Using of Chlorella Vulgaris for Livestock Wastewater Treatment and the Expression of NR Gene

Fariba Bagheri Bajestani1, Nasrin Moshtaghi*, Farhad Talebi2

1Department of Biotechnology and Plant Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.
2Genetic Department, Faculty of Biotechnology, Semnan University, Iran.

*Corresponding Author: Nasrin Moshtaghi

Abstract

Algal treatment was performed for 30 days in three autoclaved livestock wastewaters. Nitrate concentrations in the three batch cultures and autoclaved livestock wastewaters were determined by spectrophotometer method. It was found that the capacity of Chlorella vulgaris removal was reached at rates of 89.22% nitrate in cattle livestock wastewater, 89.34% in sheep livestock wastewater and 89.41% in poultry livestock wastewater. Also, we studied the growth characteristics of C. vulgaris under three wastewater treatments within thirty days. The results showed that in the lag phase, the microalgae was treated in the wastewater with 501.33, 406.66, 381 mg.l-1 nitrate with a biomass productivity of 0.1, 0.09, 0.03g/l per day. On the other hand, we studied the removal of nitrite from wastewater by C. vulgaris. The results showed that the capacity of Chlorella vulgaris for nitrite removal reaches 87.50%, 43%, 65.15%, respectively. Moreover, the expression of nitrate reductase (NR) was analysed over six days in BG11 medium and livestock wastewater treatment. The highest fold of transcription was observed 96 hours after treatment in both of wastewater and culture medium. The transcript of gene at 96 hours in cattle wastewater was higher than BG11 culture medium. These result showed high potentials of Chlorella vulgaris to gene expression related to uptake of toxic nutrient and suitable candidate for gene isolation.

Keyword: Algae, Livestock waste water, Nitrate reductase, Transcript.

Introduction

Agricultural effluents may be polluted with more than >1000 mg.l-1 of total nitrogen (N) and phosphorus (P) concentrations [1]. Release of such wastewaters without proper refinery cause serious problems such as discharge of these nutrients into aquaculture could lead to eutrophication. Stimulated growth of unwanted plants such as algae and aquatic macrophytes deplete the dissolved oxygen of an ecosystem, hence, eutrophication and resulting hypoxia are associated with the natural aquatic food chain interruption [2]. Moreover, excessive N concentrations interfere with disinfection process using chlorine residual and also are toxic for fish and other aquatic organisms in the form of non-ionized ammonia [3]. Many microalgal species such as green algae and cyanobacteria can uptake N and P into their cells and accumulate them as primary metabolites in their biomass [4-5] as it has previously mentioned by Chisti [6]. About 6.6% and 1.3% of a typical algal biomass gravimetrically are contributed by N and P, respectively. Microalgal cultivation can be considered as an binary treatment process to enhance the uptake of nutrients from wastewater [7-8]. Various forms of inorganic nitrogen, such as NH4+, NO3-, NO2- or NO can be up taken by microalgal strains [9-10] However, application of the physicochemical approaches to remove N and P residue is expensive, consumes large input of chemicals, and final removal is incomplete especially in low concentrations of pollutants.

But, biosorption using microalgae feedstock considered as more economic, safer and efficient mean to remove nutrient from different wastewaters [11].
High capacity of algae to high concentration of nutrient (N and P) surveyed in algae cell [12]. Nitrate assimilation in algae with nitrate transporter protein function is regulated by nitrate and nitrite reductase enzyme [13]. Transcription of nitrate reductase was related to environmental conditions, such as nitrogen source, light, CO₂ availability and algae species. Correlation between nitrate eliminate from culture and lipid production has been noticed in different studies [14].

Transcriptional level regulation of nitrate reductase and Acetyl-CoA carboxylase (AC Case) measured by real-time PCR reaction that showed mRNA transcripts in microalgae cell. Different condition such as starvation and nitrogen depletion or repletion, had varied responses of gene (nitrate reductase) expression. High potential of gene expression exists in microalgae cells in different condition [15].

One of the benefits of using microalgae in coupled wastewater treatment approaches is the recycling of biomass into costly outputs, such as fertilizers, resulting in justifying the overall costs of the bioremediation [16-17].

Eliminating the cost of the nutrients and the freshwater supplementation in integrated microalgae cultivation and wastewater bioremediation could result in 20–25% reduced unit cost energy Talebi and coworkers [18] reported that produced water treatment (highly-polluting wastewater of the petroleum industry) coupled with biomass production could minimize the operating expenses of wastewater treatment while concurrently offering a sustainable platform to produce algae derived be foul.

Development of renewable, carbon-neutral and economically feasible be foul is prerequisite of sustainable industrial development in a global context. Photosynthetic microalgae produce biomass which can be further transformed into wide range valuable products, such as bio ethanol and biodiesel.

To throw light on the purpose of the present study, investigation of the potentials of algal-based treatment of agricultural effluents were considered while focusing on biomass productivity and gene expression report concurrently. Moreover, effective removal by algae needs preparation good condition to benefit from highest performance with microalgae. Microalgae was high potential biologically treatment of wastewaters system with more transcripts of related gene. These articles could candidate suitable genes for isolation and transgenic studies.

Materials and Methods

Microalgae Strain Isolation and Pre-Culture Condition

Methods of sampling, isolation, and propagation of the algal material are those generally reported previously [19] In brief, purified microalgae cells were cultivated in BG₁₁ medium. The first pH of the medium was modified to 7.0. The cultures were incubated at 28º C in 1000 ml E-flasks under 4000 Lux illuminations with a photoperiod of 16/8 light/dark.

Molecular Identification

Identification of the species was performed by using basic systematic reports. First of all, to draw comprehensive conclusion, morphological identification of the sample was done by using an Olympus (BH-2) microscope at magnifications 1000 x. Taxonomic identification was completed based on the sequence analysis of 18S rDNA gene.

DNA was extracted using CTAB method Doyle and doyle [20] Specific oligonucleotides (Forward primer: 5’ AACCTCCTTGATCATGCCAG 3’; Reverse primer: 5’ CACTAGACTTGCCATACA 3’) were used to amplify 18S rDNA according to the method described by Lee and coworkers [21]. PCR amp icons were purified from agarose gel using the PCR purification kit (Denazist, Iran) according to the manufacturer’s instructions.

The purified product was ligated into a PTG19/RT plasmid vector (Fermentas), further transformed/cloned into Escherichia coli strain DH5α [22] To extract recombinant plasmids a highly pure plasmid isolation kit (Denazist, Iran) was used and the product was directly sequenced by Macrogen Company (Korea).

The determined 18S rDNA sequence was analyzed by a similar matching on the NCBI Gene Bank database using BLAST to compare with the related sequences [23].
Phylogenetic study consist of a neighbor-joining tree which was constructed using the software MEGA version 4. Maximum composite likelihood model was considered to compute the evolutionary distances by 1000 bootstrap replicates to bring reproducibility and powerful statistical support for the tree. These studies included Chlorella minutissima, as the out group.

**Waste Waters Preparation**

Cattle, poultry and sheep wastewaters were obtained from farm land located in the college of agriculture, Mashhad University. The effluent from was first faced with pre-treated to remove large, non-soluble particulate solids. Sedimentation and filtration with a filter cloth was carried out and the resultant was centrifuged at 4000rpm, for 15 min at 12°C, to remove most of the suspended solids.

The supernatant was used as substrate for the experiments which was further disinfected using an autoclave in 121°C for 15 min. The chemical composition of wastewaters were determined using spectrophotometer and ICP (induced coupled plasma) methods (Perkin Elmer Optima 3000, USA), in Central Laboratory of Ferdowsi University of Mashhad, before and after the pretreatment step. Dilution ratio in wastewaters 20% (v/v) was applied for algae treatment.

**Microalgae Growth**

*C. vulgaris* cultures were grown in different wastewaters, with varying amounts of nitrate and nitrite. During 30 days, 5 mL of the treated wastewaters, was taken to measure the growth rate. Final cell densities were measured by spectrophotometer at 680 nm in different days [24] A correlation between the optical density of *C. vulgaris* at 680 nm and the dried biomass was pre-determined. OD$_{680}$ was measured every day using spectrophotometer (UNICO 2100). The correlation is shown below:

$$R^2=0.9913$$

$$\text{Dry weight (g/l)} = 1.054 \cdot \text{OD}_{680} + 0.042$$

The specific growth rate in exponential phase of algal growth was measured by using Eq. (2) [23]:

$$\text{GR} = \ln \left( \frac{N_2-N_1}{(t_2-t_1)} \right)$$

$N_1$ and $N_2$ are defined as dry biomass (g/l) at days $t_1$ and $t_2$, respectively. The biomass productivity (P) was calculated according to the following formula:

$$P = (\text{DW}_t - \text{DW}_0)/(t - t_0)$$

Where $\text{DW}_t$ and $\text{DW}_0$ are dry biomass (g/l)$^2$ at days $t$ and $t_0$ (initial day), respectively.

**Determination of Nitrate Concentration**

The experiment was done within 7 days. Autoclaved wastewaters treated with *C. vulgaris*. After 7 days, 30 mL of the treated wastewaters were taken to measure the nitrate concentrations. The amount of the nitrate in each sample was determined and analyzed according to the standard method for examination livestock wastewaters [25].

Nitrate concentration in the culture medium and wastewaters were determined spectrophotometrically (UNICO 2100) according to the method described by Apha et al.[25]. Culture samples were collected after 30 days and centrifuged (1500 ×g, 10 min). The supernatant was collected and the absorbance measured at 220 nm after a proper dilution with deionizer water. The absorbance values were converted to nitrate concentration using a standard calibration curve made with NaNO$_3$ in the range of 0–5 mM.

**Determination of Nitrite Concentration**

Nitrite concentration in the culture medium and wastewaters were determined spectrophotometrically (UNICO 2100) according to the method described by Apha et al. [25]. Culture samples were collected after 30 days and centrifuged (1500 ×g, 10 min). The Griess-Ilosvay reaction was used for nitrite determination. The supernatant was collected and the absorbance measured at 540 nm after a proper dilution with deionizer.

**Statistical Analysis**

All experiments were managed in triplicate treatments unless otherwise stated. By one-way analysis of variance (ANOVA) were performed and the means were conducted by Tukey’s range test in JMP v8.

**Total RNA Isolation and CDNA Synthesis**

Total RNA was extracted from *C. vulgaris* cultured in BG11 medium and livestock wastewaters with RNA isolation kit.
To approve the elimination of genomic DNA from total RNA, polymerization of the control gene (18S rDNA) was carried out using RNA as PCR templates. Total RNA applied as the template to synthesize cDNA with RevertAid™ H Minus First Strand cDNA Synthesis Kit, (Fermentas # K1622) with random primers.

**Real-Time PCR**

All primer pairs of the marker genes are described in Table 1. The 18S rDNA gene of C. vulgaris was served as the internal control. Real-time PCR was done in an I Cycleriq real-time PCR detection system (Bio-Rad Laboratories, CFX 96) using SYBR® Green real-time PCR Master Mix (parstoos) same as the manufacturer's directions: one cycle of 95ºC for 30 s and then 35 cycles of 95ºC for 15 s each, followed by 60ºC for 15 s, and 72ºC for 30s. Three biological and three technical replicates were applied. Three parallel samples were applied for RNA isolation and three parallel real-time PCR analyses were done for each RNA sample.

In order to normalize the amount of tranblockeds in each sample, the relative abundance of 18S rDNA was also determined as the internal control. At the end of each run, melting curves for the amp icons were checked by raising the temperature stepwise by 0.5ºC from 55ºC to 95ºC while monitoring fluorescence. The specificity of the PCR amplification was verified by examining the melting curve for melting temperature, its symmetry and the lack of non-specific peaks. The gene expression data was analyzed using the 2^(-ΔΔCt) method. the changes were calculated according to standard curve and efficiency (E) for each primer.

<table>
<thead>
<tr>
<th>Wastewaters</th>
<th>NO$_3^-$ (mg/l) autoclaved</th>
<th>NO$_2^-$ (mg/l) autoclaved</th>
<th>NO$_3^-$ (mg/l) treatment</th>
<th>NO$_2^-$ autoclaved Remova 1(%)</th>
<th>NO$_3^-$ autoclaved Remova 1(%)</th>
<th>NO$_3^-$ Bio removal (%)</th>
<th>NO$_2^-$ Bio removal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>501.33</td>
<td>9.33</td>
<td>54.01</td>
<td>1.16</td>
<td>30</td>
<td>34</td>
<td>89</td>
</tr>
<tr>
<td>Sheep</td>
<td>406.66</td>
<td>9.33</td>
<td>43.33</td>
<td>5.83</td>
<td>32</td>
<td>29</td>
<td>89.3</td>
</tr>
<tr>
<td>Poultry</td>
<td>381.01</td>
<td>10.33</td>
<td>40.33</td>
<td>3.60</td>
<td>41</td>
<td>29</td>
<td>89</td>
</tr>
</tbody>
</table>

**Table 1: Description of studied genes and primers used for Real time-PCR amplification**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Function</th>
<th>Primer sequence (5’—3’)</th>
</tr>
</thead>
</table>
| NR      | Nitrate reductase (EC 1.7.1.1)             | Assimilatory nitrate reduction                | NR-L: TACTACAACTTCAACCACAAGC
NR-R: TATACCACGACTCCCTGTT        |
| accC    | Acetyl-CoA carboxylase BC subunit (EC 6.4.1.2) | Fatty acid biosynthesis                      | ACC-L: CAATCTAGATCAGGCTAGCG
ACC-R: AGAACTCGCTCTCGCTGCTTCT    |
| 18S rRNA| 18S rRNA (small ribosomal subunit)         | Reference gene                                | 18S-L: CCTGGCCTTTAATTGACGTC
18S-R: CGGA ACCAGCCGCTGACTATT    |

**Results and Discussion**

**Microalgae Strain Identification**

Colony formation, cell shape, growth phase, nutritional requirement and also molecular observation were simultaneously studied to determine the taxonomic classification of the studied strain. Morphological properties were evaluated by binocular and light microscope...
such as the lack of cell wall, a well-developed apical papilla.

The 18S rDNA sequence of the Chlorella vulgaris, was registered in NCBI database with an accession number of (KF661335.1). The sequence was studied using BLASTn tools. This sequence exhibited high similarities to other members of Chlorellaceae. The highest similarity was observed with Chlorella vulgaris (KF569735.1). Further analysis was performed using alignment of the studied sequence with 20 different strains whose 18S sequences were previously submitted at NCBI. These findings aligned with the morphological properties of the isolated strain and it was confirmed that the fresh water isolate strain is a member of Chlorella genus.

Table 2: The concentration of some elements were determined in wastewaters before autoclave.

<table>
<thead>
<tr>
<th>Wastewaters</th>
<th>NO$_3^-$ (mg/l)</th>
<th>NO$_2^-$ (mg/l)</th>
<th>PO$_4^{3-}$ (mg/l)</th>
<th>Fe$^{3+}$ (mg/l)</th>
<th>Ca$^{2+}$ (mg/l)</th>
<th>N/p ratio (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>720.01</td>
<td>14.33</td>
<td>30.86</td>
<td>1.24</td>
<td>123.20</td>
<td>23.33</td>
</tr>
<tr>
<td>Sheep</td>
<td>601.01</td>
<td>14.23</td>
<td>34.02</td>
<td>0.68</td>
<td>279.36</td>
<td>17.66</td>
</tr>
<tr>
<td>Poultry</td>
<td>650.33</td>
<td>14.13</td>
<td>25.4</td>
<td>0.50</td>
<td>121.12</td>
<td>25.60</td>
</tr>
</tbody>
</table>

Table 3: Different removal rate in C. vulgaris wastewaters treatments after autoclave and algae treatment.

<table>
<thead>
<tr>
<th>Wastewaters</th>
<th>NO$_3^-$ (mg/l)</th>
<th>NO$_2^-$ (mg/l)</th>
<th>NO$_3^-$ (mg/l)</th>
<th>NO$_2^-$ (mg/l)</th>
<th>NO$_3^-$ (mg/l)</th>
<th>NO$_2^-$ (mg/l)</th>
<th>NO$_3^-$ (mg/l)</th>
<th>NO$_3^-$ (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>autoclaved</td>
<td>autoclaved</td>
<td>treatment</td>
<td>treatment</td>
<td>autoclaved</td>
<td>autoclaved</td>
<td>autoclaved</td>
<td>autoclaved</td>
</tr>
<tr>
<td>Cattle</td>
<td>501.33</td>
<td>9.33</td>
<td>54.01</td>
<td>1.16</td>
<td>30</td>
<td>34</td>
<td>89</td>
<td>87.5</td>
</tr>
<tr>
<td>Sheep</td>
<td>406.68</td>
<td>9.33</td>
<td>43.33</td>
<td>5.83</td>
<td>32</td>
<td>29</td>
<td>89.3</td>
<td>43</td>
</tr>
<tr>
<td>Poultry</td>
<td>381.01</td>
<td>10.33</td>
<td>40.33</td>
<td>3.60</td>
<td>41</td>
<td>29</td>
<td>89</td>
<td>65.15</td>
</tr>
</tbody>
</table>

**Microalgae Growth**

On such a base, the growth characteristics of C. vulgaris within three cultivation media were studied. Figure 1 depicts the growth curves (GC) in a batch culture of microalgae using cattle, sheep and poultry wastewaters. The statistical comparison showed significant correlation between the initial nutrient concentration and the specific growth rate as well as the dried biomass weight.

Light/dark regime and pretreatment of centrifuge and autoclave and dilution were factors that affected biomass productivity and removal of nutrient. preparing of good and safe condition for algae treatment were main factors which were established with centrifuge and autoclave pretreatment. Solid matter was removed by centrifuge and microorganisms were eliminated with high temperature in autoclave. Suitable concentration was reached with appropriate dilution considered [26]. Considering N removal was very sensitive to N:P ratio, suitable ratio from elements existing in agricultural wastewater could prepared high removal percent. The microalgae treated in the wastewater with 34.02, 30.87 and 25.40 mg. l$^{-1}$ phosphate (dissolved in sheep, cattle and poultry wastewater), respectively. In similar observation, Ruiz and coworkers discussed the positive effect of increasing concentration of phosphate residue
on the enhanced algal cell growth. Moreover, Wang reported, [27]. Neochlorisoleoabundans in simulated wastewater and secondary municipal wastewater effluent 43.7% N removal with N/p ratio 1.33 and biomass productivity 0.63(g DCW L⁻¹ d⁻¹).

Similar trends were observed for the Ca²⁺ ion in the studied wastewater. On the other hand, studying the effect of other nutrients such as nitrate residue showed that 720, 650.33 and 601, mg. L⁻¹ nitrate included in, cattle, poultry and sheep wastewater, respectively, might negatively influenced the APAS. The possible reason is that it can increase the amount of hydrogen ions in wastewater without much acid-neutralizing function, out coming the acidification of wastewater [28]. High concentration of nitrogen could be toxic and diminish growth rate of algae. These results were in line with the previously published studies on B. braunii grew well in piggery wastewater pretreated by a membrane bioreactor (MBR), with initial concentration of nitrate 1020 (mg/l) and specific growth rate reduced to 0.024 (h⁻¹) [29].

Summarily it worth quoting that the high concentrations of some organic compounds could be inhibitory to algae. Exceeded the level of nitrate in effluents might be as toxic to Chlorella cells. The same conclusion was previously reported by Wang [30].

Nitrite and Fe⁺³ variation were not significantly meaningful therefore had not imposed such a big influence on the APAS.

What could be clearly observed from the cell density measurements as seen in Fig. 1 is that the decreasing or increasing nutrient residuals in the wastewater solely could not determine the final APAS in C. vulgaris. The results confirmed that a suitable combination of the nutrient could affect the cell growth and finally the produced dried biomass.

Table 4: C. vulgaris cultured in 20% (v/v) diluted wastewaters and BG11 medium as control

<table>
<thead>
<tr>
<th>wastewaters</th>
<th>Dry biomass (g/l)</th>
<th>Biomass productivity (g. L⁻¹ d⁻¹)</th>
<th>Growth rate (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry</td>
<td>1.03±0.14 A</td>
<td>0.08±0.007 B</td>
<td>0.00 B</td>
</tr>
<tr>
<td>Cattle</td>
<td>3.15±0.14 A</td>
<td>0.1±0.007 A</td>
<td>0.07±0.013 A</td>
</tr>
<tr>
<td>Sheep</td>
<td>2.7±0.14 A</td>
<td>0.09±0.007 A</td>
<td>0.03±0.013 AB</td>
</tr>
<tr>
<td>BG11</td>
<td>0.8±0.14 B</td>
<td>0.02±0.007 B</td>
<td>-0.00 B</td>
</tr>
</tbody>
</table>

Data showed as mean SD (n=3) during a 30-days cultivation period. Means were compared using one-way ANOVA and those with different letter are significantly different (at P<0.05).

Cattle wastewater had highest dry biomass 3.15(g/l), its biomass productivity is 0.1 (g.L⁻¹.d⁻¹) and its specific growth rate is 0.07 (g/l) (Table 4). This result was near the Wang and coworkers report 0.31 (g DCW L⁻¹ d⁻¹) biomass productivity for initial concentration 45 mg/l NaNO₃ containing 108 (mg/l) P-PO₄ ³⁻ was shown with Neochlorisoleoabundans cultured in synthetic wastewater at sodium nitrate and phosphate. They used different dilution of NaNO₃ to achieve a good removal and biomass productivity. Zhu and coworker in 2013 reported, Chlorella zofingiensis in piggery wastewater, Indoor and autoclaved condition 0.3 (g/l) specific growth rate and biomass productivity 0.2 (g. L⁻¹.d⁻¹). Pretreatment of autoclave and composition of batch and semi-continues could have effects on their biomass.

Biomass productivity 0.09±0.007 (g. L⁻¹. d⁻¹) and dry biomass 2.7±0.14 (g/l) with growth rate 0.03±0.007 (g/l) was obtained in Sheep waste water. In similar studies, 0.4 (g/l) a specific growth rate was obtained by Scenedesmus sp. of the nitrate sources along with wastewater, with air spraying to optimize the growth [31]. In Eustance’s study the effect of different factors such as pH and CO₂ spraying and nitrogen sources, without CO₂ spraying was evaluated and unsuitable concentration (3mM) of nitrate showed reduction in specific growth rate. In our study maybe it was necessary to change
nitrate concentration for the arranged N/P ratio and biomass productivity. Poultry wastewater had biomass productivity 1.03 (g.l⁻¹.d⁻¹) and dry biomass 0.03 (g/l), according to the stu [26]. This wastewater was less rich in comparison with two other wastewaters, so it might have needed CO2 spraying for this treatment to enhance biomass productivity. Wang and coworker obtained 0.5 (g/l) dry biomass in 20 days cultivation on 2% diluted poultry wastewater with 2h electrochemical pretreatment.

Nitrate Removal

Different concentrations of NO₃⁻ in wastewaters were used to screen nitrate removal ability using alive cells of microalgae C. vulgaris.

In this study, the livestock wastewaters were sterilized by autoclaving before the bioremediation treatment. The removal pattern was studied during the experiments and two steps were observed. Throughout the first step, the biomass began to settle the uptake nutrient and little assimilate of NO₃⁻ was done. As the algae grew, the NO₃⁻ amount reduced further, and finally concentrations in terminal step were stable. This step was named as steady state where the assimilation of NO₃⁻ was stable.

The wastewater concentrations after treatment depend on the initial concentrations, where the higher initial concentrations gave higher final culture concentrations. Fig. 2 shows the removal rate of the three wastewaters.

C. vulgaris could remove the nitrate at rates of 89.22% in cattle wastewater and 89.34% in sheep wastewater and 89.41% in poultry wastewater (Fig. 2). The high rate of nitrate removals was obtained among all the batch cultures (Fig. 4). In this study the nitrate removal was contemporary with the growth rate. Our results showed that growth rate increased with high initial nitrate concentration from cattle wastewater. The biomass productivity in the wastewater treatments might result from nutrient assimilate by new cells and increased biomass in batch cultures. Microalgae can uptake, organic and inorganic nitrogen in batch culture [32-33-34].

For the reason that the microalgae cells need to nitrogen for protein, nucleic acid and phospholipids production. Thus, microalgae growth is found to be essential for nitrogen removal via assimilation in cells [35].
Nitrite Removal

Simultaneous bioremoval of nitrate and nitrite from three livestock effluents using C. vulgaris were studied (Fig3). Nitrite removal in the cattle, sheep and poultry wastewaters were 14.33, 14.23, 14.13 mgL⁻¹ initial concentrations observed, the final removal in treatments could reach 87.50%, 43%, 65.15%, respectively. Nitrite removal in contrast to nitrate removal in this study was lower, for removal rate and initial concentration.

Nitrite is the most transitory form of nitrogen in the aquatic cultures [36]. In all culture systems, it observes as the least common form of inorganic nitrogen [37]. Recently cells of microalgae have been applied for nitrite removal from different sources [38]. The algae displayed relatively high growth and removal rates when cultured in medium with high amounts of nitrite ions [39].

In a study, the nitrite removal experiments with the algal biofilms were performed in modified BB medium for 40 days. The removal amount of nitrite was 0.20 mg per day [40].

High rate algal ponds (HRPs) for wastewater treatment containing nitrite have obtained great attention. In these systems, oxygen made by photosynthetic algae was applied to helping heterotrophic bacteria activity and changed wastewater containing nitrite into biomass [41].

In other study, growth features of a locally sampled were investigated by microalgae strain Chlorella sp. in aquatic cultures and its ability to remove nitrite from environment. The results showed that Chlorella removed 70% of nitrite ions [42]. In another study, nitrite removal was investigated by chitosan immobilized Scenedesmus. It was prepared to remove nitrite at a level of 70% [43].

Also, in a study, it is evaluated the adaptation of Chlorella to high concentrations of nitrite. The results of their experiments displayed that, although high amounts of nitrite totally have negatively impacts on growth and photosynthesis of Chlorella strains, the level of nitrite resistance is a strain-specific characteristic. Some Chlorella strains have an extremely higher ability to adapt to high concentration of nitrite. It is noteworthy that, Chlorella sp. C2 was discovered to have a high resistance and quickly adapt to high levels of nitrite [44].
Fig. 3: Change in nitrite concentrations of livestock wastewaters during batch culture under various initial concentrations of nitrite. Differences in mean nitrite removal rate showed after autoclaved livestock wastewater (black bars) cultures, and then autoclave treated livestock wastewater under treatment with C. vulgaris (gray bars) (P < 0.05).

There have been many new researches of algal treatment on wastewater (industrial and municipal and agricultural) and algal biofuel harvest. An algal turf scrubber (ATS) system of growing filamentous algae has been increased and considered to eliminate nitrogen, phosphorus and soluble carbon from agricultural effluent [45-46].

Gene Expression

3.5.1. Nitrate reductase expression

Nitrogen storage and carbon fixation are highly related in unicellular algae. The first step in the nitrogen uptake is the reduction of nitrate to nitrite which is catalyzed by the nitrate reductase enzyme (NR). Livestock wastewater cultures showed an important up-regulation for NR gene throughout the times compared with cultures grown under BG11 medium conditions (Fig. 5).

As well as in C. vulgaris, over-expression of NR gene under wastewater treatment has also been showed lately in other microalgae [47-48]. Besides, it is also known that in low concentration of nitrate, the NR enzyme activity decreases due to the loss of the NR protein [13]. Therefore, it might be cause that a decrease of NR enzyme content would due to the observed down-regulation of NR gene. This cell response could be a correlate mechanism to facilitate nitrogen storage if nitrate is replaced in the medium, as it has been suggested for other nitrogen synthesis-related genes [49].

Remarked cattle wastewater for high concentration of nutrient special nitrate. In this treatment, NR expression was increased until 96 hours in both wastewater and culture medium and NR transcript decreased after this time. In algae cells, gene expression related to nutrient concentration in environment, after nitrate elimination from wastewater in 96 hour, nitrate reductase activity was decreased.

The highest fold of NR transcript in both wastewater and culture medium was observed in 96 hours after treatment. Gene expression was down-regulated after 96 hours in both culture conditions. In livestock wastewater, fold changes were higher than BG11 culture medium. It might be for reason of higher nitrate concentrations in livestock wastewater than BG11 medium.
Conclusions

In summary, the analysis of the response of *C. vulgaris* to different nitrate concentration showed a two-stage response which was appeared at the chemical and molecular impacts. In the first stage, high concentration of nitrate in wastewater and medium leads to a strong assimilation of nitrate in to cell compounds. After nitrate removal in the batch culture condition, NR transcript is decreased.

The concurrent up-regulation of genes involved in nitrogen assimilation (NR) suggests the activation of some kind of molecular mechanism that will enable a fast assimilation of new nitrate by cells. Prolonged nitrate in low concentration in BG11 triggers a cell response with lower transcript regulation characterized by a major metabolic shift of NR and nitrate assimilation.

For re-use of nutrient in wastewater, we benefited from algae treatment. NR gene was selected for measuring reason of algae capacity resistant to high level of nutrient in wastewater. The outcome of this experiment can be useful for a better realization of the underlying molecular changes in response to nitrate concentrations in *C. vulgaris*. The analysis of gene expression changes is a strong tool to realize the compatibility of microalgae to variable environments, although it is also well known that changes in mRNA levels with changes in protein levels or enzyme activities [50-58].

References


