Phycoremediation of alcohol distillery wastewater with a novel *Chlorella sorokiniana* strain cultivated in a photobioreactor monitored on-line via chlorophyll fluorescence

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**A B S T R A C T**

Possibilities of alcohol distillery wastewater (ADW) bioremediation with a new *Chlorella sorokiniana* from White Sea cultivated in semi-batch mode in a high-density photobioreactor monitored on-line via chlorophyll fluorescence were investigated. Upon inoculation of the ADW, a stable alga-bacterial consortium was formed. A decrease in chemical oxygen demand (COD) of the ADW from 20 000 to ca. 1500 mg L\(^{-1}\) was achieved over four days with a decline in nitrate (>95%), phosphate (77%) and sulfate (35%). Minimal pre-treatment of the ADW (adjustment to 6.0–7.0 pH) was necessary. Kinetics of COD decline and growth of microalga on ADW as well as chlorophyll and fatty acid (FA) composition of the biomass were studied. Cultivation on ADW increased the unsaturation of the FA of the microalga cell lipids rendering the biomass of *C. sorokiniana* cultivated on ADW a suitable feedstock for biodiesel production. Measurement of variable chlorophyll fluorescence was shown to be a sensitive method for monitoring of the physiological condition of the microalgae grown on ADW. The cultivation conditions facilitating ADW bioremediation with the microalga were investigated. The advantages and limitations of the proposed process for ADW treatment are discussed in view of the findings obtained.

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1. Introduction

Production of ethanol from agricultural raw materials displays a steady growth which is based on its consumption in the form of industrial solvent and a beverage component as well as an alternative CO\(_2\)-neutral fuel [1]. The latter usage attracts increasing worldwide interest due to the shortage of non-renewable energy resources and variability of oil and natural gas prices [1,2].

At the same time, production of ethanol generates large volumes of wastewater (referred to below as alcohol distillery wastewater, ADW). Typically, the ADW features a high (20–100 g L\(^{-1}\) chemical oxygen demand; COD) content of organic (carbonic acids, sugar decomposition products, dextranes, etc.) as well as inorganic pollutants (mainly nitrate, ammonia, and phosphate ions), strong odor, brown color and low pH, making it, unless properly treated, a serious environment threat to water bodies and soils. The composition and environmental hazards of ADW are reviewed in detail by Mohana et al. [3].

The conventional treatment of ADW is carried out by means of aerobic and anaerobic processes [3–5]. Increasingly stringent environmental regulations are forcing distilleries to improve existing treatment and also explore alternative methods of effluent management. As a result, space and funds required for building the ADW treatment plants are turning to be the most serious obstacles for such investments. Additional drawbacks of traditional treatment systems include complexity and generation of waste sludge [6,7].

Though the methodology of wastewater treatment using phototrophic organisms has a long history [8], the alternative approaches for phyto-remediation of ADW emerged only over the last decade [9,10] involving mostly combinations of higher plants, microalgae [11] and/or cyanobacteria [4]. Microalgae as photoautotrophic organisms produce oxygen (providing photosynthetic aeration) which accelerates the degradation of organic pollutants in wastewater [12]. An added benefit of this approach is the production of microalgal biomass, which can be used as a raw material in many applications such as production of biofuel, feeds in aquaculture and cattle farming, fertilizers and value-added lipids and carotenoids [13–16]. The fatty acid (FA) composition of the biomass obtained as a result of cultivation of microalgae on wastewater is very important for the choice of the preferred way of its utilization. In particular, a good quality biodiesel has a high cetane number, which is associated with saturated FA such as palmitic (16:0) and stearic (18:0) acids; on the contrary, a low cetane number was observed with highly unsaturated FA such as linolenic (18:3) acid [17]. The high

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**Abbreviations:** ADW, alcohol distillery wastewater; CFU, colony forming unit; Chl, chlorophyll; COD, chemical oxygen demand; DMSO, dimethyl sulfoxide; DW, dry weight; FA, fatty acids; HRT, hydraulic retention time; LED, light emitting diode(s); PAR, photosynthetically active radiation; PFD, photon flux density.

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proportion of saturated FA in algal biomass may provide increased energy yield, superior oxidative stability, and higher cetane numbers that cause fewer problems in fuel polymerization during combustion [18]. Nevertheless, the reports on efficient microalgal photobioprocesses specifically aimed for ADW bioremediation are scarce and limited to laboratory-scale experiments [11,19]. Most of them are based on cultivation of green microalgae (Chlorophyta), in particular representatives of the genus Chlorella due to its remarkable capability of mixotrophic growth. Thus, Valderrama et al. [10] carried out research to develop a procedure for biological treatment of recalcitrant anaerobic industrial effluent from ethanol and citric acid production using first the microalga Chlorella vulgaris followed by the higher plant Lemna minuta. More recently, Yang et al. [20] cultivated Chlorella pyrenoidosa in a closed cycle tubular photobioreactor (PBR) in undiluted wastewater from ethanol fermentation using cassava powder as the raw material. High rates of organic pollutant removal were achieved with a fixed bed reactor coupled with a laboratory-scale microalgal pond [21]. It should be noted in addition that the up-scaling of microalgal photobiotechnology for wastewater remediation requires fast and reliable techniques, preferably non-destructive, for on-line monitoring of the state of algal culture [22]. This is necessary for timely and informed decisions on adjustment of illumination conditions, the rate of wastewater inflow and on the time for biomass harvesting. Often, the decisions must be taken within hours and mistakes may lead to a significant reduction in productivity or in a total culture loss.

We hypothesized that the use of the high-density microalgal culture grown in a closed system (PBR) under optimal mixing and illumination conditions would allow to shorten the hydraulic retention time (HRT) and to increase the rate of pollutant removal from ADW. We also attempted to demonstrate that on-line monitoring of variable Chl fluorescence is a useful tool for wastewater phycoremediation. This method was found to provide valuable information on the physiological condition of the microalgal culture and hence to maintain the performance of Chlorella sorokiniana during the growth in ADW. We also aimed to investigate the possibilities of treatment of ADW by a high-density culture of the newly isolated from White Sea strain of C. sorokiniana in a mixed annular PBR with simultaneous production of the microalgal biomass suitable for downstream processing.

2. Materials and methods

2.1. Strain and its cultivation conditions

A novel Chlorella sp. (Chlorophyta) strain isolated from White Sea (Russia) according to the earlier published protocol [23] and identified as C. sorokiniana (GenBank ID: KC678067) was used in this work. In pilot experiments the newly obtained C. sorokiniana strain appeared to be capable of rapid destruction of organic components of the ADW during cultivation at 27 °C and pH of ca. 7. The algal inoculum was grown in liquid ½ Tamia medium [24]. Batch cultures in 250 mL flasks with 100 mL of medium were kept at 27 °C on an orbital shaker under continuous illumination at 80 μmol PAR photons m⁻² s⁻¹. Before the experiments, the culture was acclimatized to the ADW by cultivation of the algae on the series of media composed from the ½ Tamia medium and the ADW in the proportions increasing from 1:10 to 2:3 (the ½ Tamia medium: ADW, by volume) and finally transferred to ADW prepared as described below. In some experiments, the ADW was inoculated with the microalgae and was sparged with filtered atmospheric air (150 mL L⁻¹ min⁻¹); other conditions were as described above.

The experiments on ADW bioremediation were performed in a 50-L annular PBR (Fig. 1) with a suspension layer thickness of 46 mm in semi-batch mode. The process was started at 20 g L⁻¹ COD. After the decline in COD to ca. 1.5 g L⁻¹, a portion of the suspension was removed from the PBR by the sampling cock and a portion of fresh ADW was added to restore the COD to a level of 20 g L⁻¹ and to maintain OD₆₇₈₈ in the range 2.0–3.0. Routinely the cells were harvested by means of decanting; nearly complete sedimentation was achieved within 2–3 h.

The culture was mixed by 10 vertical paddle stirrers at 120 rpm and aerated with air at a rate of 5 L min⁻¹ (0.1 v v⁻¹ min⁻¹). The PBR was kept at 27 °C under continuous illuminations with white LEDs through the inner surface of the PBR (see Fig. 1) at 180 μmol photons m⁻² s⁻¹ PAR. The axenicity of the initial microalgal culture as well as the presence of bacteria during the growth on ADW was determined by a standard plate colony forming unit (CFU) count method as described by APHA [25].

2.2. Alcohol distillery wastewater preparation

The untreated ADW (pH 3–4) was obtained from a local distillery. Prior to inoculation with the microalgal culture it was pH-adjusted with 1 M NaOH to pH 7.0, diluted if necessary, and centrifuged for 5 min at 3000 g. The supernatant with a COD of ca. 20 g L⁻¹ was used as the cultivation medium for C. sorokiniana in routine experiments.

2.3. Growth measurement

Algal growth estimation was based on cell dry weight (DW) measurements and volumetric content of Chl a. An aliquot of the cell suspension was sampled from the PBR and the cells were harvested by centrifugation for 5 min at 3000 g. The cell pellet was washed with distilled water and used for dry weight determination according to Pal et al. [26]. The wash water was pooled with the supernatant from the previous centrifugation, evaporated to dryness and weighed. In routine measurements total Chl were extracted from the biomass with dimethyl sulfoxide (DMSO) for 5 min at 70 °C with 5 mL per ca. 3.5 mg DW. The pigment concentrations were determined in DMSO extracts spectrophotometrically with an Agilent Cary 300 spectrophotometer (Walnut Creek, CA, USA) [27].

2.4. Fatty acid analysis

Capillary gas-chromatography was used for fatty acid quantification; the analysis was performed according to Cohen et al. [28]. The data shown represent mean values with a range of less than 5% for major peaks (over 10% of fatty acids) and 10% for minor peaks, of at least two independent samples, each analyzed in duplicate. Heptadecanoic (margaric) acid [29] was added as an internal standard. Identification of fatty acids was done according to retention times of standards (Sigma, USA) and by characteristic mass spectra obtained with Agilent 7890 (Agilent, USA) gas chromatograph equipped with HP5-MS.
2.5. Spectral measurements

The OD spectra of the culture samples were taken with the Agilent Cary 300 spectrophotometer equipped with an integrating sphere attachment. Prior to recording of the spectra, the cells were pelleted by centrifugation (3 min, 3000 g), and the cells were re-suspended in the fresh cultivation medium. The supernatant containing, according to the plate count (see above), the bulk of the bacterial cells, was used for the turbidity measurement (at 800 nm). The absorbance of the re-suspended microalgal cells was measured as described below. The correction of the measured OD values for uncertain and variable contribution of scattering by the cells is considered to be essential for accurate monitoring of the microalgal culture growth via OD in the long-wave band of Chl absorption [30,31]. The method developed by Merzlyak et al. [31] was used for scattering-correction in this work. This method employs OD, in the spectral region unaffected by pigment absorption, e.g., in the near infrared, to quantify the incomplete collection of light by integrating sphere due to scattering by the sample. Briefly, the spectra of C. sorokiniana cell suspension were recorded in 1 cm glass cuvette at two distances: as close as possible to and 1 cm apart from the entry window of the integrating sphere. In both cases, a cuvette with the cultivation medium was used as a reference. The scattering-corrected optical density at 678 nm then was calculated as

\[
\text{OD}_{678}^c = \frac{\text{OD}_{678}^0 - \text{OD}_{800}^0 - \text{OD}_{678}^c}{\text{OD}_{800}^0} \times (\text{OD}_{678}^0 - \text{OD}_{678}^c)
\]

where \(\text{OD}_{678}^c\) is the scattering-corrected OD678, \(\text{OD}_{678}^0\) and \(\text{OD}_{800}^0\) are the optical density values at 800 nm (where pigments do not possess detectable absorption) and 678 nm (the long-wave absorption maximum of Chl) measured 1 cm apart from the integrating sphere; \(\text{OD}_{800}^c\) and \(\text{OD}_{678}^c\) are the optical densities at the same wavelength measured as close as possible to the integrating sphere.

2.6. Chlorophyll fluorescence and light curve recording and treatment of the data

For on-line assessment of the photosynthetic activity of C. sorokiniana cells, an automatic flow-through system was developed at the Faculty of Biology of Moscow State University including a time-resolving fluorometer, a peristaltic pump (77914-10, Cole-Parmer, USA), a flow cell and the custom-made software controlling both the pump and fluorometer. At a set time interval, the software activates the pump, which purges and fills the flow cuvette with the algal suspension from PBR and, after the selected dark-adaptation period, triggers the recording of a chlorophyll fluorescence transient as detailed below.

The fluorometer induces chlorophyll fluorescence transients by a square pulse of excitation light with a duration of 2 s generated by two light emitting diodes (LED, \(\lambda_{\text{max}} = 455 \text{ nm}\)) located on the opposite sides of the measurement chamber yielding a photon flux density (PFD) of 8000 \(\mu\text{mol}\) photons m\(^{-2}\) s\(^{-1}\). The measurements were performed in a 1 mL cuvette with 3 mm path length. After passing through a cut-off filter (KS-18, 690 nm, Russia) fluorescence is detected at 90° to the axis of the excitation beam by the SD200-11-21-241 photodiode (Advanced Photonix, USA) with a time resolution of 1 \(\mu\text{s}\). Samples were dark adapted for 10 min prior to the measurement. The minimal (Fo) and maximal (Fm) fluorescence levels were identified as the fluorescence intensities at the plateaus O and P of the fluorescence transient, respectively; the maximum quantum yield of photosystem II was estimated as \(Fv/Fm = (Fm - Fo)/Fm\) [32].

The irradiance saturating photosynthesis in the inoculum cultures of C. sorokiniana was determined by measurement of the relative electron transport rate (rETR) in the electron-transporting chain of the algal chloroplast. The rETR was calculated as \(\text{rETR} = I \cdot Fv/Fm \cdot A\), where \(I\) is the actinic light intensity; \(Fv/Fm\) is the quantum yield of photosystem II in the microalgal cells adapted to the actinic light, and \(A\) is the fraction of the light absorbed by the microalgal suspension. The latter was calculated as \(A = 1 - T = 1 - 10^{-\text{OD}}\) where \(T\) is the transmittance of the suspension and OD is the optical density measured and scattering-corrected as described above. The microalgal suspension sample was pumped into the flow cell inside the fluorometer (see above) and exposed to the actinic light (\(I\)) from the built-in LED PAR light source. After 5 min of the actinic light exposition, a chlorophyll fluorescence transient was recorded as described above to obtain the \(Fv/Fm\) value at the corresponding \(I\). To record a full light curve, this procedure was repeated with \(I\) increasing stepwise from 0 (dark-adapted sample) to 700 \(\mu\text{mol}\) photons m\(^{-2}\) s\(^{-1}\).

2.7. COD measurements and inorganic ion quantification

For water analysis, 50 mL of wastewater was sampled. Nitrate, orthophosphate and sulfate ion concentrations were measured using Thermo Dionex ICS 1600 HPLC (Sunnyvale, CA, USA) with a conductivity detector and IonPac AS12A (5 \(\mu\text{m}\); 2 × 250 mm) anionic analytical column with AG12A guard column (5 \(\mu\text{m}\); 2 × 50 mm). The column temperature was maintained at 30 °C. The ions were eluted isocratically with 2.7 mM sodium carbonate/0.3 mM sodium bicarbonate buffer (flow rate of 0.3 mL min\(^{-1}\)). Determination of COD was carried out using LCK514 COD cuvette tests with a Hach Lange thermoreactor and spectrophotometer (Hach, Germany) according to the specifications of the manufacturer.

2.8. Statistical treatment

The experiments were carried out in three biological replications with at least two analytical replications per sampling point. In the figures, average values together with standard errors are presented unless stated otherwise. The significance of differences was tested using ANOVA from the analysis tool pack of the Microsoft Excel spreadsheet software.

3. Results

3.1. Determination of starting culture conditions

Preliminary tests were conducted in order to determine a suitable medium composition, a starting culture density, and a PAR irradiance for cultivation of the C. sorokiniana in ADW. Since the C. sorokiniana was found to be unable to grow in the untreated ADW (pH 3–4), all further experiments were conducted with the pH-adjusted ADW (see ‘Materials and methods’). In the pilot experiments carried out in flasks the culture growth was recorded (via OD678 and DW) in the cultures initiated on ADW with starting OD678 ranging from 0.1 to 1 (Fig. 2A) corresponding to a DW of ca. 0.2 to 2 g L\(^{-1}\). The cultures started at OD678 <0.25 failed to grow on ADW (Fig. 2A, curve 1). The cultures with starting OD678 <0.35 displayed a prolonged lag period of ca. 5 days (Fig. 2A, curve 2). The cultures initiated at 0.35 < OD678 < 0.5 featured no detectable lag period and accumulated biomass steadily (Fig. 2A, curves 3 and 4). The culture with an initial OD678 of ca. 1.0 demonstrated the highest growth rate during the first 2 days of cultivation and reached stationary phase thereafter (Fig. 2A, curve 5).

It should be noted in addition that the C. sorokiniana cultures initiated at a cell density high enough for a rapid growth on the ADW (e.g. curves 5 and 4 in Fig. 2) were characterized by neutral-to-mildly alkaline pH (7–8), which tended to increase to alkaline values (10–11). On the contrary, in the cultures that failed to grow or lagged considerably, acidification of the medium took place (to pH < 6).

The analysis of changes in the maximum quantum efficiency of PSII estimated via Fv/Fm (Fig. 2B) showed that failure to grow or pronounced lag observed in highly diluted cultures was accompanied by.

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a strong inhibition of PSII function (Fig. 2B, curves 1 and 2). The cultures initiated at OD$_{678}$ = 0.35 displayed a uniformly high PSII efficiency ($F_{v}$/$F_{m}$ ≈ 0.7; Fig. 2B, curves 3–5) with a small dip (to ca. 0.65) observed at the 2nd day of cultivation.

To determine the appropriate irradiance for growing of the *C. sorokiniana* on ADW, the relationship between irradiance and linear electron transport rate [33] was studied in the irradiance range 1–700 μmol photons m$^{-2}$ s$^{-1}$ PAR. As could be seen from Fig. 3, the rate of electron transport was saturated at a PAR irradiance of ca. 180 μmol photons m$^{-2}$ s$^{-1}$ and declined at higher irradiances.

### 3.2. Cultivation of *C. sorokiniana* on ADW in the photobioreactor

The culture of the *C. sorokiniana* was initiated in the PBR on pH-adjusted ADW with ca. 20 g L$^{-1}$ COD (Fig. 4) and considerable amounts of inorganic N and P and sulfate (Table 1). Sparging of the ADW with atmospheric air without inoculation with the microalgae did not result in a sizable decline of COD, which comprised less than 3 g L$^{-1}$ for 5 days of cultivation (ca. 0.6 g L$^{-1}$ d$^{-1}$). By contrast, cultivation of *C. sorokiniana* on ADW in the PBR brought about a dramatic (to ca. 1.5 g L$^{-1}$) decline in COD, inorganic ion content, and a nearly complete loss of odor within 2–3 days after ADW addition (Figs. 4 and 5, Table 1). The decline in COD and inorganic nutrients in the ADW was accompanied by a steady increase in biomass (average growth rate of ca. 2 g DW L$^{-1}$) and Chl, and Fv/Fm (Fig. 5). After 4–5 days of cultivation, the *C. sorokiniana* culture in the PBR attained an OD$_{678}$ of 8–10 (corresponding to a DW of 10–12 g L$^{-1}$).

The kinetics of changes in Chl content appeared to be more complex in comparison with the kinetics of COD changes (Fig. 5B). As a rule, an increase in Chl content for 1–1.5 days was observed during the first two cycles of the *C. sorokiniana* cultivation in PBR after initiation of the culture. The increase in Chl was followed by a decline, which was more pronounced during the 1st cultivation cycle (4–5 days), less

### Table 1

Bioremoval of sulfates, nitrates, and phosphates by *Chlorella sorokiniana* cells from alcohol distillery wastewater.

<table>
<thead>
<tr>
<th>Cultivation time (h)</th>
<th>Concentration (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO$_3^-$</td>
</tr>
<tr>
<td>0 (at pH 7)</td>
<td>15 ± 1.3</td>
</tr>
<tr>
<td>96</td>
<td>ND$^*$</td>
</tr>
</tbody>
</table>

$^*$ ND—not detected.

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**Fig. 2.** Growth (A) and (B) time-course of changes in maximum PSII efficiency of the *Chlorella sorokiniana* during pilot cultivation in flasks on pH-adjusted alcohol distillery wastewater (see Methods) as a function of starting culture density (see legend in panel A).

**Fig. 3.** The relationships between the irradiance and the relative electron transport rate in the *Chlorella sorokiniana* culture (OD$_{678}$ = 1.0) grown in flasks on pH-adjusted alcohol distillery wastewater (see Methods).

**Fig. 4.** Characteristic kinetics of a decline in COD of pH-adjusted alcohol distillery wastewater used as the medium for cultivation of *Chlorella sorokiniana* in the photobioreactor (see Fig. 1). The results of three representative experiments are shown. Dashed line is the best fit function.
pronounced during the second cycle (1.5–2 days), and was almost indistinguishable during the 3rd and all subsequent cycles.

The changes in the quantum efficiency of photosystem II estimated as changes in Fv/Fm are shown in Fig. 5C. At pH > 7 the Fv/Fm values of the *C. sorokiniana* cells were in the range 0.6–0.7. A sharp decline of Fv/Fm was observed in the beginning of each cycle upon addition of fresh pH-adjusted ADW. The dip in Fv/Fm was most profound at the initiation of the culture when the photosystem II quantum efficiency declined to 0.61; it was less prominent at subsequent cycles where the Fv/Fm declined to ca. 0.65. After a short lag, Fv/Fm increased showing inverse linear relationship with the residual COD of the culture medium in the PBR (Fig. 6). In the middle of a cycle the Fv/Fm of the *C. sorokiniana* cells as well as the COD leveled off. Notably, an additional drop in Fv/Fm frequently occurred during the first cycle after initiation of the culture, which coincided with a step in the COD decline and was again followed by an increase in Fv/Fm and a decline in COD (Fig. 5C).

As evidenced by the changes in the ratio of OD678 and turbidity (OD800) related to the ratio of microalgal and bacterial cell numbers, a relatively stable alga-bacterial consortium was formed after inoculation of the ADW and sustained after subsequent additions of the fresh ADW (Fig. 5D). Apart from the *C. sorokiniana* as the dominant component (90–95% of the total DW of the culture sampled from the PBR), this consortium contained heterotrophic microorganisms. The numbers of these organisms were relatively stable amounting, at the end of each cultivation cycle, to ca. (50 ± 8) × 10^7 CFU mL^-1 in total for Gram-positive
coryneformic bacteria and actinomyces (the latter accounted for ca. (20 ± 4) × 10⁷ CFU mL⁻¹).

3.3. Fatty acid composition of the *C. sorokiniana* cells grown in ADW

During the first cycle, the growth of *C. sorokiniana* in the PBR was accompanied by a steady increase in the total fatty acid (TFA) content of the microalgae from the level characteristic of the inoculum (10% of the cell DW) to 20% DW (Table 2) along with depletion in nitrate and other inorganic ions (Table 1) within 4 days. The volumetric content of TFA increased considerably reaching 2.36 g L⁻¹ culture by the end of cycle.

The main trends of changes in the FA composition of *C. sorokiniana* cells grown in ADW were comprised by an increase of saturated FA (14:0 and 16:0) and an increase in dienoic FA 16:2 and 18:2 at the expense of monoenoic (18:1) and trienoic (16:3 and 18:3) FA; the total volumetric content of saturated FA increased more than two times whereas that of unsaturated FA increased by approximately 25% (Table 2). As a result, a slight (by ca. 10%) decrease of the unsaturation expense of monenoic FA (curve 1) was observed by the end of each cultivation cycle leading to a slight decrease in TFA DW percentage (not shown); in 2–3 days it returned to 20% DW, the steady-state level attained under the nitrogen-limited growth conditions (nitrate content <2 mg L⁻¹, Table 1) in the PBR.

4. Discussion

We investigated the possibilities of ADW treatment in a PBR with the high-density semi-batch culture of a newly isolated strain of *C. sorokiniana* on-line monitored via Chl fluorescence. The ability of the microalgae to grow mixotrophically and to form a stable alga-bacterial consortium utilizing organic matter and inorganic nutrients from the wastewater made it possible to decrease the COD by more than one order of magnitude (Fig. 4), to remove nitrate nearly completely, and to decrease phosphate and sulfate ion concentration with a reasonable efficiency in a relatively short time (Table 1). It is difficult to determine precisely the contributions of the autotroph (the microalgae) and the heterotroph (the bacteria and actinomyces) components of the consortium to the destruction of organic pollutants. Nevertheless, our preliminary tests with sparging of the ADW with atmospheric air and cultivation of the heterotrophic microorganisms on the ADW demonstrated that *C. sorokiniana* is the essential component of the consortium. The mixotrophically grown *C. sorokiniana*, apart from direct consumption of the organic substances, provides the oxygen for the respiration of the heterotrophic microorganisms as well as for the direct oxidation of organic pollutants in the ADW [12].

| Table 2 |
| Fatty acid content and composition of the *Chlorella sorokiniana* cells grown on the alcohol distillery wastewater. |

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>mg L⁻¹</th>
<th>% TFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>96 h</td>
</tr>
<tr>
<td>14:0</td>
<td>2.8</td>
<td>136.5</td>
</tr>
<tr>
<td>16:0</td>
<td>16.5</td>
<td>508.0</td>
</tr>
<tr>
<td>16:1</td>
<td>8.7</td>
<td>307.5</td>
</tr>
<tr>
<td>16:2</td>
<td>5.6</td>
<td>282.7</td>
</tr>
<tr>
<td>16:3</td>
<td>5.6</td>
<td>32.3</td>
</tr>
<tr>
<td>18:0</td>
<td>6.8</td>
<td>45.3</td>
</tr>
<tr>
<td>18:1</td>
<td>10.0</td>
<td>62.8</td>
</tr>
<tr>
<td>18:2</td>
<td>6.7</td>
<td>739.7</td>
</tr>
<tr>
<td>18:3</td>
<td>6.3</td>
<td>93.6</td>
</tr>
<tr>
<td>Sum of fatty acids</td>
<td>143.5</td>
<td>2357.8</td>
</tr>
<tr>
<td>% DW</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Unsaturation index</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saturated/unsaturated fatty acid ratio</td>
<td>-</td>
<td>0.27</td>
</tr>
</tbody>
</table>

At the same time it was important to keep the COD load rate within the capacity of the efficient decomposition by the microalgal culture (Fig. 2). Under our experimental conditions, it amounted to 4 g COD d⁻¹ g DW⁻¹ of the culture (90–95% of the total culture biomass was comprised by the microalgal cells). It should be noted that, depending on the agricultural raw material and the variation of technological process used, the COD load of ADW could exceed this capacity. In this case dilution of ADW is necessary; a practical solution to this problem (dilution with the washwater originating from the same distillery) was suggested by Valderrama et al. [10]. Another option is to operate the PBR in fed-batch mode at a suitable dilution rate, e.g. as described by Yang et al. [20]; the added benefit of the fed-batch cultivation is the possibility to operate at the native ADW pH. We would like to note in addition that the COD removal rate attained in the present work (>90%; Fig. 4) appeared to be higher than recorded in a number of other systems for ADW microalgal bioremediation, e.g., 57.5% with *Scenedesmus obliquus* in flasks [11] or 76.3% with *C. pyrenoidosa* in fed-batch mode in a tubular PBR [20].

The cultures initiated in flasks at low inoculum density (OD₆₇₅ < 0.25; curve 1 in Fig. 2A) were unable to grow even in the pH-adjusted ADW under our experimental conditions. During cultivation of *C. sorokiniana* in PBR, the addion of fresh portions of ADW resulting in dilution of the culture and effectively increasing the biomass load lead to a transient drop of the Chl content (Fig. 5B). We hypothesize that after inoculation of ADW with a diluted inoculum the algal cell encounters the amount of pollutants that is too high to cope with resulting in the cell death. A higher-density inoculum obviously possesses a higher total buffer capacity of the microalgal cells that appears to be crucial for proliferation during the first several days upon transfer to ADW. Another reason of the failure of diluted microalgal culture could stem from the photooxidative damage to the cells by excess irradiation. Cultures attaining a higher cell density are indeed more robust in this regard due to mutual shading of the cells in suspension [34]. This suggestion is supported by a progressive decrease in the magnitude of the decline in Chl in the course of biomass accumulation (Fig. 5B). In view of these findings, emphasis should be placed on finding the optimal inoculum density as well as on optimization of the dilution (the ADW inflow rate).

The ADW used in this work was not particularly enriched in nitrate and almost lacked ammonium ions but contained significant amounts of phosphate and sulfate (Table 2). It was therefore expected that, at the culture density attained, the nitrate would be removed from the ADW very quickly. At the same time, disproportionately high phosphate removal was recorded in comparison with what could be expected given the nitrate amount in the ADW and the Redfield ratio. The enhanced bioremoval of phosphate could be, at least in part, explained by its accumulation in the form of inclusions in the cells of the microorganisms. We did not determine the partitioning of phosphorus between algal and bacterial biomass in the present work but electron microscopy revealed, in some instances, the presence of polyphosphate granules in the cells of *C. sorokiniana* grown in ADW (unpublished data).

The microalgae cultivated in the PBR encountered a slight nitrogen deficiency by the end of each cultivation cycle. This suggestion was indirectly confirmed by a moderate increase in TFA percentage (up to 20%) of the microalgal biomass and by changes in FA composition (Table 2) typical of nitrogen deficiency [35–37]. In particular, an increase in 16:0 and a decline in 18:3 and 16:3 fatty acids were recorded (Table 2). The nitrogen deficiency observed under our experimental conditions did not exert an obvious effect on the photosynthetic apparatus of the *C. sorokiniana* cells acclimated to ADW. Accordingly, no decline in Pₐ/Fₐ was observed by the end of a cultivation cycle excepting the first cycle after the inoculation of the PBR (Fig. 5C).

The biomass of *C. sorokiniana* obtained under the conditions of moderate nitrogen deficiency appeared to be suitable for production of biodiesel (Table 2). On the other hand, alleviation of the nitrogen deficiency by means of nitrate addition may help to accelerate the culture.
growth and to attain higher COD load rates. Higher productivity in terms of accumulation of biomass although less enriched with FA is the added benefit of nitrate feeding. The resulting biomass would be a valuable additive to animal feed. In particular, the microalgal biomass could be utilized in the production of cattle feed additives from the solid dregs generated by the same distillery.

Remarkably, *C. sorokiniana* cells cultivated under the condition described above were capable of phosphate removal from ADW at a rate commensurate to or higher than that attained by a number of other systems [10,38]. By contrast, the extent of sulfate removal was not so high (35%), probably, due to the limited rate of its involvement into metabolism of microalgae. A possible way to increase the efficiency of sulfate removal without increasing HRT is to increase the culture density of the microalgae and/or reduce the load rate of the PBR.

The efficient removal of both organic matter and inorganic ions by the high-density *C. sorokiniana* culture observed in the present work makes it a good candidate for the development for combined secondary and tertiary treatment of ADW, at least featuring the composition similar to that of ADW used in this work. Currently, the water obtained after the microalgal treatment of the ADW and harvesting of the microalgal cells is not suitable for direct discharge to water bodies but could be recycled for ethanol production. To improve the efficiency of the treatment, the density of the microalgal culture and/or the number of stages of the microalgal treatment could be increased.

To ensure the robustness of the process, one has to monitor, preferably in real time, the physiological condition of the microalgae (Fig. 5C). The latter proved to be more responsive allowing for swift corrective actions restoring the optimal pH and COD load rate of the culture. This feature is important since an ADW treatment method has to be flexible enough to overcome the constant fluctuations of the organic and inorganic ion load by timely adjusting of dilution rate, light intensity, etc. There is a ground to believe that the use of optical and Chl fluorescence-based sensors is a plausible option for automation of microalgae-based ADW treatment.

5. Conclusions

We demonstrated the possibility of efficient bioremediation of ADW with the use of the high-density semi-batch culture of the new *C. sorokiniana* strain. Nearly complete deodorization and removal of the bulk of inorganic nutrients and organic matter from the ADW were achieved within 3–4 days. The added value was comprised by production of *C. sorokiniana* biomass, a suitable feedstock for biodiesel and/or animal feed additive. On-line monitoring by means of Chl fluorescence provides an insight into the physiological condition of the microalgae essential for timely decisions regarding the culture management. The findings obtained in this work could serve as a starting point for the development of an automated process for treatment of ADW that would complement the currently used activated sludge-based processes to make it simpler and more affordable.

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Conflict of interest

The authors declare no conflict of interest.

References


