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Acclimation process of cultivating *Chlorella vulgaris* in toxic excess sludge extract and its response mechanism

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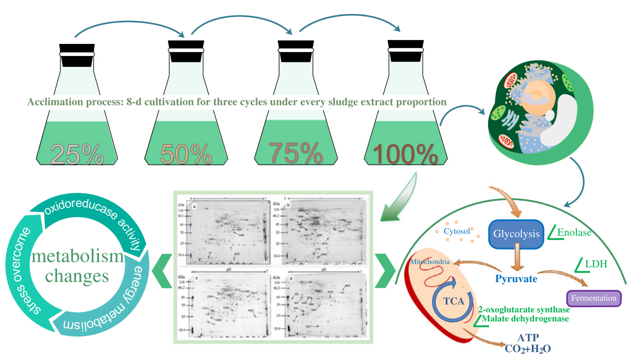
HIGHLIGHTS

- The removal of TOC and ecotoxicity could be improved after acclimation.
- The growth of *C. vulgaris* could be improved after acclimation.
- Proteomics proved that *C. vulgaris* could acclimatize itself by metabolic selection.
- Pyruvate fermentation, TCA cycle, and glycolysis were enhanced after acclimation.

GRAPHICAL ABSTRACT



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ABSTRACT

Chlorella vulgaris was cultivated in the gradually increased proportion of toxic sludge extracts for acclimation, which was obtained from SBR treated synthetic wastewater containing mixed chlorophenols (2,4,6-trichlorophenol and 4-chlorophenol). The growth of *C. vulgaris* was obviously improved after acclimation with the cell number in the 100% sludge group was $22.75 \pm 0.85 * 10^6$ cell mL^{-1}, which was relatively more than the BG11 control group's ($20.80 \pm 0.35 * 10^6$ cell mL^{-1}) and apparently over the 100% sludge group ($10.78 \pm 0.45 * 10^6$ cell mL^{-1}). Compared with the sludge control sludge group, *C. vulgaris* in the acclimation group gained 24.1% and 18.2% more relative removal rate about TOC and ecotoxicity, respectively. Proteomics analysis showed that protein spots were more clear and centralized and the clarifications of the different protein spots narrowed from 8 to 5 after acclimation. Proteins related to oxidoreducase activity and energy metabolism were over expressed and *C. vulgaris* could select the metabolic pathways, especially enhanced pyruvate fermentation, TCA cycle, and glycolysis after acclimation, by over accumulating the corresponding vital enzymes. Conclusively, acclimation was a good method to improve the removal ability and growth of *C. vulgaris* and algae could acclimatize itself to grow upon the toxic sludge extracts by metabolic selection. We suppose acclimation process was a potential method for algae wastewater treatment and algae cultivation without or reduce dilution.

1. Introduction

In recent decades, the rapid growth of economy leads to the over-development of industry which would cause industrial wastewater

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problems (Lei et al., 2010). Though industrial wastewater contains complex chemicals, biological treatment processes also can be used to treat it after some pretreatment (Wang et al., 2016) mainly due to the economy, stability and efficacy (Babaee et al., 2010). While more severe secondary pollution might be caused by the excess sludge, especially from the treatment process of industrial wastewater which contains toxic organic substances (Fang et al., 2017). The wide use of chlorophenols as fungicides, herbicides and wood preservatives ensure them can be found in industrial wastewater (Lim et al., 2013; Monsalvo et al., 2009). Chlorophenols could be biodegraded by activated sludge (Sahinkaya and Dilek, 2002, 2005), especially by some dominant microorganisms after acclimating (Diez et al., 2012; Kong et al., 2014). During the biodegradation of 4-CP, sludge toxicity was positively related to microbial diversity (Zhao et al., 2016) and extracellular polymeric substances (EPS) toxicity (Bao et al., 2016). Excess sludge toxicity was also induced by secondary metabolites from the microorganism and the by-product of chlorophenols (Zhao et al., 2015). On the other side, excess sludge contains complex organic materials which could be considered as nutrients (Jia et al., 2013; Qian et al., 2016). Attention should be paid to the sludge from chlorophenols wastewater treatment, for toxicity needs to be removed properly while the nutrients in excess sludge could be utilized as resources.

Microalgae, widely existing in both land and ocean, have been used to deal with environmental problems for about 80 years. *Chlorella* sp. was reported as one of the most tolerant eight genera according to the previous study (Palmer, 1969) and highly tolerant to soluble organic compounds (Kumar et al., 2010). *Chlorella vulgaris* is one of the most popular strains for its good performance on waste and toxicity, which earned a lot of researchers' concern. However, there always is an upper limit of the waste for this species. When treating with the single or mixed substances, cell number, protein (enzyme activities) and pigment rates of *C. vulgaris* would be inhibited by too high chemical concentration (Chen and Pei, 2016; Sahinkaya and Dilek, 2009; Saygideger and Okkay, 2008; Xiong et al., 2017) and the inhibition to algae varied with different kinds of substances and their toxicity (Geiger et al., 2016). In order to let *C. vulgaris* perform more potentially in overcoming the undesirable and mixed circumstance and remain good performance on removal, the wastes need to be diluted or add at a certain proportion (Table 1).

While in the dilution or addition process, the wastewater volume would be enlarged and the cultivation cost would be increased accordingly due to the mix with the conventional medium or fresh water. So it would make the application of algae to the environmental field more feasible and lower the cost/secondary pollution by finding a proper way to cultivate algae for wastewater treatment without dilution.

For microorganism, especially unicellular lower organisms, could improve their abilities to survive and accommodate the undesirable condition after orient acclimation (Los and Murata, 2004). *C. vulgaris* is one of the unicellular planktonic eukaryotes which are easily and fast acclimated. In Li's study, many strains of chlorella species were adapted to high nitrite concentrations, which was achieved through a three-step process of restrict, acclimation, and thriving (Li et al., 2016). Under strong light conditions, yellow coloration could be observed in *Chlorella* and the “molecular sunglasses” mechanism would be stimulated (Grudzinski et al., 2016). Under the high-temperature exposure, the photosynthetic machinery of *Chlorella saccharophila* would change to cope with this stress (Patil et al., 2017). The symbiotic alga *Symbiodinium* spp. could adjust the PSII mechanism under thermal acclimation to ameliorate the high temperature-induced photoinhibition and photobleaching (Takahashi et al., 2013). During the acclimation process of *Nannochloropsis oceanica* from seawater to freshwater, the mechanism changes in algae caused the final slowdown in cell growth (Guo and Yang, 2015). To use algae for wastewater treatment, the ability of algae to cope with the environmental stress and what the changes in algae mechanism should be explored. Proteomics analysis is a proper method that could help us to reveal the differences of molecular pathways and metabolism in algae while the cultivation medium changes (Li et al., 2014; Perez-Garcia et al., 2011).

Therefore, in this study, *C. vulgaris* cultivation was performed in the mixed culture medium with BG-11 and toxic sludge extracts from the activated sludge-treated synthetic wastewater containing 2,4,6-trichlorophenol (2,4,6-TCP) and 4-chlorophenol (4-CP). According to the previous study (Wang et al., 2017), the proportion of toxic sludge extracts increase from 25% to 100% in the process of acclimation at each 25% interval; the incubation period was 8-days with 3 cultivating cycles under each proportion. Also, the mixed mediums came into four percentages of sludge extracts (25%, 50%, 75% and 100%) were taken as the control group for comparison. TOC and ecotoxicity inhibition rate were analyzed for evaluating the acclimation effects. Proteomics analysis of *C. vulgaris* was tested for indicating its changes in molecular pathways and response mechanism.

2. Materials and methods

2.1. *C. vulgaris* strain and pre-culture conditions

The strain, *C. vulgaris* (FACHB-31, Chinese Academy of Sciences, Wuhan Institute of Aquatic Organisms), was preserved in 250 mL flasks containing 100 mL BG-11 medium (Wang et al., 2017) in a Boxun light growth chamber (SPX-250B-G; Shanghai Boxun Industry & Commerce Co., Ltd., China). The algal preservation conditions were as follows: temperature: 25 ± 1 °C; light density: 2000 lx; light: dark = 14:10.

2.2. Cultivation medium procedure

2.2.1. Excess sludge

Sequencing batch reactors (SBR) were set for synthetic wastewater (20 mg L^{-1} 2,4,6-TCP and 20 mg L^{-1} 4-CP) treatment (dissolved oxygen: 1.5 ± 0.5 mg L^{-1}; hydraulic retention time: 8 h; sludge retention time: 20 d; Temperature: 25 ± 1 °C). The excess sludge (after 2 h of precipitation) was taken from SBR reactor during the stable phase (after 60 days operation) when 2,4,6-TCP and 4-CP were undetectable in both aqueous and sludge phrase.

2.2.2. Sludge solids

The excess sludge (with the equal volume of the algal cultivation sludge medium) was centrifuged (5000 r min^{-1}, 5 min) to obtain the sludge solids. The sludge solids were cleaned thrice by Milli-Q (MQ, equal volume with the excess sludge) water to dispose of the extracellular component effect.

Table 1
The dilution conditions of wastewater for *C. vulgaris* cultivation.

Algal Species	Wastewater	Dilution	Influences	Ref.
C. vulgaris	Digested piggery	**200 mL to 10 L**	Algal production	Kumar et al. (2010)
C. vulgaris JSC-6	Swine wastewater	**20-fold**	Nutrient removal rate	Wang et al. (2015)
Arthrospira platensis & C. vulgaris	Raw poultry litter leachate	**10–25 X**	Intracellular component	Markou et al. (2016)
C. vulgaris (C9-JN2010)	Citric acid effluent	**20% diluted**	Algal growth & nutrients removal	Li et al. (2013)
C. vulgaris (UTEX 2714)	Thermophilic anaerobic digestion swine manure	**3×**	Algal growth & nutrients removal	Deng et al. (2017)
C. vulgaris (FACHB-31)	Toxic Sludge extracts	**50%**	Algal growth, nutrients and toxicity removal	Wang et al. (2017)