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Biosorption Analysis and Penoxsulam Herbicide Removal Efficiency by Transgenic *Chlamydomonas reinhardtii* Overexpression the Cyanobacterial Enzyme Glutathione-*s*-transferase

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Abstract

Penoxsulam, a new post-emergent rice herbicide, is widely used in rice fields in Egypt. Penoxsulam has been shown to have adverse effects on plant growth; hence, it prevents plants from producing an important enzyme, acetolactate synthase**.** Glutathione-*s*-transferase (GST) plays a vital role in the detoxification of xenobiotics including Herbicides. Genetically-engineered algae can provide feasible and environmentally safe approaches for the phycoremediation of herbicides. In the current study, Cyanobacterial GST was transferred to *Chlamydomonas reinhardtii*, a model unicellular alga, via the *Agrobacterium tumefaciens*-mediated transformation method. The GST enzyme activity was confirmed in three transgenic *C. reinhardtii* lines. To evaluate the removal capacity of penoxsulam by transgenic *C. reinhardtii*, the microalgae were treated with penoxsulam at 5, 10, and 20 µg ml^{-1} for twenty-four hours. The percentage of the herbicide removal from 5 and 20 µg ml^{-1} reached maximally 93.6 % and 54 % in the case of transgenic alga, whereas in the case of the wild type, the percentage of removal reached 52 % and 21 %, respectively. Langmuir and Freundlich adsorption isotherm models and the sorption kinetics have been applied to the experimental data to check the effectiveness of the removal process. The equilibrium data were well-fitted with the Langmuir isotherm model. The results showed that GST transgenic microalgae are effective in the removal of penoxsulam from aqueous solutions and it can be used in phycoremediation systems of herbicides.

Keywords: Penoxsulam- phycoremediation- Glutathione-*s*-transferase (GST) - Isotherm- Langmuir

1. Introduction

Xenobiotics are naturally-occurring compounds (animal poisons, toxins, antibiotics, drugs, and toxic products from plants) or synthetic compounds (insecticides, pesticides, herbicides, and inorganic fertilizers) (Bulucea *et al*., 2012). These compounds may cause damage to living organisms resulting in deformities, DNA damage, poisoning or at the very least, a feeling of discomfort (Shamaan, 2005; Bulucea *et al*., 2012). Nowadays, pollution and soil contamination are among the major concerns, and biofertilizers play a very significant role in that pollution. These contaminants limit the growth and development of many microorganisms that play a role in mineralization process (Mishra *et al*., 2013).

Herbicides are considered as pollutants in aquatic environments, including ground water, estuaries, rivers, lakes, and coastal marine waters (Prado *et al*., 2009). The different types of herbicides are designed to kill plant tissues. However, this is accomplished through two basic methods referred to as selective (Systemic) and non-selective (contact) herbicides. Penoxsulam is a new post-emergent rice herbicide in Egypt. Penoxsulam is a member of the triazolopyrimidine sulfonamide family of herbicides. It is considered to be a systemic herbicide. It moves throughout the plant tissues and prevents plants from producing an important enzyme, called acetolactate synthase (ALS) (Whitcomb, 1999). Microalgae are essential to provide energy and primary substances for survival in most aquatic ecosystems (Ma *et al*., 2002; Prado *et al*., 2009). Hence, there is a need to estimate the effect of the toxic herbicide on microalgae. *Chlamydomonas reinhardtii* has attracted more attention as a model for studying biological systems because this organism is the most biologically characterized one (Harris, 2001). The genomes in *C. reinhardtii* (chloroplast, nuclear and mitochondrion) are well characterized (Merchant, 2007; Popescu and Lee, 2007), and provide wealth of information for genetic manipulation and studies of this microalgae. New strategies have been established for developing algae or plants that are highly tolerant to herbicides through biotechnological tools. In this context, glutathione-*s*-transferases (GSTs) are members of the transferases family that quench reactive molecules and catalyze the conjugation of GSH to an array of

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hydrophobic and electrophilic substrates, protecting the cells from oxidative burst. Members of this family were first discovered for their potential of metabolizing an array of toxic exogenous compounds, that is xenobiotics via GSH conjugation (Cummins *et al*., 2011). GSTs have been implicated in several cellular processes (Nianiou- Obeidat *et al*., 2017). Studies suggest that GSTs could protect the plants from different abiotic stresses (Ding *et al*., 2017), heavy metal stress (Zhang *et al*., 2013), damage of ultra-violet light (UV) and radiations (Liu and Li, 2002). Studies suggest that GSTs safeguard the cells against chemical-induced toxicity and provide tolerance by catalyzing S-conjugation between the reduced thiol group of GSH and electrophilic moiety in the hydrophobic and toxic substrate (Deavall *et al*., 2012). After conjugation, the new molecules are sequestered into the vacuoles or are exported from the cells by putative membrane ATP-dependent pump systems. Although the involvement of members of GST family has been reported in plant development and biotic stresses (Nianiou- Obeidat *et al*., 2017), limited information is available regarding the involvement of this family in combating stress. Among the different classes of GSTs, the role of Lambda class GSTs has been reported in stress responses (Kumar *et al*., 2013a, 2013b) as well as in plant growth and development. Thus, functional characterization and analysis of regulatory aspects of gene expression of the GST gene family members can help enhance the understanding of their role in detoxification. Apart from herbicide detoxification, the involvement of GSTs in hormone biosynthesis, tyrosine degradation, and peroxide breakdown (Oakley, 2011), stress-signaling proteins (Loyall *et al*., 2000), and non-catalytically acting as flavonoid-binding proteins (Mueller *et al*., 2000) has been reported. In the current study, the cyanobacterial glutathione-*s*-transferase gene (GST, gi451779298, sll0067)) was genetically cloned and transferred into *C. reinhardtii* cells. The overexpression of GST in transgenic *C. reinhardtii* showed an enhanced tolerance to the penoxsulam herbicide compared to wild types.

2. Materials and Methods

2.1. Chemicals.

All chemicals used were of the analytical grade and were obtained from Sigma-Aldrich (Munich, Germany). Th stock solution of penoxsulam was freshly-prepared daily with deionized water throughout the tests.

2.2. Chlamydomonas reinhardtii Culture Conditions.

Chlamydomonas reinhardtii, was obtained from Prof. Mohammed Ismaeil (Botany Department, Faculty of Science, Mansoura University, Egypt). *C. reinhardtii* was aseptically grown in a Tris Acetate Phosphate (TAP) medium (Gorman and Levine, 1965). The pH of the medium was adjusted to 7.4. For kanamycin (5 mg/mL) selection of transformed *C. reinhardtii* colonies, solid TAP medium supplemented with 1.5 % (w/v) agar was used. The algal cultures were incubated in growth chamber at 25±2 ˚C under long day conditions (16 h light/8 h dark), and were kept under a light intensity of 80 µmol m^{-2} s^{-1} with continuous shaking (75 rpm) in case of liquid cultures.

2.3. Experimental Procedures

2.3.1. Gene Cloning and Plasmid Constructs

The Glutathione-*S*-transeferase gene (GST) coding sequence (gi451779298, sll0067) was amplified by PCR using genomic DNA isolated from *Synechoccous elongatus PCC6803* (kindly provided by Botany Department, Faculty of Science, Cairo University, Giza, Egypt) as template. A forward primer with extension for *Nco*I site (5´-ACTGCCATGGGTATCAAACTATACG-GTGC-3´), and a reverse primer (5´-AATTTCTAGAT-CAGCGGGCACCGATG-3´) with extensions for *Xba*I site were used. The amplified GST PCR fragments were column-purified and then ligated into the binary expression vector pTRAK, a derivative of pPAM (gi13508478). This cloning step results in a single-plant expression construct (pTRAK-GST) for GST (Figure 1). The GST gene expression cassette was flanked by the scaffold attachment region (SAR) of the tobacco RB7 gene (gi3522871). The nptII cassette of pPCV002 was used for the transgenic *C. reinhardtii* selection on kanamycin (Koncz and Schell, 1986). The GST gene expression is designed to be under the control of CaMV-35S promoter (Reichel *et al*., 1996).

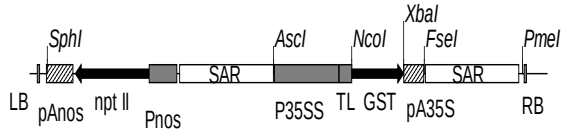

Figure 1. Schematic representation of the binary expression vector pTRAK-GST. P35SS/PA35S; promoter/Terminator sequence of Cauliflower Mosaic virus. TL (Cab22L); 5'UTR of the tobacco leader peptide. LB and RB; Left and Right border sequences of Nopalin-Tiplasmids. NptII; Neomycin phosphor-transferase type II that confers resistance to kanamycin. Pnos/PAnos; Promoter/Terminator of Nopaline synthase gene from *Agrobacterium tumefaciens*. GST gene is flanked by the scaffold attachment region (SAR) of the tobacco RB7 gene (gi3522871).

2.3.2. Transformation and Generation of Transgenic C. reinhardtii

The pTRAK-GST construct was transformed into *C. reinhardtii* following the *Agrobacterium tumefaciens* (GV3101)-mediated co-cultivation transformation protocol as previously described (Kumar *et al*., 2004). A single colony of *C. reinhardtii* was inoculated into a liquid TAP medium and was allowed to grow till reaching a log phase of growth. Cells were then plated on a solid TAP medium and incubated under continuous light for forty-eight hours until forming a lawn of cells. An *Agrobacterium* culture transformed with pTRAK-GST plasmid was grown in a liquid LB medium containing appropriate antibiotics (25 mg/l rifampicin and 50 mg/l kanamycin) at 28 ˚C till OD_{600} reaches 0.6. The *A. tumefaciens* cells were then spun down (4000 × g for 5 min at 4 ˚C) and resuspended in a 250 µl liquid TAP medium containing 100 µM acetosyringone. The bacterial suspension was then co-cultivated with *C. reinhardtii* cells grown on the agar plates for two hours at 28 ˚C followed by overnight incubation at 25 ˚C in dark. The *C. reinhardtii* cells were harvested and washed twice with a liquid TAP medium supplemented with 500 mg/l cefotaxime in order to completely kill and/or remove the *Agrobacterium* cells. For the selection of the transformed *C. reinhardtii* cells,

the washed cells were cultivated on solid TAP agar plates containing 500 mg/l cefotaxime and 5 mg/ml kanamycin, and were incubated at 25 ℃ in growth chamber for eight days until the appearance of the transformed colonies. The presence of the GST transgene in the selected *Chlamydomonas* colonies was confirmed by the colony PCR test using GST gene specific primers.

2.3.3. Quantitative Real-time RT-PCR Analysis

The quantitative real-time RT-PCR was performed as described previously (Kebeish *et al*., 2015). RNA was extracted from the *C. reinhardtii* cells following the 1-bromo-3-chlorpropane (BCP protocol) of Chomczynski and Mackey (1995). First-strand complementary DNA (cDNA) was synthesized as described by Niessen *et al.* (2007). An ABI PRISM1 7300 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) was used following the manufacturer's instructions. PCR amplifications were performed using SYBR Green Reagents (SYBR1 GreenERTM qPCR Super Mixes; Karlsruhe, Germany). Primers were purchased from Intron Biotechnology Inc. (Kyungki-Do, South Korea). For the detection of GST transcripts, primers 5′-GCGATCGCCATTTATCCTC-3′/5′-ACCGCAGGATA-GTCGTCAAA-3′ were used. Primers 5′GCGATGTGGA-CATCCGCAAG-3′ and 5′-GGGCCGTGATCTCCT-TGCTC-3′ were used for the detection of ACTIN transcripts. Primer concentration in the reaction mixture was adjusted to 200 nM. The amplification program used for the amplification of both of the GST and ACTIN fragments was a ten-minute primary denaturation at 95 ˚C, followed by forty cycles consisting of fifteen seconds of denaturation at 95 ˚C and one minute of combined annealing and extension at 60 ˚C.

2.3.4. Growth Assay of Transgenic and Wild C. reinhardtii

The algal strains were aseptically grown in a TAP medium according to Gorman and Levine (1965). The wild type (WT) as well as transgenic strain were allowed to grow in a TAP medium under a photoperiod of light: dark (16:8 hrs) for twenty-six days. The optimum light intensity (80 µmol m^{-2} s^{-1}) was supplied with cool white fluorescent tubes. The growth curves of both WT and transgenic were spectrophotometrically estimated at different intervals (2 days) at 650 nm according to Robert (1979) through the incubation periods. As the incubation period reached a mid-log phase (12 days), the algal cells of either WT or transgenic strains (C.GST3) were harvested by centrifugation at 5000 xg for five minutes and were double-washed thoroughly with 10 mM Na_2-EDTA, and then by sterile distilled water. The algal tissue was used for further analyses.

2.3.5. Estimation of Glutathione-s-Transferase

A determined amount of each treatment was ground to a fine powder in liquid N_2 and was then homogenized in 1 ml of an extraction buffer (50 mM potassium phosphate buffer (pH 7.4) containing 10 mM EDTA, 0.1 % Triton X-100, 20 % sorbitol and 2 % Polyvinyl pyrrolidone). The homogenate was centrifuged for fifteen minutes at (8000 xg /4˚C). The supernatant was collected and made up to a known volume and used to estimate the enzymes activity.

The GST activity was determined according to Habig *et al*. (1974), using 1-chloro -2, 4-dinitrobenzene (CDNB) as substrate. Assay of GST activity, 3 mL of the reaction mixture (100 mM Potassium phosphate buffer (pH 7.0), 0.03 % Reduced glutathione (GSH), 0.02 % CDNB and 0.0372 % Na-EDTA) were mixed with 100 µL of the enzyme extract. The enzyme activity was calculated by monitoring the reaction mixture for 120 minutes (20 minutes intervals) at 340 nm in a spectrophotometer.

2.3.6. Analysis of Adsorption Equilibrium Isotherm

2.3.6.1. Isotherm Process

To Erlenmeyer flask, 100 mL containing 25 mL of sterilized TAP medium, a suitable volume of penoxsulsm 2.5 % stock was taken and inoculated to reach the final concentration (5, 10 and 20 µg mL^{-1}). Three flasks for each concentration were prepared. All the flasks were inoculated with 5 ml of healthy algae under aseptic conditions. (1 mL = 0.6 optical density) and incubated at 25±2 ℃ under a photoperiod 16 light: 8 dark for twenty-four hours. At the end of the incubation period, different samples were taken from both treated and untreated alga. The algal cells were separated by centrifugation at 10000 rpm, and the clear solutions were used to estimate the herbicide residue according to Nour El-Dien *et al.* (2010).

2.3.6.2. Determination of Penoxsulam Residue

2 mL of 2 % (w/v) of an ammonium molybdate solution was added to 6 mL of 4M H_2SO_4, 0.75 mL of ammonium thiocyanate (10%, w/v) and 0.1 mL of ascorbic acid (10%, (w/v)). The solutions were placed in a 100 mL separating funnel. The mixtures were left for fifteen minutes at room temperature (25±5 ˚C). 1 mL of the herbicide solution was added, and the reaction mixture was left for another fifteen minutes. The ion-pairs were extracted with dichloromethane twice with 5 mL portions after shaking for one minute. The ion-pairs were collected in a 10 mL measuring flask and methylene chloride was dried over anhydrous sodium sulphate. The absorbance of the filtered extract was measured at 470 nm, against a reagent blank, prepared similarly without the herbicide (Nour El-Dien *et al*., 2010).

The removal percentage was determinated by the following equation

$$\text{Removal \%} = (C_0 - C) \times 100/C_0$$

Where C_0 is the initial concentration of penoxsulam (µg mL^{-1}) in the solution, and C is the final concentration of penoxsulam (µg mL^{-1}).

2.3.6.3. Adsorption Isotherm

Studies of the adsorption equilibrium isotherm were conducted using varying concentrations of the penoxsulam herbicide (5, 10, and 20 µg mL^{-1}) for twenty-four hours at room temperature. These data were evaluated by Langmuir and Freundlich adsorption isotherm equations to interpret the efficiency of the bioremediation of the penoxsulam sorption (Freundlich, 1906; Langmuir, 1918).

Langmuir's isotherm model assumes monolayer adsorption, and is presented by the following equation.

Langmuir model: $qe = Q_{max} * K_L * Ce/(1+K_L * Ce)$

Langmuir model in linear form: $Ce/qe = 1/(Qmax*KL) + 1/ Qmax *Ce$

Where qe is the penoxsulam amount adsorbed per unit mass of adsorbent (mg g^{-1}), Ce is the equilibrium concentration of penoxsulam in the solution (mg mL^{-1}), Q_{max} is the maximum adsorption capacity (mg g^{-1}), and K_L is the constant related to the free energy of adsorption. A straight line is obtained by plotting

Ce/qe against Ce and the slope and intercept are used to calculate the Q_{max} and K_L, respectively.

The Freundlich model is presented by the following equation, which indicates that the surface of the adsorbent is heterogeneous (Freundlich, 1906).

Freundlich model: $qe = K_F * Ce\ 1/n$

Freundlich model in linear form: $\log qe = \log K_F + 1/n * \log Ce$

Where K_F is a parameter of the relative adsorption capacity of the the adsorbent related to the temperature, and n is a characteristic constant for the adsorption system. A plot of log qe against log Ce gives a straight line; the slope and intercept correspond to 1/n and log K_F, respectively.

2.4. Statistical Analysis

The data were represented in figures as mean ± stander error (SE) of three independent samples for each genotype. The obtained data were analyzed statistically using Student's *t*-test to determine the significant differences among the data. Differences were considered significant when (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All statistical analyses were carried out using Microsoft Excel software (Microsoft corporation, USA).

3. Results

3.1. Gene Cloning and Generation of Transgenic C. reinhardtii.

GST plays a vital role in various detoxification processes of xenobiotics under biotic and abiotic stresses. The over-expression of cytosolic GST in algae enhances the tolerance to herbicide. Therefore, glutathione-*s*-transferase was genetically cloned and transferred into the nuclear genome of the model microalga, *C. reinhardtii.* The binary expression vector, pTRAK-GST (Figure 1) was used for the transformation of the green microalga, *C. reinhardtii* via the *Agrobacterium tumefaciens*-mediated co-cultivation method (Kumar *et al.*, 2004) in order to test the efficacy of the transformed alga in the removal of herbicide. Transgenic *Chlamydomonas* lines were selected on a solid TAP medium supplemented with kanamycin antibiotic (Figure 2). Three independent lines of *Chlamydomonas reinhardtii* transgenic for GST, named C.GST-1, C.GST-2, and C.GST-3, were generated and used for further molecular and biochemical analyses.

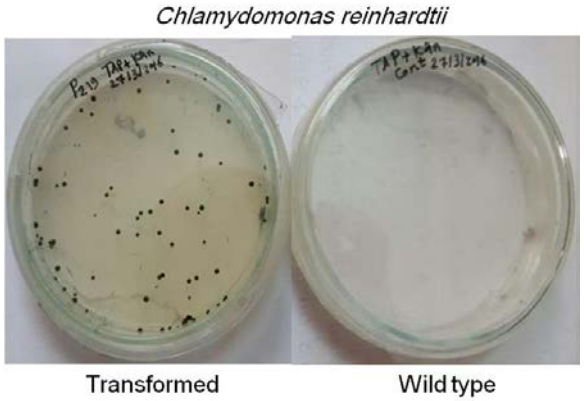

Figure 2. Photograph representing the selection of the transformed colonies of *C. reinhardtii* and the wild type cells on solid TAP medium supplemented with kanamycin.

3.2. RT-PCR and Glutathione-S-Transeferase Enzymatic Assays.

Quantitative real-time polymerase chain reaction (qRT-PCR) systems for the screening of GST gene expression level using TaqMan 7300 series were established. Primer systems for GST and Actin (as a house-keeping gene) were designed and optimized for the RT-PCR analysis using serial dilutions of plasmid DNA. This test was used for testing the expression level of GST. The results of the qRT-PCR analysis for the expression of the GST gene in transgenic *C. reindardtii* are shown in Figure 3A. Three transgenic *C. reinhardtii* lines of GST were tested. Variable expression levels for the GST gene were observed in all of the tested lines. All the tested lines revealed a high expression level of the mRNA transcript accumulation of both GST compared with Actin. C.GST-3 and C.GST-2 show a high expression level for both transgenes compared to the other tested algae.

In vitro, the biochemical reaction was prepared to determine the activity of GST in the case of the transgenic and wild types. The results in Figure 3B show that the different transgenic lines under investigation have a more or less similar increase with a highly significant response with respect to the wild type. The previous increases reaching about 5x fold over the WT. C.GST-2 and C.GST-3 lines show more a GST activity compared to GST-1 line. C. GST-3 exhibited the highest expression level for GST, thus this line was chosen for the herbicide biosorption and removal analyses.

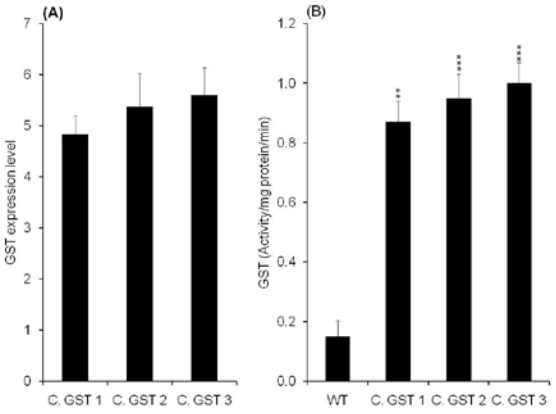

Figure 3. (A) Accumulation of GST mRNA transcripts in the GST transgenic *C. reinhardtii* alga. (B) Activity of glutathione-*s*-transferase.

3.3. Growth Assay of Transgenic and Wild-Type C. reinhardtii.

The growth of transgenic (C.GST-3) and wild-type *C. reinhardtii* was varied (Figure 4). Short-lag phases were observed, which indicates that the algae have a good adaptability to these growth conditions. The growth patterns of both algae have log phases extended for eighteen days followed by the stationary phase. There were significant differences in the cell density of the transgenic and wild-type *C. reinhardtii* due to the overexpression of GST in the transgenic line.

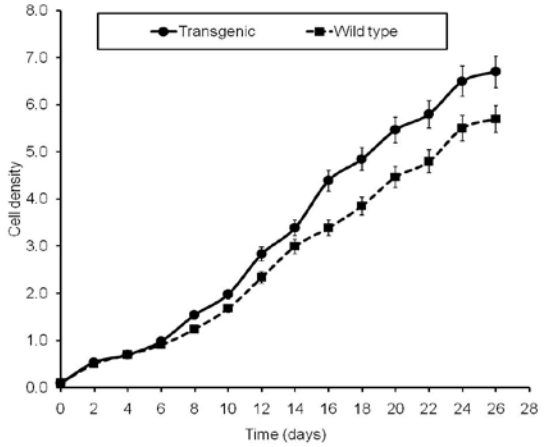

Figure 4. Growth curve of transgenic (C. GST3) and wild type *C. reinhardtii*.

3.4. Sorption and Desorption Efficiency of Penoxsulam

The Penoxsulam (Triazopyrimidine sulfonamide) herbicide removal was checked by transgenic *C. reinhardtii* for the GST gene against the wild type. Two sets of different concentrations of penoxsulam each comprised of (5, 10, 20 µg mL^{-1}) were prepared. Each concentration inoculated by 50 mg fresh weight of transgenic and wild cells, and all sets were maintained for twenty-four hours at room temperature. The results in Table 1 reveal that transgenic alga has a promising efficiency to remove the herbicide compared to the wild type. The percentage of herbicide removal attained from the culture was maximally 93.6 % as the transgenic alga treated with 5 µg mL^{-1}, whereas, in the case of the wild type, the percentage of removal reached 52 %. As the concentration of penoxsulam increased, the rate of its removal decreased to reach 54 % and 21 % for the transgenic and wild type, respectively.

Table 1. Percentage of removal of penoxsulam by transgenic (C. GST3) *Chlamydomonas* against wild type.

Concentration of penoxsulam (µg mL^{-1})	Residual concentrations of penoxsulam (µg mL^{-1})		Removal percentage of penoxsulam (%)	
	C.GST	WT	C.GST	WT
5	0.32	2.4	93.6	52.0
10	4.26	6.5	57.4	35.00
20	9.2	15.7	54.00	21.50

Table 2 shows that the adsorption co-efficient isotherm for penoxsulam fitted the Langmuir equation quite well. The values of Langmuir constants, q_{max} and k_L obtained from the linear plot of C_e/q_e against C_e, and their correlation coefficient (R^2), k_L and q_{max} revealed that the Langmuir isotherm model best fitted the experimental data for the sorption of herbicide by both the wild and transgenic alga (Figure 5A and 6A). This is because the experimental q_{max} in the case of transgenic type was 10.8 µg mg^{-1} and the calculated value was 12.5 µg mg^{-1} and R^2 reached 0.80. Meanwhile, in the case of the wild type, the calculated q_{max} (4.8 µg mg^{-1}) was also coincident with the experimental one (q_{max}= 4.89 µg mg^{-1}); R^2 also reached 0.96 and the Langmuir constant was 3.52. The major difference between the sorption capacities of both the transgenic and wild type was due to the difference in their overexpression of GST.

Table 2. Langmiur and Frundlich isotherm constants for penoxsulam adsorption by *C. reinhardtii.*

Species	Langmuir Isotherm				Freundlich Isotherm		
	R^2	K_L	Q_{max} Experimental (µg mg^{-1})	Q_{max} Calculated (µg mg^{-1})	R^2	K_F	1/n
C. GST3	0.88	0.05	11.7	12.5	0.69	0.89	4.87
WT	0.98	3.52	4.38	4.8	0.44	0.342	4.27

On the other hand, the Freundlich isotherm model did not fit the experimental data for the biosorption of the penoxsulam due to slope n<1 and other parameters (R^2 and K_F) was also low (Figure 5B and 6B). So, the overexpression of glutathione-*s*-transferase enables the transgenic alga to remove the herbicide compared with the wild type. In the future, phycoremediation would be a promising and eco-friendly process to reduce the risks of xenobiotics.

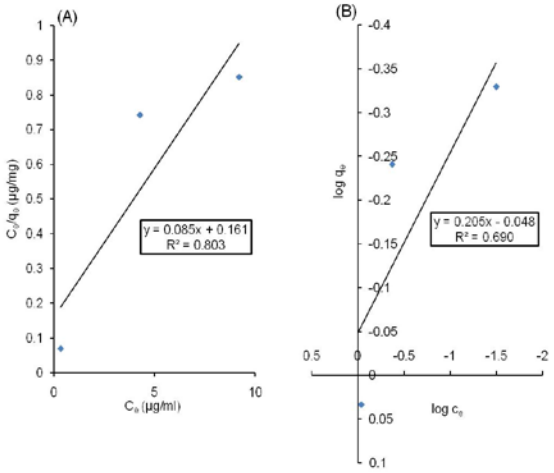

Figure 5. The amount of penoxulam adsorbed onto the trasnsgenic *C. reinhardtii* (C. GST3) at various equilibrium penoxsulam concentrations, (A) Langmuir adsorption isotherm, (B) Freundlich adsorption isotherm. *P*-value <0.05 for transgenic line (C. GST3) compared to wild type.

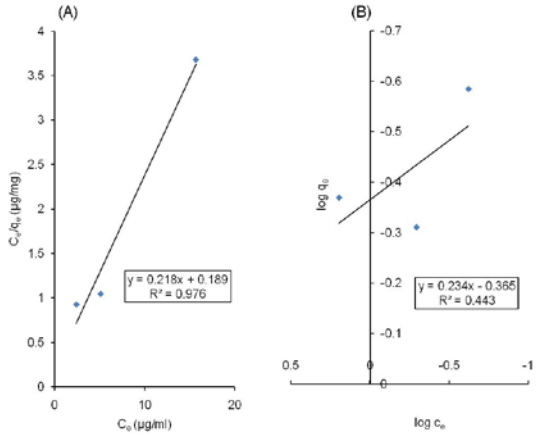

Figure 6. The amount of penoxulam adsorbed onto the Wild *Chlamydomonas* at various equilibrium penoxulam concentrations, (A) Langmuir adsorption isotherm, (B) Freundlich adsorption isotherm

4. Discussion

4.1. Gene Cloning and Generation of Transgenic C. reinhardtii Lines.

This study presented the overexpression of the antioxidant enzyme, glutathione-*s*-transferase, (GST, from *Synechococcus elongatus*) in *C. reinhardtii*. *C. reinhardtii* was shown to accumulate a large quantity of penoxsulam. Such uptake and decay of penoxsulam resulted in the rapid removal of penoxsulam from the medium due to the overexpression of GST in transgenic alga. This observation suggests that *C. reinhardtii* may be useful in molecular breeding designed to improve the environmental stress tolerance to the herbicide or remediation of contaminated aquatic ecosystems. To achieve this purpose, the gene- encoding GST was cloned into a binary expression construct in the current study. The construct was transferred into *Chlamydomonas* via an *Agrobacterium tumefaciens*-mediated transformation (Kumar *et al*., 2004). GST was overexpressed in *Arabidopsis* to increase tolerance to salt (Qi *et al*., 2004). It was also overexpressed in tobacco separately or in combination with CYND to maintain the redox homeostasis and improve the cyanide remediation capacity of tobacco (Kebeish *et al*., 2017).

4.2. RT-PCR and Enzymatic Assay.

The activity of the GST enzyme was tested in the algal extract isolated from the GST transgenic algal cells. As control, an extract from the wild-type (WT) *Chlamydomonas* was used. There was a 5x fold increase in GST expressing *Chlamydomonas* lines over WT (Fig. 3B). This indicates that GST is functionally active in the GST lines. The background activity observed for the GST enzyme in the wild-type alga may be related to the endogenous *Chlamydomonas* glutathione-*s*-transferase activity. The results of this study are in accordance with Kebeish *et al*. (2017) who found a three- fold (GST) to a four-fold (CYND) increase in the activity over background in GST and CYND expressing tobacco lines when GST was cloned separately or in combination with CYND. Similarly, about a 5x-fold GST expression level over actin was observed for the GST transgenic algae (Figure 3A). The data obtained from the qRT-PCR analysis (Figure 3A) and the GST enzymatic activity (Figure 3B) indicate that GST is functionally active in the transgenic lines.

There are three phases of biotransformation. Phase I consists of enzymes that catalyze reactions which modify the functional groups of the pollutant. In phase II, those enzymes catalyze transfer reactions of whole groups or biomolecules to pollutants. Phase III includes translocation processes that render pollutants or their metabolites non bioavailable. For bioremediation purposes, biotransformation enzymes mainly belong to four biochemical types: oxidoreductases, hydrolases, transferases and translocases (or pumps). Among all known transferases, Glutathione *S*-transferase (GST) is mainly involved in biodegradation for bioremediation purposes. GST includes a superfamily of enzymes that have been found in bacteria, fungi, algae, plants and animals (Herve *et al*., 2008; Toribio *et al*., 1996). Even though they catalyze the transference of glutathione to electrophillic pesticides, they can also show hydrolytic and peroxidase activities (Toribio *et al*., 1996). Interestingly, GST can also catalyze the dehalogenation of rings e.g. Lindan (Habig *et al*., 1974). In addition, there is evidence that GST has a role in the regulation of plant growth and development (Jiang *et al*., 2010).

4.3. Sorption and Desorption Efficiency of C. reinhardtii to Penoxsulam

The biosorption isotherm models (Figure 5 and 6) are extensively used to evaluate the maximum biosorption capacity, the concentration of treated effluent, the distribution of polluted agents in the bulk solution and on the biomass, which could all be described by one or more isotherms such as the Langmuior model and Freundlich that are the most commonly used.

The Langmuir's isotherm model describing the Adsorption of Adsorbate onto the surface of the Adsorbent requires three assumptions (Langmuir, 1981):

1. The surface of the adsorbent is in contact with a solution containing an adsorbate which is strongly attracted to the surface.
2. The surface has a specific number of sites where the solute molecules can be adsorbed.
3. The adsorption involves the attachment of only one layer of molecules to the surface, i.e. monolayer adsorption.

The Langmuir model assumes a monolayer adsorption with a homogenous distribution of the adsorption sites and adsorption energies without interaction between the adsorbed molecules. The Langmuir adsorption isotherm model describes quantitatively the formation of a monolayer adsorbate on the outer surface of the adsorbent; hence, no further adsorption takes place. On the other hand, in the Freundlich model, the energetic distribution at the sites is heterogeneous due to diversity of adsorption sites and the diverse nature of the adsorbate.

The Langmuir isotherm model best fitted the experimental data for the sorption of herbicide by both the wild and transgenic alga. The results in the current study are concomitant with Ramadoss and Subramaniam (2018) who found out that the Langmuir isotherm model shows clear data about the experimental adsorption of chromium on blue green marine algae with an R^2 value of 0.90. R^2 values of Langmuir are highly significant with the experimental data. In the present study, the value of the Langmuir adsorption equilibrium constant k_L is 0.05 for transgenic alga. This shows quantitatively the clear affinity between the herbicide and transgenic alga. These results are also in accordance with Ramadoss and Subramaniam (2018) who observed that the Langmuir adsorption equilibrium constant k_L was 0.0354. Suwannahong *et al.* (2014) found out that fly ash may possess a homogeneous as well as a heterogeneous surface energy distribution that describes the formation of monolayer and heterolayer solute (Herbicide; Paraquat, Alachlor) coverage on the surface of the adsorbent (fly ash). Ho Kyong Shon (2008) has found that the adsorption of metsulfuron-methyl onto titanium oxide and powdered activated carbon fitted the Langmuir isotherm model with a reasonable degree of accuracy with higher R^2 values than those of the Freundlich isotherm model.

In conclusion, transgenic alga C. GST3 plays an effective role in the biosorption and bioremediation of the herbicide penoxsulam. The overexpression of GST improved the phycoremediation capacity of transgenic alga

through the reduction of the stress generated by xenobiotics.

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