide formation more efficiently than β-carotene, which possesses no oxo groups. The authors suggested that astaxanthin could be a more effective antioxidant than β-carotene because of stabilizing the trapped radicals. Matsushita et al. [2000] demonstrated that the antioxidant activity of polar carotenoids, i.e. zeaxanthin, astaxanthin and astaxanthin-β-glucoside, in free radical-mediated oxidation of phosphatidyl choline liposomes depends not only on their ability to trap free radicals, but also on their location and orientation in a structured liposome system. In contrast, oxidation of ethyl linoleate in a chloroform solution measured based on hydroperoxide formation was strongly protected by tocopherol, while carotenoids were less effective. A similar tendency was observed in the $DPPH^{\bullet}$ scavenging effect, in which tocopherol was a stronger scavenger than carotenoids. Lim et al. [1992] reported also a stronger antioxidant activity of astaxanthin and zeaxanthin than that of β-carotene in egg yolk liposomes. It was demonstrated that polar astaxanthin and zeaxanthin could be oriented parallel to the hydrocarbon chains of phosphatidyl choline in liposomes and this orientation is favourable for these carotenoids to trap the free radical attack at the liposome bilayer surface. Low antioxidant activity of astaxanthin could be explained by its possible oxidative deterioration. Results reported by Chen and Djuric [2001] showed a substantial depletion of carotenoids after incubation with free radicals generated by Fe^{2+} or AAPH, although no protection against lipid peroxidation in unilamellar liposomes was found. Nishigaki and coworkers [1994] observed that astaxanthin, as the naturally occurring derivatives of vitamin A, suppressed lipid peroxidation in the rat liver microsomal system. The following effect was more potent than that of α-tocopherol.

Bulk lipid system – sunflower oil

Antioxidant stability in bulk lipid was evaluated with the use of three different methods: Rancimat, Oxidograph and Schaal oven tests.

Rancimat test. Sunflower oil with an addition of carotenoids showed oxidative stability, depending on the concentration used. The best antioxidant activity under conditions applied in the Rancimat test was recorded in samples added at a concentration of 0.1% in relation to the lipid content (Table 2). Both the *Phaffia rhodozyma* extract and

Table 2. Antioxidant activity of *Phaffia rhodozyma* extract and astaxanthin in sunflower oil under Rancimat and Oxidograph test conditions

Sample	Concentration %	Rancimat		Oxidograph	
		induction period h	protection factor	induction period h	protection factor
Phaffia rhodozyma extract	0.1	20.98 [c]	3.70	5.20 [a]	1.41
	0.05	6.07 [d]	1.07	4.80 [ab]	1.30
	0.02	5.57 [d]	0.98	4.65 [b]	1.26
Astaxanthin	0.1	25.80 [a]	4.55	4.00 [c]	1.08
Control	0	5.67 [d]	1.00	3.68 [c]	1.00

a, b, c, d – mean values with different letters differ statistically in the column ($p < 0.05$).

astaxanthin showed high activity. The longest induction period of a lipid sample was found for astaxanthin in case of a 0.1% sample (25.80 h) and the *Phaffia rhodozyma* extract at 0.1% (20.98 h). Almost a four time lower activity was observed in other samples. Such induction periods resulted in the recording of equal protection factors, which similarly to induction periods were highest in samples with a 0.1% addition of astaxanthin (4.55). A protection factor above 1.00 indicates antioxidant effect of a given additive on examined lipids. Results of analyses showed that all additives except for the *Phaffia rhodozyma* extract at 0.02% (Pf = 0.98) protected sunflower oil from deterioration.

Oxidograph test. Antioxidant activity of the carotenoid extract and astaxanthin showed a protective effect of additives on sunflower oil under Oxidograph test conditions. Among additives used the *Phaffia rhodozyma* extract exhibited a significantly higher activity than astaxanthin did (Table 2). It was found that its activity depended on the concentration used ($p < 0.05$), and it decreased with a decrease in concentration. The induction period of a lipid sample with a 0.1% addition of the *Phaffia rhodozyma* extract was found at 5.20 h. Protection factor values confirmed results concerning the induction period of lipid samples. Results showed a weaker protection of additives in the Oxidograph test than in Rancimat. Such a difference resulted from specific evaluations that are basic for the tests used. Rancimat makes it possible to measure carboxylic acids as an increase in the level of oxidation products, while in the Oxidograph test measures the oxygen consumption by lipid samples.

Behaviour of lipids and antioxidants in accelerated oxidation tests such as Rancimat or Oxidograph does not always correspond with their behaviour under normal conditions. Sometimes different tests could lead to different a result that is why more experiments are needed to confirm antioxidant activity of examined constituents in bulk lipids. To show a wider potential of the *Phaffia rhodozyma* extract antioxidant activity was analysed using the Schaal oven test. The above mentioned test shows the kinetics of oxidation in sunflower oil by presenting changes in primary and secondary oxidation products. Antioxidant activity of tested additives was presented as the Totox value, which is a total content of primary and secondary lipid oxidation products.

The content of primary oxidation products, presented as the peroxide value (PV), increased regularly in sunflower oil (Fig. 4). The highest lipid oxidation protection values

were recorded in samples with an addition of the *Phaffia rhodozyma* extract (0.1%); however, the astaxanthin standard exhibited a significantly lower activity. For the lipid sample with an addition of the carotenoid extract the peroxide value was 50 meq O_2/kg in a longer time (> 120 h) than in case of astaxanthin (96 h), which was not different from with the figures for the unprotected sunflower oil sample.

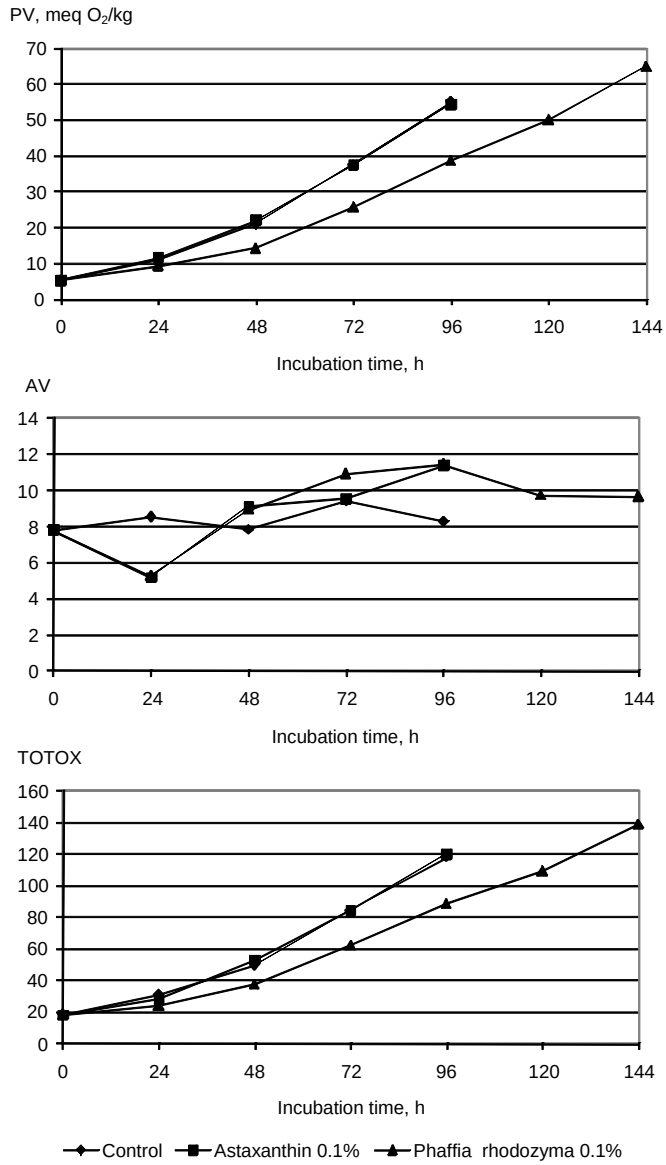

Fig. 4. Oxidative stability of sunflower oil incubated for 144 h with addition of *Phaffia rhodozyma* extract and astaxanthin

Anisidine value (AV) is an indicator of contents of secondary oxidation products in a lipid sample. Results showed that in all examined samples the accumulation of secondary oxidation products varied (Fig. 4). During the first 24 hours of incubation the content of secondary oxidation products decreased in all samples except for the control sample, whereas further incubation resulted in a sudden increase of their content. It showed that the protective action of additives was noticeable at the beginning of the incubation period, further incubation showed no lipid protection whatsoever.

Totox value (TOTOX) shows an overall oxidation degree, characterizing both primary and secondary lipid oxidation products. Results of Totox value confirm results concerning trends for peroxide and anisidine values (Fig. 4). The longest accumulation time of oxidation products was evaluated in a sample with an addition of the carotenoid extract from the *Phaffia rhodozyma*. The Totox value for the astaxanthin standard was similar to that of the control sample with no carotenoids added, proving an non--significant effect of lipid protection. No prooxidative activity of additives was found under examined conditions. Results of protection factor (Pf) evaluations showed that a 0.1% addition of the *Phaffia rhodozyma* extract protected sunflower oil 1.5 times longer than it was in case of the sample with a 0.1% addition of the astaxanthin standard.

Becker et al. [2007] examined the synergistic effect of antioxidants in bulk oil under Rancimat test conditions. It was found that quercetin and astaxanthin showed an additive effect at low concentrations and an antagonistic effect at high concentrations. The induction period for purified sunflower oil was close to zero at all examined astaxanthin concentrations (0.25-2 μM/g oil). In the same publication the authors observed astaxanthin in emulsified methyl linoleate. Astaxanthin was expected to orientate close to the interface of the liposome lipid bilayer and protect lipids, but no protection was observed. It was suggested that astaxanthin antioxidant activity in bulk oil was correlated with the polarity of the antioxidants, with a higher effectiveness of hydrophilic antioxidants in bulk lipid systems. However, in emulsions more lipophilic antioxidants protected the lipid phase more effectively, indicating proper localization of the antioxidant at the oil-water interface, where oxidation takes place. The limited efficacy of astaxanthin in bulk oil was explained by its hydrophobicity, due to its localization in the oil and not in the air-oil interface where lipid oxidation is initiated. It was also expected that in multiphase systems, astaxanthin due to the diketo-group would be close to the interface and thus to peroxy radicals. That activity was observed by the protection of methyl linoleate in emulsions, but not in liposomes [Becker et al. 2007, Frankel et al. 1994]. Concluding the differences in the antioxidant activity of compounds in the organised (liposomes or emulsion) or the homogeneous (bulk oil) systems may be explained by the antioxidants partitioning into different phases. Unfortunately, the relationship between partition of antioxidants and their effectiveness in multiphase systems is not very clear [Becker et al. 2007, Frankel et al. 1994].

Literature sources present many studies conducted on natural tissues, concerning astaxanthin content and its influence on lipid oxidative stability. Aubourg et al. [2005] reported a relatively high content of the endogenous antioxidant – astaxanthin in chilled Coho salmon. It was found that in spite of astaxanthin susceptibility to oxidative damage, its content was stable until the end of the experiment and could have possibly contributed to the lipid stability observed in chilled samples. Similar results were presented by Rodriguez et al. [2007], who examined lipid changes related to quality loss in Coho salmon during frozen storage for up to 15 months. Endogenous antioxidants, astaxan-

thin and tocopherol isomers, were remarkably stable throughout the experiment and this might have also contributed to the oxidative stability of frozen farmed Coho salmon lipids.

In another study Jensen et al. [1998] examined antioxidant stability of female rainbow trout (*Oncorhynchus mykiss*) fed with astaxanthin. Frozen storage of raw fish for 18 months significantly reduced astaxanthin and α-tocopherol levels, while lipid oxidation, measured as TBARS, was limited. Astaxanthin did not affect lipid oxidation in the chill-stored smoked product, in contrast to the frozen, raw fish. Astaxanthin seems to protect against the very early stages of lipid oxidation, while α-tocopherol is more important as an antioxidant at more advanced stages of lipid oxidation. Results of the above analysis suggest a complexity of the antioxidative mechanism involved in yeasts extracts antioxidative potential evaluations.

SUMMARY

Phaffia rhodozyma yeast could be a potential source of compounds that might be active in lipid systems. Research showed a potential antioxidant effect of *Phaffia rhodozyma* carotenoid extracts and the astaxanthin standard under examined conditions of emulsified linoleic acid and bulk sunflower oil systems. Results presented a significantly higher ability to scavenge the $DPPH^{\bullet}$ radical in comparison to the $ABTS^{\bullet+}$ radical. Evaluations of lipid oxidative stability with an addition of carotenoids again showed a higher antioxidant effect of the *Phaffia rhodozyma* extract than that of astaxanthin, which did not differ significantly in emulsified linoleic acid incubated for 96 hours. In bulk sunflower oil the protective effect of additives was observed. In both Rancimat and Oxidograph tests antioxidant activity measured using the induction period was high in samples with both additives, depending on their concentration. However, results of the Schaal oven test indicated that a 144-hour incubation of sunflower oil significantly better protected the lipid against oxidation when the *Phaffia rhodozyma* extract was added.

Research on the *Phaffia rhodozyma* antioxidant potential indicated its potential, which is why further precise analyses of culturing and carotenoid extraction procedures are needed. The present study is an element in a wider set of experiments on the antioxidant potential of microbial antioxidants. As far as complex interactions in different lipid systems are concerned, further research aiming at the evaluation of astaxanthin and *Phaffia rhodozyma* carotenoid extracts needs to be conducted.

CONCLUSIONS

On the basis of received results the following statements were formed:

- *Phaffia rhodozyma* yeast could be a source of compounds potentially active in lipid systems;
- according to antiradical activity better properties of *Phaffia rhodozyma* extract were found in presence of $DPPH^{\bullet}$ than $ABTS^{\bullet+}$ radical;
- evaluations of lipid oxidative stability with an addition of carotenoids showed a higher antioxidant effect of the *Phaffia rhodozyma* extract than that of astaxan-

thin in bulk sunflower oil, no statistically significant different results were evaluated in emulsified linoleic acid;
– research on the *Phaffia rhodozyma* antioxidant activity indicated its moderate potential, which is why further precise analyses are needed.

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POTENCJAŁ PRZECIWUTLENIAJĄCY EKSTRAKTU KAROTENOIDÓW Z DROŻDŻY *PHAFFIA RHODOZYMA*

Wstęp. Karotenoidy są związkami pełniącymi ważną rolę w układach biologicznych: chronią przed promieniowaniem słonecznym, są związkami przeciwnowotworowymi oraz wykazują potencjał przeciwutleniający. Rynek dodatków do żywności jest opanowany przez substancje syntetyczne, jednakże dzięki wzrastającej świadomości konsumentów nastąpiło zwiększenie wykorzystania przeciwutleniaczy naturalnych. Astaksantyna, karotenoid pochodzący ze świata mikroorganizmów, wykazuje potencjalne właściwości przeciwutleniające. Przeprowadzone badania miały na celu określenie potencjału przeciwutleniającego ekstraktów karotenoidów z drożdży *Phaffia rhodozyma* oraz standardu astaksantyny w układach zawierających syntetyczne rodniki oraz tłuszcze zemulgowane i w masie.

Materiał i metody. Do badań wykorzystano karotenoidy ekstrahowane z *Phaffia rhodozyma* oraz standard astaksantyny. Zdolność zmiatania wolnych rodników oznaczono metodami z wykorzystaniem $DPPH^{\bullet}$ i $ABTS^{\bullet+}$. Potencjał przeciwutleniający w układach lipidowych analizowano za pomocą testów Rancimat, Oxidograph oraz termostatowego Schaala.
Wyniki. Rezultaty wskazują na większą zdolność zmiatania rodnika $DPPH^{\bullet}$ w porównaniu z rodnikiem $ABTS^{\bullet+}$ przez badane ekstrakty. Efekt ochronny zanotowano także w układach zawierających zemulgowany kwas linolowy (19 h inkubacji) i olej słonecznikowy. W teście Rancimat i teście Oxidograph okres indukcyjny prób z dodatkiem karotenoidów był dłuższy niż w próbie kontrolnej i zależał od ich stężenia w układzie. Wyniki analiz stabilności oksydacyjnej tłuszczu na podstawie testu Schaala wskazują na większą stabilność prób z dodatkiem ekstraktu z drożdży *Phaffia rhodozyma* w porównaniu ze standardem astaksantyny.
Wnioski. Na podstawie uzyskanych wyników badań stwierdzono, że ekstrakt karotenoidów z drożdży *Phaffia rhodozyma* wykazuje potencjalne właściwości przeciwutleniające, zależne od stężenia oraz warunków prowadzonych analiz.

Słowa kluczowe: *Phaffia rhodozyma*, karotenoidy, astaksantyna, utlenianie tłuszczów, zdolność wygaszania rodników

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