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Research Article

RNA ISOLATION, CDNA SYNTHESIS AND ARSENIC METHYLTRANSFERASE GENE (*ARSMT*) EXPRESSION STUDIES BY RT-PCRINTHREE DIFFERENT MICROALGAL SPECIES *Chlorella vulgaris*, *SCENEDESMUS ACUTUS* AND *OSCILLATORIA ACUMINATA*

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ABSTRACT

Microalgal species have evolved the ability to tolerate and detoxify the toxic arsenic (AS) substances in their environments, often by producing metabolic enzymes that efficiently detoxify the arsenic toxicant. Arsenic is a redox sensitive metalloid that can also be methylated by different Microorganisms.AS methylation involves sequential transformation of inorganic ASto mono, di and tri-methylated species. Arsenic biotransformation was considered as a major pathway for arsenic detoxification, which includes the processes of oxidation, reduction and methylation. Arsenic methyltransferase (*Arsmt*) is a key enzyme that catalyzes the transfer of a methyl group to the acceptor (AS) in the presence of the methyl group donor like the methyl-cobalamin, S-adenosylmethionine. In this research, the RNA Isolation, cDNA synthesis and arsenic Methyltransferase Gene (Arsmt) expression was studied by using RT-PCR in Three Different Microalgal Species such as *Chlorella vulgaris*, *Scenedesmus acutus* and *Oscillatoria acuminata*. The test samples of *Chlorella vulgaris*, *Scenedesmus acutus* and *Oscillatoria acuminata* expressed up regulations on Arsmt gene respectively over the control samples while the 16s gene was used as internal control (Housekeeping gene) in gene expression studies for the normalization. The test samples of *S.acutus*, *O. acuminata* and *C. vulgaris* treated with 50ppm arsenic was showed up regulations on *Arsmt*gene expression by 2.24,2.03 and 3.04fold increase respectively over control samples. The microalgal species could be used to detoxify the arsenic from drinking water samples as ecofriendly, low cost and potent method for arsenic detoxification.

INTRODUCTION

Arsenic

Arsenic is a carcinogenic chemical element with the symbol *As* and its atomic number is 33. Arsenic is a metalloid, found in several minerals, generally in conjunction with sulfur, metals and can also be occur as pure elemental crystals. Groundwater contamination of arsenic is the serious environmental problem that affects more than 100 million of people all over the world. Arsenic is also one of the most toxic heavy metals and it is regarded by World Health Organization (WHO) as the first priority pollutant (Saha, 1995;Jie and Waalkes, 2008;Bhaskar*et al*., 2009).

Drinking Water Problems

Water is very precious commodity of the world and most of the water (97%) in the earth is salty (sea) water and cannot be used for drinking. Only 2.5% of the earth water is fresh water and two third of that water is frozen in ice caps and glaciers. Only 0.01% of the total earth water is accessible for drinking purpose. Water without any contamination is one the basic human needs. More than one in six people lack reliable access to get pure drinking water in the developing world. All drinking water

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samples especially from deep bore tube wells must be tested frequently for arsenic and other chemical contaminants (Meharg, 2004). Since there has been many research works were done in arsenic water pollution and suggests that the drinking water has higher arsenic concentration beyond the permissible limit Indian Standard of 50µg/l and WHO Standard 10µg/l in several districts of West Bengal, India (Johnston and Heijnen, 2001;Tokunaga *et al*., 1999).

In arsenic affected areas, the Government of West Bengal has painted the arsenic contaminated tube wells in red color which are having more than 50ppm (50mg/l) of arsenic. The safe tube wells (<50ppm) were painted in green color. Despite the many attempts has made to educate the communities about arsenic contamination, large numbers of people are now also continuing to drink water the from the tube wells which are contaminated by arsenic at more than 50ppm concentrations (Imamul Huq *et al.,* 2006). This is because in many arsenics affected areas of West Bengal only less safe tube well water is available, hard to compete with the low cost and easy maintenance. Filtering tube well water to remove arsenic is not feasible over the long term in West Bengal, India and also in Bangladesh with a per capita income of less than four hundred rupees in a day. The arsenic removing filters are relatively expensive, cumbersome to maintain and requires regular testing for timely replacement. Treatment of the surface water through community systems is an alternative solution favored by the West Bengal government. Various group of researchers are continuing to work on low-cost treatment systems for arsenic removal from drinking water (Cairncross and Kinnear, 1991). Treatment of arsenic contaminated water with biological systems especially with microalgal systems will be a key model to detoxify arsenic from drinking water habitats.

Arsenic Water Pollution in West Bengal, India

Heavy metals are naturally occurring at Ganges-Sindh plains. The problem is not only in West Bengal, but also in other parts of the sub-continent, including Punjab (east and west) and Rajasthan (Sindh) have the same problem. Several countries are objecting spices, basmati rice and other farm products imports from South Asian countries because of arsenic contamination. Millions of people in West Bengal, India are sick due arsenic poisoning. According to recent report 1.04 cr people are affected by arsenic contamination in West Bengal (The Hindu, 2017). West Bengal has sufficient rainfall throughout the year even though lots of people are not getting arsenic free and pure drinking water. Provision of arsenic free drinking water for the people in West Bengal is the basic necessity. People from rural areas of West Bengal are not testing the water obtained from the water habitats to see what harmful chemicals it contains, because the water samples may have arsenic obtained from strata's and do contain several minerals, many of which may be harmful to the health. The people from rural areas of West Bengal are needs to be aware of dangers posed by the arsenic water pollution before it is getting too late. The researches even can't provide arsenic free water and food, the minimum requirement for the life of the West Bengal people.

The tube well water affected by arsenic contamination and making water from these pumps to drink leads to get many health effects. The village in Gaighata block in North 24 Parganas, West Bengal has got arsenic contamination and its groundwater recording for arsenic levels are above 0.05 mg/liter that is five times greater than the World Health Organization's permissible limit of 10micrograms per liter. Wide occurrence of arsenic in West Bengal has attracted much attention since it was first reported in 1976. A large portion of eastern, northern and northeastern parts of India, a part of the Ganga-Meghna-Brahmaputra stretch, has reported to be at risk from drinking water arsenic contamination, which caused many health disorders among the village people on this stretch.

Arsenicosis

Arsenic is of important concern in drinking water treatment systems because it has many health effects to the Human beings (Tokar*et al*., 2011; EPA, 1988). Appearance of the threatened and dangerous disease, "Arsenical Dermatitis (ASD)" or "arsenical toxicity" or "arsenicosis" is rampant in several villages of West Bengal, India having drinking water contaminated with arsenic more than 50 micrograms per liter (Mazumder*et al*., 2010). A major impediment to a quicker diagnosis of arsenicosis is that, it takes a decade or longer for the symptoms to be appeared (Tseng *et al*., 1961). The people with arsenic poisoning in West Bengal and Bangladesh is a social stigma and are shunned within their villages as shown in the Figure. 1.

Figure 1 Some of the arsenic-affected people from 1.04 crore arsenic affected Patients byarsenic Contamination in West Bengal, India (Baba, Dawn News, March, 2015).

Bioremediation of arsenic by Microalgae

Studies on distribution and behavior of *As* in the environment, metabolism and resistance mechanisms of *As* in microorganisms and bioremediation of the *As* polluted environment have gained considerable attention (Foster *et al*., 2008;Karadjova*et al*., 2008;Velizarov*et al*., 2004;Yin *et al*., 2012;Zhang *et al*., 2014). In both prokaryotes (e.g., Cyanobacteria) and eukaryotes (e.g., Chlorella sp.), *As* (V) is absorbed into cells via phosphate transporters, while *As* (III) moves across plasma membrane via aquaglyceroporins (AQP) and hexose permeases. The use of microalgae in wastewater treatment and the systematic examination of algae forbiologically active substances, particularly for antibiotics were also initiated after 1950's (Borowitzka, 1995).

Arsenic Methyltransferase

Microorganisms have evolved the ability to tolerate toxic substances in their environments, often by producing metabolic enzymes that efficiently detoxify the toxicant. Arsenic is a redox sensitive metalloid that can also be methylated by different Microorganisms. The *As* biogeochemical cycle involves various redox and methylation reactions. Inorganic *As* can be transformed by microbial methylation, which constitutes an important part of the AS biogeochemical cycle (Jun *et al*., 2015). It is thought that *As* methylation involves sequential transformation of inorganic *As* to mono, di and trimethylated species. Arsenic biotransformation was considered as a major pathway for arsenic detoxification, which includes the processes of oxidation, reduction and methylation. Arsenic methyltransferase is a key enzyme that catalyzes the transfer of a methyl group to the acceptor (arsenic) in the presence of the methyl group donor like the methyl- cobalamin, S-adenosylmethionine (Duncan *et al*., 2015;Maeda *et al*., 1990; 1992; 1993). The *ars* gene responsible for the organoarsenical detoxification includes *arsM,* which encodes an As(III) S-adenosylmethionine methyltransferase, *arsI*, which encodes a C-As bond lyase and *arsH*, which encodes a methylarsenite oxidase. Several important genes related to sulfate assimilation and GSH metabolism were induced for detoxification of *As*.

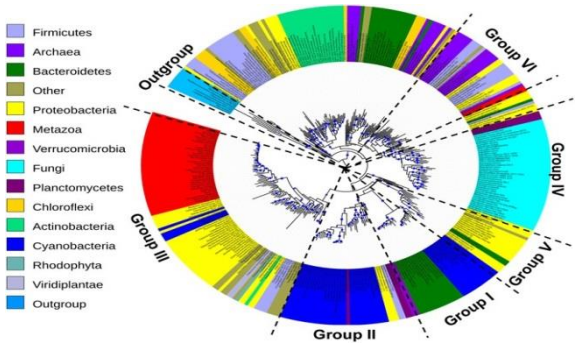


Figure 2 Phylogeny of ArsM As(III). (Ref. Nature.com)

Arsenic methylation is a sequential reaction, where toxic inorganic arsenic methylates into less toxic pentavalent mono, di and tri-methylated arsenicals. Trivalent methylarsonous acid (Mas(III)) and dimethylarsinous acid (DMAs(III)) are highly toxic intermediates of the methylation reaction and are easily oxidized into less toxic methylarsenate (MAs(V)), dimethylarsenate (DMAs(V)) and trimethylarsine oxide (TMAs(V)O) (Cullen, 2014; Drobna *et al*., 2009; Shikha *et al*., 2016). Higher accumulation of arsenic in edible plants cause increased human health risks, including cancer, especially in Southeast Asia. In microbes, TMA (III), a volatile metabolite, elaborates the arsenic detoxification pathway by converting the inorganic arsenic into organic arsenic. Soil microbes thus play a crucial role in environmental arsenic detoxification by biotransformation of inorganic arsenicals into innocuous organic forms. Many bacteria, archaea, fungi, microalgae and animals are able to methylate As Figure 2. Arsenic methylation is advantageous to organismsfor example; mice with a knock-out mutation of AS3MT retain much higher concentrations of arsenic in different tissues than do wild-type mice (Drobna *et al*., 2009).

MATERIALS AND METHODS

Microalgal Sample Collection, Isolation and Biomass Production

Microalgal samples were collected in sterile eppendorf tube by scrubbing and using sterile spatula from various arsenic contaminated sites of West Bengal, India. Based on the dominance species in all the arsenic contaminated sites three microalgal species such as *Chlorella vulgaris*, *Scenedesmus acutus* and *Oscillatoria acuminata* were isolated by serial dilution technique. The purified microalgal samples such as *Chlorella vulgaris*, *Scenedesmus acutus* and *Oscillatoria acuminate* were grown for 21 days in liquid BBM medium with 50ppm arsenic concentration and also grown without arsenic in BBM medium for control samples. The Biomass was harvested by Centrifugation at 5000rpm for 5 minutes at 4°C.

RNA Isolation, cDNA Synthesis & Gene expression analysis

Total RNA was extracted from the microalgal samples using RNA isolation reagent (TRIzol-Sigma) and the isolated RNA was assessed for its quality and quantity using Nanodrop (ThermoFisher Scientific). Total RNA (500ng) was used for the conversion of cDNA synthesis. The RNA and hexa primer were used for the first strand cDNA synthesis by Reverse transcriptase (RT) using kit method (Thermo scientific). The Real Time PCR amplification carried out in a reaction volume of 20µl containing 2µl of cDNA and 10µl of SYBR Green Supermix (Bio Rad, USA) for 35 cycles followed by denaturation 95ºC for 30sec, annealing 52ºC - 58ºC (Gradient) for 30sec extension for 72ºC for 15sec using Bio-Rad CFX96 system. The RNA expression levels were normalized to that of housekeeping gene and the results were analyzed.

Statistical analysis

Each parameter was analyzed with at least three replicates and a Standard Deviation (SD) was calculated and data are expressed in Mean ± SD of three replicates. The statistical works were done by Microsoft excel version 2003.

RESULTS AND DISCUSSION

RNA Isolation for gene expression studies

The total RNA molecules were extracted and purified from the microalgal samples (Both treated with 50ppm arsenic and control samples) such as *Chlorella vulgaris*, *Scenedesmus acutus* and *Oscillatoria acuminata*. The quality of total RNA analyzed by agarose gel electrophoresis is presented in Figure 3.The clear bands of RNA were observed between 500 base pairs to 2500 base pairs. RNA concentration of the test samples of *Chlorella vulgaris*, *Scenedesmus acutus* and *Oscillatoria acuminata* were determined by taking optical density at 260 / 280nm by Nano drop method (Table. 1).

Table 1 RNA concentration of the Microalgal Species samples by Nano drop method.

S. No	Microalgal Species	Samples	RNA Conc. ng/ µL	260 / 280nm
1	*S.acutus*var. Obliquus Rabenh	Control	3323.7	2.04
		Treated	4517.8	2.04
2	*O. acuminata* Gom	Control	2360.5	2.02
		Treated	3844.2	2.04
3	*C. vulgaris* Beyerinck	Control	2816.5	2.03
		Treated	2819.5	2.04

The RNA concentrations of the samples were described below. The isolated RNA samples were subjected to cDNA synthesis (reverse transcription) and qPCR gene expression was performed using arsenic methyltransferase (***Arsmt***) gene specific primers. The ***Arsmt*** gene expression levels in three microalgal samples (control and treated) were given Table. 2.

Table 2 Primer information of Three species

S. No	Microalgal Sp.	Gene	Forward Primer	Reverse primer
1	*S. acutus*	16s	AGAGTTTGATCCTGGCTCAG	CCGTCAATTCMTTTRAGTTT
		Arsmt	ATGACCTATTTAGAAACAGCCGC	CAGCAACCACCACCGTTATA
2	*S. acuminata*	16s	AGAGTTTGATCCTGGCTCAG	CCGTCAATTCMTTTRAGTTT
		Arsmt	ATGACCTATTTAGAAACAGCCGC	CAGCAACCACCACCGTTATA
3	*C. vulgaris*	16s	AGAGTTTGATCCTGGCTCAG	CCGTCAATTCMTTTRAGTTT
		Arsmt	ATGACCTATTTAGAAACAGCCGC	CAGCAACCACCACCGTTATA

Assay for Gene Expression in Scenedesmus acutusby Real-Time PCR (CFX96 – Bio-Rad)

The effect of the test samples on its gene expression was tested by using quantitative Real Time PCR. The test samples of *Scenedesmus acutus* var. Obliquus Rabenh Control and Treated were analyzed for the gene expression. RNA was isolated from the samples and subjected to cDNA synthesis (reverse transcription) and qPCR gene expression was performed using gene specific primers. The results revealed that the arsenic treated test samples of *Scenedesmus acutus*was expressed up regulation on Arsmt gene respectively over control sample. The 16s gene was used as internal control (Housekeeping gene) in gene expression studies for the normalization.

Arsenic MethytransferaseGene Expression Study of Scenedesmus acutusvar. Obliquus Rabenh

The mRNA expression levels were normalized to the level of housekeeping gene (16s gene). The Ct values of the test samples were calculated and the data was expressed in terms of fold change over Control sample. The arsenic methytransferase gene expression was increased in the microalgal samples of *Scenedesmus acutus* which is confirmed by observing strong bands than control samples **(**Figure. 4**).** The test sample of *Scenedesmus acutus*treated with 50 ppm arsenic was showed up regulation on *Arsmt*gene expression by 2.24-fold increased over Control sample. The bands were observed between 250 bp to 500 bp. The control samples were had light bands while the arsenic treated samples had thicker bands.

Assay for Gene Expression in Oscillatoria acuminata by Real-Time PCR (CFX96 – Bio-Rad)

The effect of the test samples of *Oscillatoria acuminata* on its arsenic methytransferase gene expression was tested by using quantitative Real Time PCR. The test samples of *Oscillatoria acuminata* 1) Control 2) Treated were analyzed for the arsenic methytransferase gene expression. RNA was isolated from the samples and subjected to cDNA synthesis (reverse transcription) and qPCR gene expression was performed using gene specific primers. The results reveal that test samples Treated-2 expressed up regulation on Arsmt gene respectively over Control-2 sample. The 16s gene was used as internal control (Housekeeping gene) in gene expression studies for the normalization(Figure. 5 and 6)**.**

Arsenic Methytransferase Gene Expression Study of Oscillatoria acuminata Gom

The mRNA expression levels were normalized to the level of housekeeping gene (16s gene). The Ct values of the test samples were calculated and the data was expressed in terms of fold change over Control sample. The arsenic methytransferase gene expression was increased in the microalgal samples of *Oscillatoria acuminata* which is confirmed by observing strong bands than control samples (Figure. 4). The test sample of *Oscillatoria acuminata*treated with 50 ppm arsenic was showed up regulation on Arsmt gene expression by 2.03-fold increased over Control sample. The bands were observed between 250 bp to 500 bp. The control samples were had light bands while the arsenic treated samples had thicker bands.

Table 3 Normalized *Arsmt* gene expression analysis of the test samples of *S. acutus, S. acuminate* and *C. vulgaris* over control samples.

S. No	Normalized *Arsmt* gene expression analysis	Samples	Ct value		Normalized Gene expression (Fold change over control)	Inference
			House keeping Gene 16s	Gene of Interest *Arsmt*		
1	*S. acutus*	Control	19.95	27.82	1.00	The test sample Treated showed up regulation on *Arsmt* gene expression by 2.24 fold increased over Control sample.
		Treated	19.36	26.07	2.24	
2	*O. acuminata*	Control	19.36	26.07	1.00	The test sample Treated showed up regulation on Arsmt gene expression by 2.03 fold increased over Control sample.
		Treated	21.15	26.83	2.03	
3	*C. vulgaris*	Control	17.49	23.04	1.00	The test sample Treated showed up regulation on Arsmt gene expression by 3.04 fold increased over Control sample.
		Treated	17.25	21.19	3.04	

Figure 3 RNA samples Isolated for gene expression studies. 01 - *Chlorella vulgaris*, (Control), 2 - *Oscillatoriaacuminate* (Control), 03 -*Scenedesmus acutus* (Control), 04 - *Chlorella vulgaris*, (Treated with 50 ppm arsenic), 05 - *Oscillatoriaacuminata* (Treated with 50 ppm arsenic), 06 - *Scenedesmus acutus* (Treated with 50 ppm arsenic).

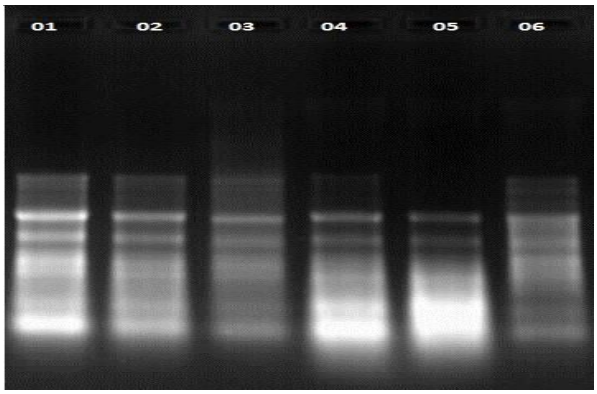
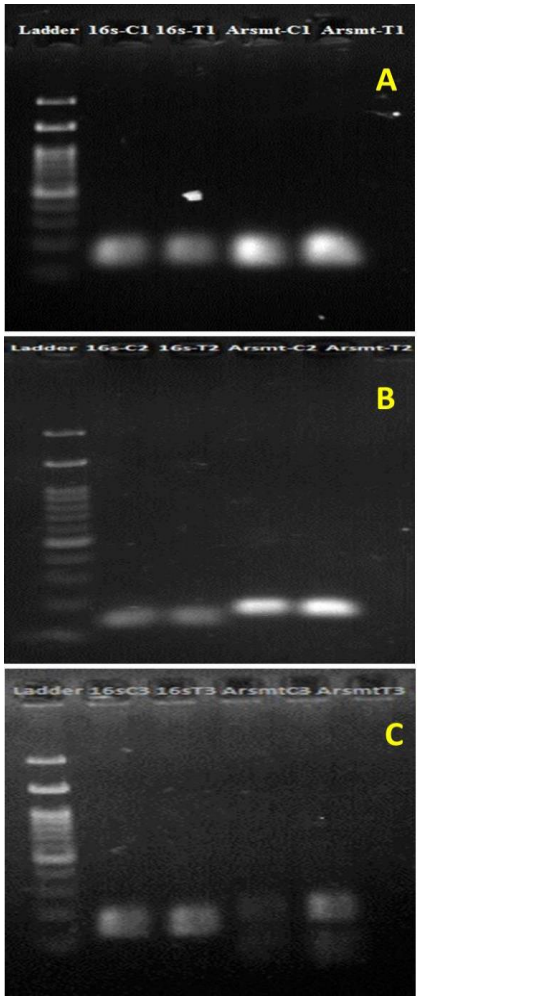


Figure 4 Normalized *Arsmt* gene expression analysis of the test samples A).*Scenedesmus acutus*B).*O. acuminata* and C). *C. vulgaris* over control sample.

Assay for Gene Expression in Chlorella vulgaris by Real-Time PCR (CFX96 – Bio-Rad)

The effect of the test samples of *Chlorella vulgaris* on its gene expression was tested by using quantitative Real Time PCR. The test samples of *Chlorella vulgaris* 1) Control 2) Treated were analyzed for the gene expression. RNA was isolated from the samples and subjected to cDNA synthesis (reverse transcription) and qPCR gene expression was performed using gene specific primers. The results reveal that test samples Treated-3 expressed up regulation on Arsmt gene respectively over Control-3 sample. The 16s gene was used as internal control (Housekeeping gene) in gene expression studies for the normalization (Figure. 5 and 6)**.**



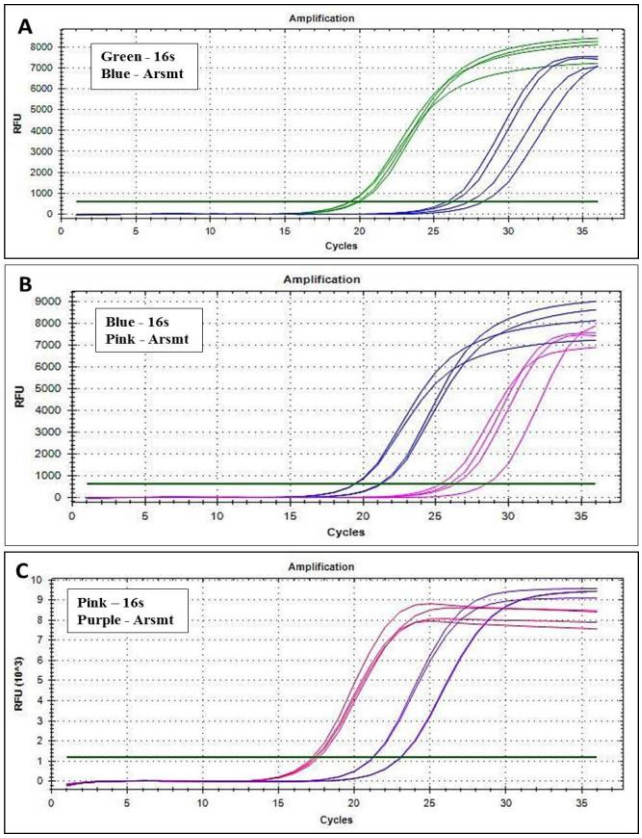


Figure 5 Gene Expression profile of test samples A). Relative normalized expression of *Scenedesmus acutus*, B). Relative normalized expression of*Oscillatoria acuminate* and C). Relative normalized expression of*Chlorella vulgaris.*

Arsenic Methytransferase Gene Expression Study of Chlorella vulgaris

The mRNA expression levels were normalized to the level of housekeeping gene (16s gene). The Ct values of the test samples were calculated and the data was expressed in terms of fold change over Control sample (Figure. 5 and 6)**.**

The arsenic methytransferase gene expression was increased in the microalgal samples of *Chlorella vulgaris*which is confirmed by observing strong bands than control samples (Figure. 4)**.** The test sample of *Chlorella vulgaris*treated with 50 ppm arsenic was showed up regulation on Arsmt gene expression by 3.04-fold increased over Control sample. The bands were observed between 250 bp to 500 bp. The control samples were had light bands while the arsenic treated samples had thicker bands.

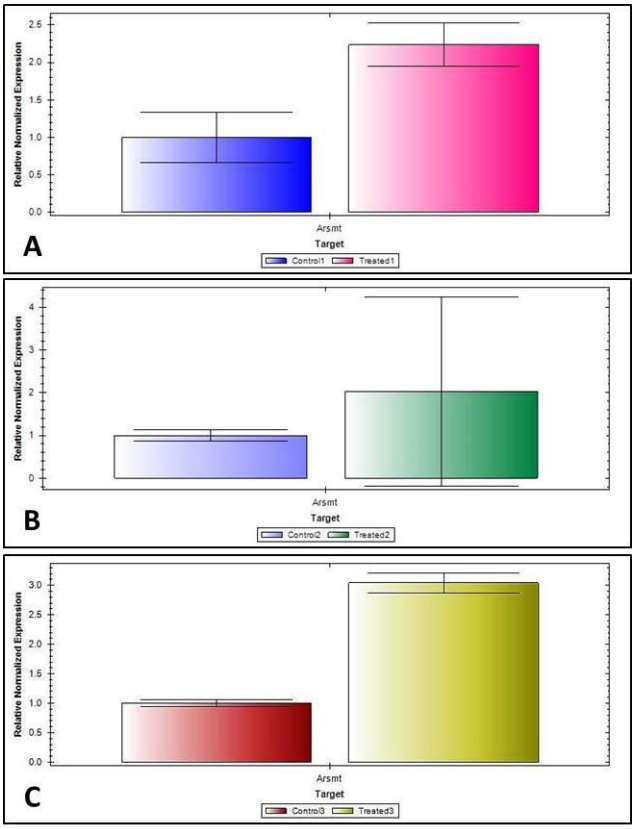


Figure 6 The test sample A). *S. acutus*, B). *O. acuminata* and C). *C. vulgaris* treated with 50ppm arsenic showed up regulation *Arsmt* gene over Control.

CONCLUSION

Scenedesmus acutus, *Oscillatoria acuminata* and *Chlorella vulgaris*treated with 50 ppm arsenic was showed up regulations on *Arsmt*gene expression by 2.24,2.03 and 3.04 fold increase respectively over Control samples.The microalgal species could be used to detoxify the arsenic from drinking water samples as eco-friendly, low cost and potent method for arsenic detoxification.

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