Surface modification of Chlorella vulgaris cells using magnetite particles

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Abstract

Expensive cell concentration procedures represent one of the bottlenecks of large-scale microalgal biotechnological processes as many industrially attractive species have a small cell size and sustain in suspension. An economically effective solution is to alter the process conditions for the cells to form aggregates, which sediment faster. The use of magnetic agents binding to the cell surface and forming larger complexes, that sediment very fast upon application of an external magnetic field, is a rarely explored possibility in this area. We used commercially available, finely pulverized magnetite (Sigma Aldrich) as a potential harvesting agent and studied its surface interactions with an industrially important microalgal strain (Chlorella vulgaris). Firstly, we characterized the interacting surfaces in model environments by zeta potential and contact angle measurements, which were followed by particle size determination. Secondly, we applied the XDLVO theory to predict favorable experimental conditions for a successful magnetic cell modification, which would lead to an effective biomass separation. The hypotheses were then tested by using various ratios of magnetic agent and microalgal biomass under different environmental conditions. Obtained results were in good accordance with the predictions and we achieved an excellent separation efficiency of over 90% within a few minutes at a ratio of microalgae to magnetite 1:26 (w/w). We can conclude that magnetite successfully modifies the microalgal surface under certain conditions and is a promising agent for harvesting C. vulgaris, enabling high separation efficiencies in a very short period of time, but further research is necessary to optimize the process.

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1. Introduction

Microalgae represent an enormous, but largely untapped resource of various biocatalytic activities, and their biotechnological applications are indeed broad. Currently they are used in the production of human food supplements, aquaculture or animal feed, syntheses of valuable bioactive compounds, bioaccumulation/biosorption of heavy metals, biodegradations of various chemicals or their biotransformation [1-3]. Additionally, microalgae have been proposed as a renewable energy source for decades, whereas the pressing reasons to promote the use of carbon neutral fuels are the constant increase crude oil prices, climate change and global warming [4]. The major advantages of microalgal processes are high productivities, low nutrient requirements (use of flue gases, wastewaters and sunlight), fast responses to environmental changes (effective metabolism regulation through nutrient limitation), and the possibility of successful genetic modification [3, 5-7]. The overall economics of microalgal processes, particularly in the aspect of biofuel production, is always an issue as many industrially attractive microalgal species have a small cell size and sustain in suspension. This leads to costly cell concentration procedures (e.g. centrifugation, microfiltration). An economically friendly possibility is to alter the process conditions for the cells to form aggregates or flocs, which sediment faster [8-10]. The use of magnetic Fe₃O₄ particles, which can attach to the cell surface and form very fast sedimenting larger complexes upon application of an external magnetic field, is a rarely explored and very simple possibility in this area [11, 12].

Generally, the use of magnetic particles is especially valuable when the desired product occurs in a difficult-to-handle environment including raw extracts, blood and other body fluids, cultivation media, etc. [13]. The surface of the particles can be coated with specific functional groups, thus ensuring product specific surface interactions. Magnetic separation techniques are successfully applied in many biotechnological aspects for decades, including cell separations [14]. They prove to be good alternatives to gravitational, centrifugal or filtration separation methods and enable a simple magnetic manipulation with the magnetized materials using an external magnetic field [13]. Magnetic surface modification of whole microalgal cells has been successfully performed, but the cells were either in a dry state [15] or the magnetic agent was prepared via a synthetic procedure under high temperatures and a N₂ atmosphere [11], which can lead to high costs considering large-scale applications. We were interested in finding another possibility of microalgae harvesting by using a naturally occurring magnetic agent and, simultaneously, describing the non-covalent surface interactions by applying a physicochemical approach, the XDLVO theory.

Physicochemical approaches are helpful in shedding some light in the aspect of assessing the Lifshitz–van der Waals (LW), Lewis acid–base (AB) and electrical double layer (EL) forces between microbial cells and (a)biotic surfaces [16-19] and to our current knowledge have not been used in the case of microalgal surface modification and/or harvesting. In the presented study we would like to contribute to this aspect. As magnetite is non-toxic and occurs in nature in the form of a stable magnetic ore that can be pulverized to nanoparticles, we tested it as a potential harvesting agent for the unicellular green alga Chlorella vulgaris. In order to achieve successful binding of the magnetic agent onto the cells, we characterized the interacting surfaces in model environments (10 mM KCl, pH 2-12). Together with particle size
determination we were able to predict favorable conditions for adhesion by the use of the XDLVO theory and the obtained results were then verified in appropriate experiments in test tubes.

2. Materials and methods

2.1. Microorganism, cultivation and preparation of algal suspension

*Chlorella vulgaris* Beijerinck strain P12 was obtained and maintained according to previously described procedures [5]. Photobioreactor batch cultivation proceeded in glass tubes situated in a water bath at 30°C under continuous illumination and feeding of a 98:2 air-CO₂ mixture (v/v) at 250 ml/min per tube. Each tube contained 300 mL of mineral medium based on the elementary composition of algal biomass reported in literature [7]. 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, i.e. it was not sterilized, but distilled water was used nevertheless. Upon reaching a biomass concentration of approximately 5 g/L, the microalgal cells were centrifuged and washed twice with distilled water (4,000 rpm, 5 min.) and used to prepare algal suspensions of a defined absorbance (750 nm) for surface characterizations or magnetic cell separations as described in the following chapters.

2.2. Magnetite

Magnetite was obtained from Sigma Aldrich (product code: 637106) in the form of iron(II,III)oxide nanoparticles (<50 nm declared particle size). If it is suspended in an aqueous environment, magnetite forms heterogeneous mixtures as to the particle’s size (see Chapter 3.1).

2.3. Physicochemical surface characterization of cells and magnetite

For contact angle measurements *C. vulgaris* cells were prepared in the form of a concentrated cell suspension in distilled water and filtered under negative pressure (Whatman filter, 0.45 μm pore size, nitrate cellulose). The resulting uniform microbial lawn on the filter had 7.10⁶ cells/mm². The lawns were then fixed with double-sided adhesive tape onto glass slides. Flat surfaces of magnetite were obtained by preparing 13 mm pellets in an evacuable pellet press (Pike Technologies) at 80 kN. In order to remove the pellet from the press easily, powdered DEAE cellulose (1.0 g) was pressed first and served as “bedding” for the subsequently pressed magnetite (0.1 g). Care must be taken in all steps of pellet manipulation as the magnetite surface is very brittle. Contact angles of three tested liquids (water, formamide and 1-bromnaphtalene) on the cell lawns and magnetite pellet were determined with the CAM 200 goniometer (KSV Instruments, Finland). Prior to measurement the microbial lawns had to be dried for 50 minutes in order to achieve a plateau region ensuring stable contact angle values [17, 19-21]. Individual components of surface tensions and Gibbs energies were calculated according to Kuřec et al. [17].

The Zeta-potentials of *C. vulgaris* cells and magnetite particles were measured at 25°C using the Zetasizer Nano-ZS (Malvern, UK) and calculated according to the Smoluchowski equation. All measurements were carried out in model solutions (10 mM KCl, pH 2-12). The solution’s ionic strength of 10 mM was chosen as representative for most freshwater microalgae culture media. Tested cell suspensions had an absorbance of 0.1 (750 nm). In the case of magnetite, 1% (w/v) was added into each tested solution and the suspension was then placed in an ultrasonic bath for 15 minutes. For zeta potential measurements only the supernatant containing fine particles, which remain in suspension, was used. All experiments were performed in duplicate.
2.4. Cell and particle size determination

Cell and magnetite size determination was carried out using image analysis (NIS Elements, Laboratory Imaging) after observing the studied particles by light microscopy (Nikon Eclipse E400, digital camera Nikon D300s).

2.5. Magnetic separation of microalgal biomass

Prepared microalgal suspensions (10 mL, 10 mM KCl, pH 4 - 12) of defined absorbance (750 nm) were mixed with various, specific amounts of magnetite for 10 minutes (Hulamixer Sample Mixer, Invitrogen). After exposure to an external magnetic field (NdFeB magnets, Neomag) the formed microalgae-magnetite complexes settled in a matter of minutes. The absorbance of the supernatant (3 mL) was then measured at 750 nm and the separation efficiency (E, %) was calculated as follows: \( E = [(A_0 - A_1)/A_0] \times 100 \), where \( A_0 \) is the initial absorbance of the microalgal suspension and \( A_1 \) the absorbance of the supernatant after the magnetic cell separation.
3. Results and discussion

3.1. Physicochemical surface characterization of cells and magnetite

The physicochemical properties of cell and magnetite interacting surfaces were evaluated by the zeta potential (Fig. 1) and contact angle measurements (Table 1). The zeta potentials reveal that *C. vulgaris* cells maintain a negative charge, independently on the pH value. Similar results were obtained by Hadjoudja et al. [22], who also explained that the anionic characteristics of the algal cell wall were caused by deprotonation of surface functional groups (carboxylic, phosphoric, phosphodiester, hydroxyl and amine). Observed zeta potentials of the magnetite particles are pH-dependent, being positive in lower pH values and vice versa, which is in accordance with literature [23]. The ability to change the surface charge of the magnetite by altering the pH of the solution is fundamental in the so called Sirofloc process, where magnetite is used for water treatment [24]. Gregory et al. [24] stress that the actual value of the magnetite’s isoelectric point depends on its purity and that magnetite materials from different origins have different performances. Generally it can be concluded, that magnetite is positively charged in an acidic environment and as a result, negatively charged particles are attracted to its surface. In alkaline environments magnetite possesses a negative charge and therefore the negatively charged particles are repelled [25].

![Zeta potential measurement of C. vulgaris cells (black square) and magnetite particles (hollow diamond) in 10 mM KCl (pH 2-12)](image)

Contact angle values of the polar (water and formamide) and nonpolar (1-bromnaphtalene) liquids show the hydrophilic nature of the magnetite surface, which has also been stated by Sagakuchi et al. [26], although their tested liquid was artificial seawater and thus, slight deflections must be considered. The contact angles (Table 1) were used to calculate individual components of surface tensions and Gibbs energies of the microalgae and magnetite (Table 2) according to Kuřec et al. [17]. The obtained results were then applied in the XDLVO theory together with the zeta potentials and particle size (Chapter 3.3).
Table 1. Average contact angles and their standard deviations of three tested liquids on *C. vulgaris* cell lawn and the surface of magnetite

| Contact angle (°) | *C. vulgaris* | Magnetite |
|------------------|---------------|-----------|
| Water            | 20.7 ± 2.2    | 5.4 ± 0.1 |
| Formamide        | 42.2 ± 1.1    | 27.8 ± 0.2|
| 1-Bromnaphtalene | 31.2 ± 2.7    | 7.8 ± 3.3 |

Table 2. Surface tensions of *C. vulgaris* cells and magnetite calculated from contact angle values followed by components of Gibbs free energies of interaction ($\Delta G^{\text{LW}}, \Delta G^{\text{AB}}$) between the studied surfaces in water obtained according to the thermodynamic approach.

| Surface       | Surface tension (mJ.m$^{-2}$) | $\gamma^{\text{LW}}$ | $\gamma^+$ | $\gamma^-$ | $\gamma^{\text{AB}}$ | $\gamma^{\text{TOT}}$ |
|---------------|-------------------------------|-----------------------|------------|------------|-----------------------|------------------------|
| *C. vulgaris* | 38.2                          | 0.0                   | 70.0       | 2.2        | 40.4                  |
| Magnetite     | 44.0                          | 0.0                   | 65.6       | 2.4        | 46.4                  |

Thermodynamic approach:

| Gibbs free energy of interaction (mJ.m$^{-2}$) | $\Delta G^{\text{LW}}$ | $\Delta G^{\text{AB}}$ |
|-----------------------------------------------|------------------------|------------------------|
|                                               | -5.9                   | 62.5                   |

### 3.2. Cell and particle size determination

Image analysis was performed for 780 *C. vulgaris* cells resulting in a cell size range 6.5-14.6 μm with a mean value of 11.3 ± 1.5 μm. Similar results were obtained also in other studies [27-30]. Microscopic observation displayed that if magnetite is suspended in an aqueous environment, it forms heterogeneous mixtures as to the particle’s size. Image analysis of 2560 analyzed magnetite particles resulted in a very broad range of sizes: 0.2 – 690 μm.

### 3.3. Predicting microalgae-magnetite adhesion (XDLVO theory)

The extended version of the DLVO theory (XDLVO) was used to obtain the profile of total free Gibbs energy of interaction vs. the separation distance (Fig. 2) and therein predict the cell (sphere)-magnetite (flat plate) interactions under different conditions (10 mM KCl, pH 2-12). The occurrence of energy barriers preventing close contact (adhesion) of the cells with magnetite is foreseen especially at higher pH values. But in spite of these potential energy barriers, adhesion may be favorable at a certain distance of the interacting particles in the secondary potential minimum [17]. The depth of the secondary minimum is most profound in the case of pH 4 and so it can be predicted, that this condition will be the most favorable for cell-magnetite interactions and that the higher the pH value, the lower the probability of cell-magnetite contact. The prediction was in good accordance with performed magnetic cell separations (Fig. 3) and the results indicate a strong influence of electric double layer forces as has been also shown by Xu et al. [11]. This is not in accordance with Sakaguchi et al. [26], who proposed hydrophilic interactions to be the leading forces; the reason is that the authors focused on marine environments, where the higher ionic strengths contribute to the lesser impact of double layer electrostatic interactions. However, culture media for freshwater microalgae have low ionic strengths and therefore electrostatic interactions cannot be neglected even at bigger distances [31]. Furthermore, we observed, that the separation efficiency can be promoted by adding a higher amount of magnetic agent, which is in accordance with literature [32]. In
the case of 10 mM KCl pH 4 the ratio of microalgae to magnetite which ensured a separation efficiency of over 90% was 1:26 (w/w) (data not shown).

Fig. 2. Total free Gibbs energy of interaction as a function of the separation distance between *C. vulgaris* cells and magnetite under different conditions (10 mM KCl, pH 2-12). In the calculations the following parameters were applied: ionic strength of KCl solution in range from 10 – 20 mM according to pH adjustment, Hamaker constant 1.37 kT, decay length 0.6 nm, diameter of cells 11.3 μm, 20°C.

Fig. 3. Separation efficiencies (E) at different ratios of magnetite to microalgal biomass in 10 mM KCl of various pH values.
4. Conclusion

Understanding physicochemical properties of the studied cell/particle surface is essential for explaining cell-surface adhesion phenomena associated with biofilm formation, cell aggregation or separation etc. We focused on using the XDLVO theory to model surface interactions occurring between microalgae *C. vulgaris* and magnetite. An agreement was found between the prediction of favorable conditions for successful magnetic cell modification and magnetic cell separation experiments, highlighting as such the significance of electrostatic interactions in this process. Thus, physicochemical approaches can serve as an effective tool for designing the optimal cell separation process. Furthermore, we can conclude that cell separation with pulverized magnetite is a fast and simple method, dependent on the environment and the amount of magnetic agent added. However, for large-scale applications care must be taken, because magnetite consumption was very high in this study. We propose that the ideal magnetic harvesting agent should be non-toxic, stable, cost-effective, displaying similar physicochemical surface properties as our tested magnetite and have a high specific surface area and colloidal stability.

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References

[1] Harun R, Singh M, Forde GM, Danquah MK. Bioprocess engineering of microalgae to produce a variety of consumer products. *Renew Sust Energ Rev* 2010;14:1037-1047.

[2] Della Greca M, Pinto G, Pistillo P, Pollio A, Previdera L, Temussi F. Biotransformation of ethinylestradiol by microalgae. *Chemosphere* 2008;70:2047-2053.

[3] León-Bañares R, González-Ballester D, Galván A, Fernández E. Transgenic microalgae as green cell-factories. *Trends Biotechnol* 2004;22:45-52.

[4] Greenwell HC, Laurens LML, Shields RJ, Lovitt RW, Flynn KJ. Placing microalgae on the biofuels priority list: a review of the technological challenges. *J R Soc Interface* 2009;7:703-726.

[5] Brányiková I, Maršálková B, Brányik T, Bišová K, Zachleder V, Vítová M. Microalgae as novel highly efficient starch producers. *Biotechnol Bioeng* 2011;108:766-776.

[6] Chisti Y. Biodiesel from microalgae. *Biotechnol Adv* 2007;25:294-306.

[7] Divakaran R, Sivasankara Pillai VN. Flocculation of algae using chitosan. *J Appl Phycol* 2002;14:419-422.

[8] Salim S, Bosma R, Vermuë M, Wijffels R. Harvesting of microalgae by bio-flocculation. *J Appl Phycol* 2010;23:1-7.

[9] Vandamme D, Foubert I, Meesschaert B, Muylaert K. Flocculation of microalgae using cationic starch. *J Appl Phycol* 2010;22:525-530.

[10] Xu L, Guo C, Wang F, Zheng S, Liu CZ. A simple and rapid harvesting method for microalgae by in situ magnetic separation. *Biores Technol* 2011;102:10047-10051.

[11] Kuřec M, Brányik T. The role of physicochemical interactions and FLO genes expression in the immobilization of industrially important yeasts by adhesion. *Colloid Surf B* 2011;84:491-497.

[12] Bayoudh S, Othmane A, Mora L, Ben Ouada H. Assessing bacterial adhesion using DLVO and XDLVO theories and the jet impingement technique. *Colloid Surfaces B* 2009;73:1-9.

[13] Šafařík I, Šafaříková M. Use of magnetic techniques for the isolation of cells. *J Chromatogr B* 1999;722:33-53.

[14] Šafařík I, Šafaříková M. Magnetically modified microbial cells: A new type of magnetic adsorbents. *China Part* 2007;5:19-25.

[15] Sharma PK, Rao KH. Analysis of different approaches for evaluation of surface energy of microbial cells by contact angle goniometry. *Adv Colloid Interfac* 2002;98:341-463.

[16] Hadjoudja S, Deluchat V, Baudu M. Cell surface characterisation of *Microcystis aeruginosa* and *Chlorella vulgaris*. *J Colloid Interf Sci* 2010;342:293-299.

[17] Sun ZX, Su FW, Forsling W, Samskog PO. Surface characteristics of magnetite in aqueous suspension. *J Colloid Interf Sci* 1998;197:151-159.

[18] Gregory R, Maloney RJ, Stockley M. Water treatment using magnetite: a study of a Sirofloc pilot plant. *Water Environ J* 1988;2:532-544.
[25] Hencl V, Mucha P, Orlikova A, Leskova D. Utilization of ferrites for water treatment. *Water Res* 1995;29:383-385.

[26] Sakaguchi F, Akiyama Y, Izumi Y, Nishijima S. Fundamental study on magnetic separation of aquatic organisms for preservation of marine ecosystem. *Physica C* 2009;469:1835-1839.

[27] Killam A, Myers J. A special effect of light on the growth of *Chlorella vulgaris*. *AJ Bot* 1956;43:569-572.

[28] Khummongkol D, Canterford GS, Fryer C. Accumulation of heavy metals in unicellular algae. *Biotechnol Bioeng* 1982;24:2643-2660.

[29] Yin HC. Effect of auxin on *Chlorella vulgaris*. *P Natl Acad Sci USA* 1937;23:174-176.

[30] Oliver RL, Kinnear AJ, Gannf GG. Measurements of cell density of three freshwater phytoplankters by density gradient centrifugation. *Limnol Oceanogr* 1981;26:285-294.

[31] Van Oss CJ. Long-range and short-range mechanisms of hydrophobic attraction and hydrophilic repulsion in specific and aspecific interactions. *J Molecular Recognit* 2003;16:177-190.

[32] MacRae IC, Evans SK. Factors influencing the adsorption of bacteria to magnetite in water and wastewater. *Water Res* 1983;17:271-277.