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Chlorella vulgaris biomass enriched by biosorption of polyphenols

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A B S T R A C T

Cell walls of microalgae are variable and contain non-specific domains where different molecules can bind. The enrichment of microalgal biomass with nutrients through adsorption can be an interesting process for the food and feed industry. In this study, naturally occurring polyphenols ((+)-catechin, (−)-epicatechin, quercetin, rutin and xanthohumol) were adsorbed onto nonliving cells of freshwater microalgae *Chlorella vulgaris*. The essential adsorption parameters such as biomass dose and contact time were examined and the adsorption was quantified with Langmuir, Sips and Dubinin–Radushkevich adsorption isotherms. The evaluation of isotherms proved the highest affinity towards *Chlorella vulgaris* biomass for xanthohumol and quercetin. The biosorption mechanism of *Chlorella vulgaris* biomass was well described by a pseudo second order kinetic model, with a high regression coefficient. The polyphenol-enriched microalgal biomass was also evaluated for its antioxidant activity. The highest antioxidant activity was detected in the case of biomass enriched with quercetin (77.5% of decolorized DPPH).

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1. Introduction

Polyphenols represent a broad and diverse group of plant secondary metabolites commonly occurring in fruits, vegetables and beverages (beer, vine, tea etc.). In recent years, many positive effects of these substances have been proved on human health, for example cardio-protective [1], anti-carcinogenic [2] and anti-diabetic [3]. From the first decade of this century an increased attention has been dedicated to the research of hop prenylflavonoids (xanthohumol and 8-prenylnaringenin). The strong antioxidant [4], estrogenic [5], antiviral and cancer chemopreventive properties [6] of these compounds have been demonstrated.

Microalgae represent a group of microorganisms that are easy to culture since they achieve high growth rates and productivities under appropriate conditions. Consequently their biotechnological applications continuously expand. The use of microalgae (mostly *Chlorella* or *Spirulina*) as sorbents in terms of living and non-living biomass has been broadly described for several cases, e.g., in the case of biomass enrichment with microelements (e.g., Se, I, Fe, and Mn) in order to obtain a product of higher nutritional value [7]. Such biomass is marketed as functional food, having human health benefits or as mineral feed additives that are used for example to enrich rotifers as larval fish food in aquaculture, maintain healthy livestock, piggeries and poultry, or to increase the quality of eggs [8,9]. Furthermore, microalgae are effective as low-cost adsorbents for removing toxic metals (e.g., Cu, Cd, Zn, and As) or organic dyes from the environment [10–12].

It is apparent that cell surface structures of microalgae display a rich variety of binding possibilities for a whole range of chemical compounds. Nevertheless, applying microalgal biomass for sorption/accumulation of bioactive compounds for either compound separation or biomass enrichment is still unexplored. The present study focuses on a novel use of nonliving freshwater *Chlorella* biomass as a biosorbent of phenolic compounds, which are ordinarily present in plants, fruits, beverages as well as in agro-industrial wastes. The enrichment of microalgal biomass by a phenolic compound was evaluated by the increased antioxidant activity of *Chlorella vulgaris* biomass.

2. Methods

2.1. Characterization of biosorbent

Chlorella vulgaris Beijerinck strain P12 was obtained and maintained according to previously described procedures [13]. Batch cultivation in the photobioreactor proceeded in glass tubes situated in a water bath (30 °C) under continuous illumination at 100 μmol/m^2/s (PAR sensor QSL-2101, Biospherical Instruments Inc., USA) and feeding of a mixture of air with 2% CO_2 (v/v) at 15 L/h per tube. Each tube contained 300 mL of mineral medium, having the initial composition (mg/L): 1100 $(NH_2)_2CO$, 238 KH_2PO_4, 204 $MgSO_4 \cdot 7\,H_2O$, 40 $C_{10}H_{12}O_8N_2NaFe$, 88 $CaCl_2$, 0.832 H_3BO_3, 0.946 $CuSO_4 \cdot 5\,H_2O$, 3.294 $MnCl_2 \cdot 4\,H_2O$, 0.172 $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$, 2.678 $ZnSO_4 \cdot 7\,H_2O$, 0.616 $CoSO_4 \cdot 7\,H_2O$, and 0.0014 $(NH_4)VO_3$. The pH value was adjusted to 6.5–7.0 using 1 M KOH prior to inoculation from an agar plate. The medium was treated as for outdoor culture so it was not sterilized, but distilled water was used nevertheless. After 144 h of cultivation ensuring linear growth a

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biomass concentration of 5 g/L was obtained. Subsequently, the microalgal cells were centrifuged and washed twice with distilled water (7800 *g*, 5 min), lyophilized (Heto Power Dry LL3000, Thermo Fisher Scientific, USA) and stored at 4 °C before use. Prior to sorption experiments the lyophilized *Chlorella* biomass was treated with 5% ethanol (v/v) in Erlenmeyer flasks for 24 h at 20 °C and 115 rpm (Minitron, Infors HT, Switzerland).

2.2. Preparation of polyphenol solutions

Stock solutions (100 mg/L) of phenolic compounds: *p*-hydroxybenzooic acid (99% purity), protocatechuic acid (97% purity), gallic acid (99% purity), *p*-coumaric acid (98% purity), caffeic acid (98% purity), *trans*-ferulic acid (99% purity), (+)-catechin (98% purity), (−)-epicatechin (90% purity), quercetin (95% purity), rutin (94% purity) from Sigma-Aldrich, and xanthohumol (98% purity) from Hopsteiner (Mainburg, Germany) were prepared by dissolving the desired amount of polyphenol standard in 10% (v/v) ethanol in distilled water. Due to their limited solubility in 10% (v/v) ethanol, the stock solutions of xanthohumol, quercetin and rutin were prepared in 50% (v/v) ethanol. The solutions for adsorption experiments (1–30 mg/L) were prepared by diluting the stock solutions. The ethanol concentration of these solutions (pH 4) was adjusted to 5% (v/v) for (+)-catechin and (−)-epicatechin, and 25% (v/v) for xanthohumol, quercetin and rutin.

2.3. Adsorption experiments

All adsorption experiments were carried out in 250 mL of polyphenol solution (pH 4) with nonliving *Chlorella vulgaris* biomass. The mixture was shaken at 20 °C and 115 rpm (Minitron, Infors HT, Switzerland). Subsequently, the biomass was centrifuged and the supernatant analyzed by HPLC. All results are presented as a mean from three independently conducted experiments.

The pre-selection adsorption experiments were carried out in polyphenol solutions (10 mg/L) with 625 mg of dry *Chlorella vulgaris* biomass. The mixtures were shaken for 2 days.

The experiments aimed at the effect of contact time were carried out in polyphenol solutions (10 mg/L) with 625 mg of dry *Chlorella vulgaris* biomass. The mixtures were shaken for 2 days.

The experiments aimed at the effect of biomass dosage were carried out in polyphenol solutions (10 mg/L) with 125–750 mg of dry *Chlorella vulgaris* biomass. The mixtures were shaken for 2 days.

Adsorption isotherms were measured in polyphenol solutions (1–30 mg/L) with 625 mg of dry *Chlorella vulgaris* biomass. The mixtures were shaken for 2 days.

Experiments without biomass were carried out for all studied polyphenols (10 mg/L, 2 days shaking). A decrease in individual polyphenol concentration caused by oxidation and adsorption to glassware was observed in the case of caffeic acid (<5%), gallic acid (ca. 20% rel.), catechin, epicatechin and quercetin (all ca. 10%). The presented results were corrected for these losses. The results of adsorption are presented as a mean from three independently conducted experiments. The maximum standard deviation was 3% rel.

2.4. HPLC analyses

Separations of phenolic compounds were performed using an Agilent Eclipse XDB-C18 column (5 μm; 4.6 × 150 mm) and the HPLC system Agilent 1100 equipped with a photodiode array detector working in the range of 180–810 nm. In all cases the volume of the injected sample was 20 μL and column temperature was 30 °C. The mobile phase in the case of quercetin, rutin and xanthohumol consisted of solvent A (0.02% v/v of *o*-phosphoric acid in acetonitrile) and B (0.05% v/v of *o*-phosphoric acid in ultrapure water). Gradient conditions applied in the separation were performed as follows: from 10 to 90% of solvent A in the first 25 min and from 90 to 10% of solvent A in the next 5 min. Each run was followed by an equilibration period of 5 min. Quercetin and rutin were analyzed at 253 nm, and xanthohumol at 368 nm. For the analyses of phenolic acids, catechin and epicatechin the mobile phase consisted of solvent A (0.02% v/v of *o*-phosphoric acid in methanol) and B (0.02% v/v of *o*-phosphoric acid in ultrapure water). Gradient conditions used in the separation were performed as follows: from 5 to 10% of solvent A in the first 5 min, from 10 to 25% of solvent A in the next 20 min, from 25 to 90% of solvent A in another 5 min and from 90 to 5% of solvent A in the last 5 min. Each run was followed by an equilibration period of 5 min. Protocatechuic acid and *p*-hydroxybenzoic acid were analyzed at 253 nm, gallic acid, (+)-catechin and (−)-epicatechin at 280 nm and caffeic acid and *trans*-ferulic acid at 330 nm.

2.5. Langmuir, Sips and Dubinin–Radushkevich adsorption isotherms

Adsorption isotherm equations are used to describe the experimental sorption data and can lead to the elucidation of how adsorbates interact with the adsorbents. Three isotherm equations have been tested in the present study.

Adsorption of polyphenols onto the microalgae cell surface was described by the Langmuir [14], Sips [15] and Dubinin–Radushkevich [16] adsorption isotherms.

The linearized form of Langmuir adsorption isotherm is expressed as:

$$\frac{1}{q_e} = \frac{1}{b \cdot Q_0 \cdot C_e} + \frac{1}{Q_0} \tag{1}$$

where q_e (mg/g) is the amount of adsorbed polyphenol per unit weight of microalgae sorbent at equilibrium, C_e (mg/L) is the equilibrium concentration of polyphenol in work solution, Q_0 (mg/g) is the Langmuir constant, which gives an information about the amount of adsorbed polyphenol per unit weight of microalgae adsorbent (maximum monolayer adsorption capacity) and b (L/mg) is the Langmuir constant related to the affinity of the binding sites (energy of adsorption).

The Langmuir constant b can be used for the prediction of adsorbent to adsorbate affinity using the dimensionless separation factor R_L [17]:

$$R_L = \frac{1}{1 + b \cdot C_0} \tag{2}$$

where C_0 (mg/L) is the initial polyphenol concentration. Values of $R_L < 1$ reflect favorable adsorption. If the values of R_L are 0 or >1, the adsorption is irreversible or unfavorable, respectively.

Sips [15] proposed an equation that can be expressed by:

$$Qe = \frac{K_s \cdot C_e^{\frac{1}{b_S}}}{1 + a_S \cdot C_e^{\frac{1}{b_S}}}. \tag{3}$$

K_S (L^{bS} mg^{1-bS}/g), a_S $(L/mg)^{bS}$ and b_S are the Sips isotherm parameters. This equation is also called Langmuir–Freundlich isotherm and the name derives from the limiting behavior of the equation. At low adsorbate concentrations it effectively reduces to a Freundlich isotherm and thus does not obey Henry's law. At high adsorbate concentrations, it predicts the monolayer sorption capacity characteristics of the Langmuir isotherm.

Sorption data were also subjected to Dubinin–Radushkevich modeling [16] and this is represented by the following linearized equation:

$$\ln\ Q_e = \ln\ Q_d - 2B_d \cdot R \cdot T \cdot \ln\left(1 + \frac{1}{C_e}\right) \tag{4}$$

where Q_d is the theoretical saturation capacity (mg/g), B_d is a constant related to adsorption energy (mol^2/kJ^2), R is the gas constant (kJ/mol·K) and T is the temperature (K). The apparent energy (E_d) of adsorption

from Dubinin–Radushkevich isotherm model can be calculated by the following equation.

$$E_d = \frac{1}{\sqrt{2B_d}}. \quad (5)$$

If the value of E_d is less than 8 kJ/mol, the adsorption process is physical in nature. If it ranges between 8 and 16 kJ/mol, it consists mainly of ion-exchange and higher values indicating strong chemisorption between the adsorbent and the adsorbate [18].

2.6. Pseudo-first and pseudo-second order adsorption kinetics

The kinetic behavior was evaluated by pseudo-first and pseudo-second order models. The linearized forms of both models are shown below as Eqs. (6) and (7), respectively:

$$\mathrm{Log}(Q_e - q_t) = \mathrm{Log}Q_e - k_1 \cdot t \quad (6)$$

$$\frac{t}{q_t} = \frac{1}{k_2 \cdot Q_e^2} + \frac{t}{Q_e} \quad (7)$$

where Q_e is the amount of adsorbate at equilibrium per mass unity of adsorbent (mg/g) as previously defined, q_t the amount of adsorbate at time t per mass unity of adsorbent (mg/g), k_1 the pseudo-first order rate constant (1/h) and k_2 is the pseudo-second order rate constant (g/mg·h).

2.7. Antioxidant activity

The nonliving *Chlorella vulgaris* biomass (625 mg in dry weight) was shaken in 250 mL of polyphenol solution (10 mg/L) for 2 days (20 °C, 115 rpm) and then it was centrifuged and freeze-dried (Heto PowerDry LL 3000, Denmark). Subsequently the biomass was crushed using a pestle and mortar and ultrasound-extracted (Sono Swiss SW 3H, Switzerland) with 5 mL of 90% v/v ethanol for 30 min. Next, the suspension was centrifuged and the pellet was re-extracted two more times (5 mL of 90% v/v ethanol for 30 min). The extracts were merged and stored at −20 °C. The antioxidant activities (AA) of microalgal biomass extracts, both with and without adsorbed polyphenols, were determined by a modified method according to Kaneda et al. (1995) [19] based on the reaction between 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing substances (polyphenols in microalgal extracts), which is accompanied by a color change. The antioxidant activity (% of decolorized DPPH) of the samples was expressed as a relative decline of the absorbance (525 nm) of the DPPH solution. In order to compare the AA of pure polyphenols, the decolorization of DPPH was determined also for polyphenol solutions (10 mg/L dissolved in 90% v/v ethanol). The results of AA are presented as a mean from three independently conducted experiments. The maximum standard deviation was 4% of decolorized DPPH.

3. Results and discussion

The nonliving biomass of green microalgae *Chlorella vulgaris* was evaluated for its ability to adsorb 11 commonly occurring plant polyphenols, belonging to five structurally diverse subclasses (Table 1). As can be seen from the results of pre-selection experiments, significant differences were observed in the adsorption of flavonoids and phenolic acids. The binding of phenolic compounds on the surface of microalgal cells is apparently strongly affected by the molecular structure, particularly the number of aromatic rings in the polyphenol molecule. Medium or high sorption ability was proved for the large molecules of flavonoids with two aromatic rings, while the small phenolic acids with one aromatic ring were characterized by low or inconclusive polyphenol uptake (Table 1). For this reason, the following experimental campaign was carried out only with flavonoids.

Table 1
Polyphenol uptake (initial conc. 10 mg/L) by nonliving *Chlorella vulgaris* biomass (2.5 g/L) as biosorbent during pre-selection experiments.

Class	Subclass	Compound	Polyphenol uptake[a]
Phenolic acids	Benzoic acid derivates	*p*-Hydroxybenzoic acid	Low
		Protocatechuic acid	Low
		Gallic acid	Low
	Cinnamic acid derivates	*p*-Coumaric acid	Low
		Caffeic acid	Low
		Trans-ferulic acid	Low
Flavonoids	Flavan-3-ols	Catechin	Medium
		Epicatechin	Medium
	Flavonols	Quercetin	High
		Rutin	Medium
	Prenylflavonoids	Xanthohumol	High

[a] Low (<5% rel.); medium (5–50% rel); high (>50% rel.).

The effect of pH on the adsorption of polyphenols onto nonliving *Chlorella vulgaris* biomass was not studied given the fact that polyphenols are substances whose structure and behavior is strongly influenced by pH [20]. The compounds used in this work are stable at acidic pH, and therefore the sorption tests were carried out at pH 4.

3.1. Effect of contact time and biomass dosage

The uptake of polyphenols, as shown in Fig. 1, is influenced by contact time. The sorption of phenolic compounds was characterized by an initial fast stage (ca. 2 h), followed by a gradual increase in polyphenol uptake observed until 48 h of contact time (Fig. 1). The fastest adsorption was observed in the case of xanthohumol when 87.5% of the total adsorbed xanthohumol was removed from the solution (10 mg/L) during the first 2 h. In the case of the remaining polyphenols, the initial adsorption (first 2 h) contributed to the total amount of adsorbed polyphenols only by approximately 50% and equilibrium was reached after 48 h. The initial rapid phase may involve adsorption at the cell surface. In contrast, the subsequent slower phase may involve other mechanisms, such as saturation of binding sites. In order to achieve maximum polyphenol accumulation by the nonliving *Chlorella vulgaris* biomass, further adsorption experiments were carried out with 48 h of contact time. Long contact times (180 h) were applied also in Cr(IV) removal by waste *Chlorella vulgaris* biomass [21]. Conversely, the adsorption of malachite green onto a chlorella-based biosorbent reached equilibrium within 1 h [22]. The explanation of this difference in adsorption kinetics can be the cationic character of malachite green leading to a strong electrostatic interaction with

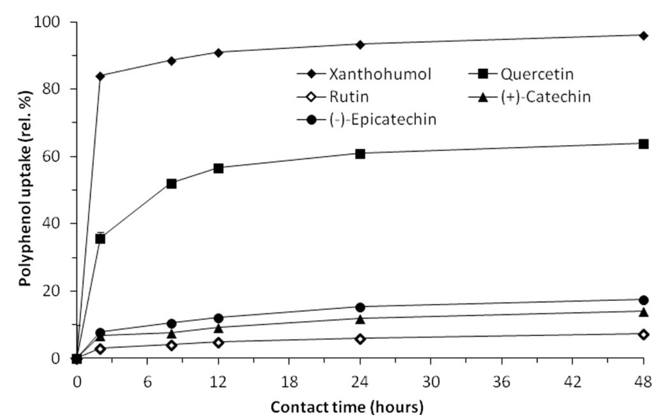

Fig. 1. Effect of contact time on polyphenol uptake (initial conc. 10 mg/L) by nonliving *Chlorella vulgaris* biomass (2.5 g/L) as biosorbent.

Table 2
Parameters of Langmuir, Sips and Dubinin–Radushkevich adsorption isotherms and correlation coefficients (R^2) for the adsorption of polyphenols onto nonliving *Chlorella vulgaris* biomass.

Subclass	Compound	Langmuir parameters			R_L [a]
		R^2	Q_0 (mg/g)	b (L/mg)	
Prenylflavonoids	Xanthohumol	0.994	19.16	0.22	0.31
Flavan-3-ols	Catechin	0.994	7.06	0.18	0.36
	Epicatechin	0.995	7.68	0.10	0.49
Flavonols	Quercetin	0.982	10.60	0.19	0.34
	Rutin	0.996	7.63	0.09	0.53
Subclass	Compound	Sips parameters			
		R^2	K_s (L^{bS} mg^{1-bS}/g)	a_s $(L/mg)^{bS}$	b_S
Prenylflavonoids	Xanthohumol	0.998	4.79	0.32	1.08
Flavan-3-ols	Catechin	0.994	1.17	0.18	1.18
	Epicatechin	0.994	0.69	0.11	1.29
Flavonols	Quercetin	0.993	1.44	0.09	1.17
	Rutin	0.999	0.68	0.14	1.22
Subclass	Compound	Dubinin–Radushkevich parameters			E_d (kJ/mol)
		R^2	Q_d (mg/g)	B_d (mol^2/kJ^2)	
Prenylflavonoids	Xanthohumol	0.94	8.07	0.002	16.22
Flavan-3-ols	Catechin	0.81	4.70	0.007	8.57
	Epicatechin	0.91	4.11	0.011	6.77
Flavonols	Quercetin	0.88	5.56	0.004	10.66
	Rutin	0.92	3.74	0.012	6.57

[a] Calculated for initial polyphenol concentration of 10 mg/L.

negatively charged *Chlorella* cells [23]. Simultaneously, the studied polyphenols do not possess positively charged moieties and therefore their adsorption will be governed by different interactions. In spite of the different kinetics of malachite green and polyphenol adsorption onto *Chlorella vulgaris* biomass, the maximum monolayer adsorption capacity (Q_0) was comparable for malachite green (18.7 mg/g) and polyphenols (7.06–19.16 mg/g).

As can be seen in Fig. 2, the variation of microalgal biomass dose in contact with constant initial polyphenol concentration (10 mg/L) caused different uptakes. Approximately 0.5 g/L of microalgal biomass was sufficient to remove nearly 100% rel. of xanthohumol. The amount of removed quercetin (max. 64% rel.) increased with the concentration of microalgal biomass until 2.5 g/L and then it reached equilibrium. A similar behavior was observed in the case of rutin (quercetin glycoside) and both flavan-3-ols (catechin and epicatechin) but their maximum uptake was significantly lower as compared to xanthohumol and quercetin (Fig. 2). At the initial polyphenol concentration of 10 mg/L, the optimal dose of microalgal biomass was found at 2.5 g/L, resulting in maximum uptakes for all studied polyphenols.

3.2. Adsorption isotherms

The adsorption isotherms were designed to describe the distribution of adsorbate between the solid phase (adsorbent) and liquid phase at equilibrium [24]. Through the modeling of equilibrium data it is possible to characterize biosorbents under various operational conditions and this point is essential for future practical applications. In this study, Langmuir, Sips and Dubinin–Radushkevich adsorption isotherms were used to determine the adsorption mechanism of flavonoids onto the microalgal surface.

The fit of the adsorption equilibrium data for the five pre-selected phenolic compounds was carried out by three isotherms and the related parameters are in Table 2. The best fit was obtained using the Sips equation (Fig. 3) due to the fact that it incorporates the features of Langmuir and Freundlich equations. The Langmuir isotherm also fits the data satisfactorily, which implies a monolayer adsorption mechanism. The Langmuir constant (b) was used to predict the adsorbent to adsorbate affinity through dimensionless separation factor R_L. As can be seen in Table 2, the lowest value of R_L (high adsorbent to adsorbate affinity)

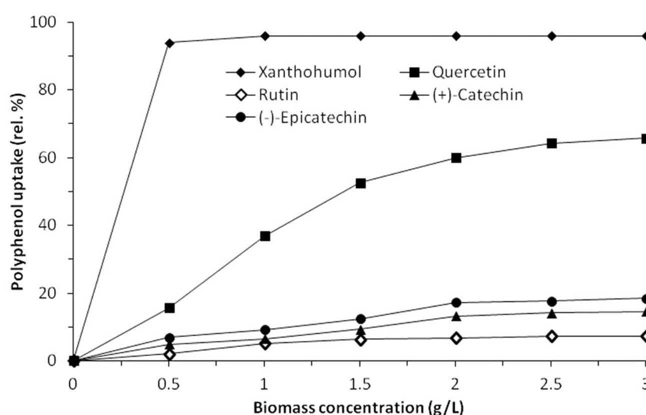

Fig. 2. Effect of biomass dosage on polyphenol uptake (initial conc. 10 mg/L) by nonliving *Chlorella vulgaris* biomass as biosorbent.

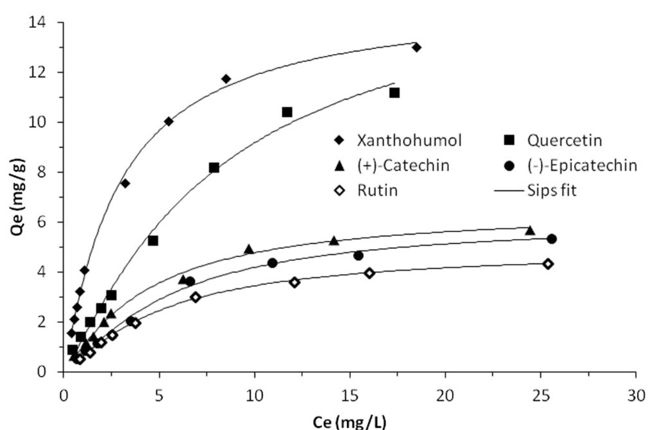

Fig. 3. Isotherm equilibrium data and attempted fitting by Sips model for polyphenol adsorption onto nonliving *Chlorella vulgaris* biomass as biosorbent.

was found for xanthohumol (0.31). The glycosylation of quercetin resulted in an increase of separation factor from 0.34 (quercetin) to the 0.53 (rutin). The separation factors (R_L) of flavan-3-ols were comparable, i.e., 0.87 for catechin and 0.92 for epicatechin. The worst fit was obtained for the Dubinin–Radushkevich isotherm. No matter this fact, the apparent energy of adsorption (E_d) was calculated and its values indicate that the adsorption process is mainly physical in nature for catechin, epicatechin and rutin, while a more significant participation of electron donor/acceptor interactions can play a role in the adsorption of xanthohumol and quercetin onto *Chlorella* biosorbent (Table 2). Xanthohumol and quercetin differ from the flavan-3-ols by the carbonyl group. It can be speculated that the additional carbonyl group can strengthen the adsorption by providing hydrogen bonds with proton donor functional groups of *Chlorella* biosorbent.

As can be seen from the results above, the adsorption of polyphenols is strongly influenced by their molecular structure. It is obvious, that the molecules of xanthohumol, flavonols and flavan-3-ols can interact with the functional groups on the surface of *Chlorella vulgaris* biomass through different mechanisms [25]. The widest variety of interactions is offered by xanthohumol. There can be hypothesized hydrogen bonds (carbonyl and hydroxyl groups), hydrophobic interaction (prenyl group, aromatic rings), π-bond overlap (delocalized electrons of aromatic rings) and van der Waals bonds (dipole–dipole interaction) [26, 27]. The main difference between xanthohumol and other studied polyphenols is in the presence of a prenyl group capable of interacting with hydrophobic components of microalgal biomass such as structural and reserve lipids [28]. Thus the highest affinity of xanthohumol to nonliving *Chlorella vulgaris* biomass can be most probably ascribed to the hydrophobic interactions.

The effect of glycosylation is illustrated with the pair of quercetin and rutin. The substitution of the quercetin C_3 hydroxyl group by a disaccharide rutinose resulted in a considerable reduction of both Langmuir constants (Table 2). This is in accordance with the literature and the reason for this behavior is the hydrophilic saccharide group of polyphenol glycoside [29,30]. This can prevent the possibility of hydrophobic interactions between the aromatic rings of the polyphenol and biosorbent [26].

The adsorption parameters for compounds with a similar molecular structure, such as (+)-catechin, (−)-epicatechin and quercetin, suggested that structural dispositions were of significant importance for adsorption. Catechin and its stereoisomer epicatechin, which differ only in the orientation of the hydroxyl group at C_3, exhibited similar adsorption isotherm parameters (Table 2).

The adsorption disposition of polyphenols to *Chlorella* biosorbent can be compared with that of polyvinylpolypyrrolidone (PVPP), a commercially available agent used for colloidal stabilization of beverages. The comparison of Langmuir (equilibrium) constants clearly demonstrates the higher affinity of the phenolic compound to PVPP [29]. Nevertheless, as the results demonstrated the enrichment of microalgal biomass by the adsorption of polyphenols from weak alcoholic solutions is possible. In addition, as sources of polyphenols for enrichment, natural materials of residual origin or their alcoholic extracts could be exploited [31]. Naturally, the sorption of phenolic substances from natural materials would occur in a more complex system consisting of a mixture of compounds similar in structure.

3.3. Adsorption kinetics

Adsorption kinetics was investigated to understand the dynamics of polyphenol sorption onto *Chlorella* biosorbent. According to Pandey et al. [32], adsorption kinetics is expressed as the removal rate that controls the residence time of the adsorbate at the solid–liquid interface. The popular kinetic models, pseudo-first order and pseudo-second order were applied to the obtained kinetic data and the rate constant values obtained, k_1 and k_2 are presented in Table 3. The correlation coefficient (R^2) values show that the pseudo second-order model fits better for all polyphenols. The pseudo-second order rate assumes that the adsorption process is controlled by surface reaction, with chemisorption involving valence forces, through the sharing or exchange of electrons between biosorbent (algae) and the adsorbed compound [33]. The calculated Q_e values (3.9, 2.7, 0.3, 0.8, 0.6 mg/g) agree with the experimental data (3.8, 2.6, 0.3, 0.7, 0.6 mg/g) for xanthohumol, quercetin, rutin, epicatechin and catechin, respectively. Ofomaja [34] studied the relationship between pseudo-second order parameters and biosorption performance. This author established a relationship, described as the approaching equilibrium factor (R_w), between the pseudo-second order model constants and the characteristic kinetic curve. This approaching equilibrium factor (R_w) is defined as:

$$R_W = \frac{1}{1 + k_2 Q_e \cdot t_{ref}} \tag{8}$$

where t_{ref} is the longest operation time (based on kinetic experiments), Q_e is the uptake value and k_2 is the pseudo-second order constant. Wu et al. [35] refer to four different situations depending on the R_w value: (i) $R_w = 1$, type of kinetic curve: linear, not approaching equilibrium; (ii) $1 > R_w > 0.1$, type of kinetic curve: slightly curved, approaching equilibrium; (iii) $0.1 > R_w > 0.01$, type of kinetic curve: largely curved, well approaching equilibrium; and (iv) $R_w < 0.01$, type of kinetic curve: pseudo-rectangular, drastically approaching equilibrium. When $R_w = 1$, as in the first situation, biosorption is ineffective as equilibrium cannot be reached. In this study, the values for R_w were found to range between 0.03 and 0.23 confirming the good performance of the system used. It means that the kinetic curve is largely curved with a good approach to equilibrium for the lower values (xanthohumol, quercetin) and slightly curved, approaching equilibrium for the higher values (rutin, epicatechin and catechin).

3.4. Antioxidant activity of polyphenol-enriched biomass

Recently, the antioxidant potential of microalgae was demonstrated by various authors [36–38]. It is obvious, that the adsorption of phenolic compounds onto the microalgal cell surface can further increase the natural antioxidant activity (AA) of microalgal biomass (Table 4). The final AA of enriched biomass depends on the amount of adsorbed

Table 3
Constant parameters of pseudo-first and pseudo-second order kinetic models for the adsorption of polyphenols (initial conc. 10 mg/L) onto nonliving *Chlorella vulgaris* biomass.

Subclass	Compound	Pseudo-first order		Pseudo-second order		
		k_1 (h)	R^2	k_2 (g/mg·h)	R^2	R_w
Prenylflavonoids	Xanthohumol	0.03	0.98	0.40	~1	0.03
Flavan-3-ols	Catechin	0.07	0.93	0.26	0.98	0.23
	Epicatechin	0.08	0.96	0.24	0.99	0.20
Flavonols	Quercetin	0.12	0.92	0.19	~1	0.08
	Rutin	0.06	0.91	0.49	0.98	0.23

Table 4
Antioxidant activity (AA) of polyphenols and nonliving *Chlorella vulgaris* biomass with/without polyphenol enrichment.

Material/Compound	AA of pure compound[a] (% of decolorized DPPH)	AA of biomass (% of decolorized DPPH)
Chlorella vulgaris	–	14.6
Xanthohumol	10	17.0
Catechin	42	17.4
Epicatechin	37	17.6
Quercetin	63	77.5
Rutin	35	15.2

[a] Determined for polyphenol concentration of 10 mg/L (dissolved in 90% v/v ethanol).

polyphenol as well as on its antioxidant property. Although xanthohumol has the highest affinity towards *Chlorella* biomass, AA of *Chlorella* biomass increased only by 16.4% after xanthohumol enrichment. This is due to the weak AA of pure xanthohumol (Table 4). On the other hand, the maximum monolayer adsorption capacity (Q_0) of quercetin by *Chlorella* biomass is only half of Q_0 for xanthohumol (Table 2), but the antioxidant activity of the quercetin enriched biomass was the highest among the studied compounds (Table 4). The increase in AA of biomass enriched by xanthohumol, catechin, epicatechin and rutin was lower than it would be proportional to Q_0 and AA for pure compounds. The reason may lie in blocking the H-donating groups of *Chlorella* biomass molecules interacting with polyphenols. Conversely, in the case of quercetin adsorbed onto *Chlorella* biomass, the AA increased more than it would be expected from mass balance. An enhanced AA was already observed for the quercetin–β-cyclodextrin complex and a proposed explanation for this is by the modified redox behavior of the ortho-diphenol group (B-ring) inside a less polar β-cyclodextrin cavity [39,40]. Similar mechanisms can be assumed for quercetin interacting with *Chlorella* biomass molecules, but further experiments would be necessary to confirm this hypothesis.

Based on these results, quercetin seems to be the most suitable phenolic compound to increase the AA of *Chlorella vulgaris* biomass. In addition, quercetin enrichment can confer *Chlorella vulgaris* biomass a whole range of health benefits [41]. Nevertheless, increased antioxidant activity is only one of the manifestations of biomass enrichment by polyphenols. The health benefit of *Chlorella vulgaris* biomass with adsorbed polyphenols should be evaluated comprehensively and the selection of adsorbed polyphenols should be done intentionally with respect to the intended application of microalgal biomass.

4. Conclusions

The nonliving *Chlorella vulgaris* biomass has higher affinity to large polyphenols than to small monoaromatic phenolic acids. Xanthohumol and quercetin are good candidates to increase the nutritional value of *Chlorella* biomass, given their well documented health effects. The different affinity of polyphenols to *Chlorella* biomass has also practical implications in that to increase the polyphenol content of microalgal biomass, some agro-industrial residues or their extracts would be more suitable than others. Presumably the most suitable sources for polyphenol enrichment of *Chlorella* biomass would be the xanthohumol and quercetin rich residues from hop and onion or apple processing, respectively.

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