Detoxification of tannery effluent using marine bacterial and microalgal consortium

A Thesis submitted to Goa University for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Marine Sciences

BY

Cindrella Prabal Das

Research Guide

Dr. N. Ramaiah

Goa University,

Taleigao Goa

2017

CERTIFICATE

This is to certify that **Ms. Cindrella Prabal Das** has duly completed the thesis entitled **'Detoxification of tannery effluent by marine bacterial and microalgal consortium'** under my supervision for the award of the degree of Doctor of Philosophy.

This thesis, being submitted to the Goa University, Goa for the award of the degree of Doctor of Philosophy in Marine Sciences, is based on original studies carried out by her.

The thesis or any part thereof has not been previously submitted for any degree or diploma in any University or Institutions.

Date: June 20, 2018
Place: Dona Paula, Goa

N. Ramaiah
Research Guide

DECLARATION

As required under the University ordinance OB-9.9 (i-iv), I hereby declare that the present thesis entitled **"Detoxification of tannery effluent using marine bacterial and microalgal consortium"** is my original work carried out at the CSIR-National Institute of Oceanography, Dona Paula, Goa and the same has not been submitted in part or in full elsewhere for any other degree or diploma.

The literature related to the problems analyzed and investigated has been appropriately cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Cindrella Prabal Das

Acknowledgements

"Appreciation is a wonderful thing. It makes what is excellent in others belong to us as well." –Voltaire

Culmination of my thesis work allows me the pleasant task of expressing my heartfelt gratitude towards all those who contributed in many different ways in my journey and made it a memorable experience for me.

First and foremost, my praises and thanks to Almighty God for His blessings throughout my research work.

I am highly indebted to my research guide Dr. N. Ramaiah, Scientist, National Institute of Oceanography, Goa for his continued guidance and immense patience. His constant encouragement and support helped me bring this research work to fruition. I will be forever thankful for his constructive criticisms and valuable advices, not only in matters of work but also in life. His dynamism, vision, and earnestness have deeply inspired and motivated me throughout.

I thank Dr. Satish Shetye and Dr. S.W.A. Naqvi, former Directors; Dr. Prasanna Kumar and Dr. VSN Murty, Acting Directors, CSIR-National Institute of Oceanography, for giving me the opportunity to be associated with this prestigious Institute.

I wish to thank my FRC members - Dr. Savita Kerkar, Dept. of Biotechnology, Goa University for her kind consideration to be my VC's nominee and Dr. Janarthanam, Dean, Life Sciences, Goa University.

I thank Professor G.N. Nayak, Former HOD and Professor C.U. Rivonker, HOD, Department of Marine Sciences, Goa University, for their constant help and support.

Special thanks to my friends and colleagues Elroy, Naseera, Nadine, Late S. SatheeshBabu and Areena for their kind help in sample collection and analyses during my research work. Thanks to Ram Murti Meena for helping me with sequencing of samples and Dr. Sanitha for guiding me in statistical analyses of data.

I gratefully acknowledge my friends Prachi and Mandar for their help and continued support. Thanks to my colleagues Jasmine, Ujwala and Pankaj for their good wishes along the way.

I dedicate this thesis to my parents Mr. Prabal Kumar Das and Mrs. Nicky Das for their abundant and unconditional love, care, prayers and sacrifices throughout and for educating me and preparing me for the future. I thank my in-laws Mr. Nisith K. Mandal and Mrs. Anita Mandal for their love, care and consideration. They supported me with immense patience during my research.

Special thanks to my loving husband, Suman Kumar Mandal, for his unequivocal support and care through all the hardships as well as triumphs during my research term. My siblings, Maria, Annie, Martin and Clare were also ever-present with their love and encouragement.

Last but not least, I thank my furry friends - my pets, that were my source of happiness, companionship and joy in stressful times. Blessings be unto everyone!

-Cindrella Das

Dedicated to my beloved family

Table of contents

Chapter 1

General Introduction

1.1 Introduction

Life inherited the Earth. Its evolution from primordial to prominence is shaped, nurtured and sustained by the Mother Earth. Having traversed through umpteen millennia, the diversified life that kept adapting to, and enduring the vagaries is the pride possession by Her. Her eco-habitats -bountiful yet limiting, accommodating yet constricting, providing yet prohibiting- are innumerable and varied. Anthropogenic activities alter them, mostly disadvantageously to Her teeming, countless and many yet to be reported life-forms.

Human, the hegemonic species in the ladder of life, is modestly kind but largely unkind. This is because, its needs soon turned to desires along the traverse. During the last two millennia, with material usage escalating, the eco-health became the least priority. This one species has impacted Her hydrosphere, lithosphere and atmosphere to often pitiful statuses. While mankind has itself advantaged in some aspects, the inflicted damage impacts are suffered by both its active and passive fractions, apart from other forms of life.

Water, known also as blue gold, is one of the priceless gifts of nature. It is also regarded as the life-line on earth as the evolution of life, growth and development of human civilization and crop production could not have been possible without water. Rapidly increasing population, indiscriminate urbanization and rapid industrialization have placed tremendous pressure on the natural water resources and their quality.

One of the grievously challenging issues facing mankind at this juncture is aquatic, terrestrial and atmospheric pollution by various anthropogenic activities. Industries are consuming large volumes of water and release effluents loaded with many toxic and

hazardous pollutants. The current scenario is such that the higher concentration of contaminants even in the treated effluents, reach the surface and ground water systems thereby affecting vegetation, humans and animals.

With this overarching essence, this thesis is an account of research work done on detoxification of tannery effluent using marine bacterial and microalgal consortium with the preset objectives listed later on.

1.2 The tanning industry

In India more than 55% of the industries do not have any type of effluent treatment plant and just around 20% have partial treatment facilities (Yelda et al., 2002). All of these industries drain off black liquor without chemical recovery or treatment of effluent due to economic reasons (FAO, 1999). Among the industries, the leather tanning industry is a significant contributor to the economy in many low income and middle income group of nations. It provides large scale employment opportunities, in particular to unskilled, illiterate and/or semiskilled-literates that are economically quite disadvantaged. Leather industries, or tanneries, deal with the conversion of putrescible material such as skin/hide to non-putrescible material. During the conversion, series of chemical processes and physical operations are carried out to attain the final desired properties of leathers. Pre-tanning, tanning and post-tanning are the major steps involved in leather processing. One major challenge in leather processing is the generation of voluminous solid and liquid wastes which are environmental hazards.

Among the many steps, pre-tanning involves the preparation of skin material for tanning during which the raw hides undergo extensive cleaning and removal of unwanted materials. The next battery of steps within pre-tanning involves soaking, liming, unhairing, reliming, fleshing, deliming, bating, and pickling. The resultant wastes such as hair, lime sludge, interfibrillary materials, and adipose tissues are the major contributors to solid waste (Covington, 2009). Toxic gases such as hydrogen sulphide and ammonia emanate during this process. Liquid wastes are generated in all the above listed pre-tanning processes loaded with chemicals which are used. Chrome tanning is widely practiced globally due to the unique properties it imparts to leather in terms of durability. However, generation of Cr(VI) in the waste stream causes a serious threat to the environment due to its high carcinogenicity (Dayan and Paine, 2001). Alongside chrome, the vegetable tanning, considered as an alternate to chrome tanning, is practiced owing to its non-toxic nature. However, the vegetable tanned leathers are unable to match the properties of chrome-tanned leathers. Several other tanning agents such as phosphonium, oxazolidine, and silica-based tannings are in use. Tanning process along with the release of toxic substances in the effluent (Fig. 1.1) can be of significant ecological concerns.

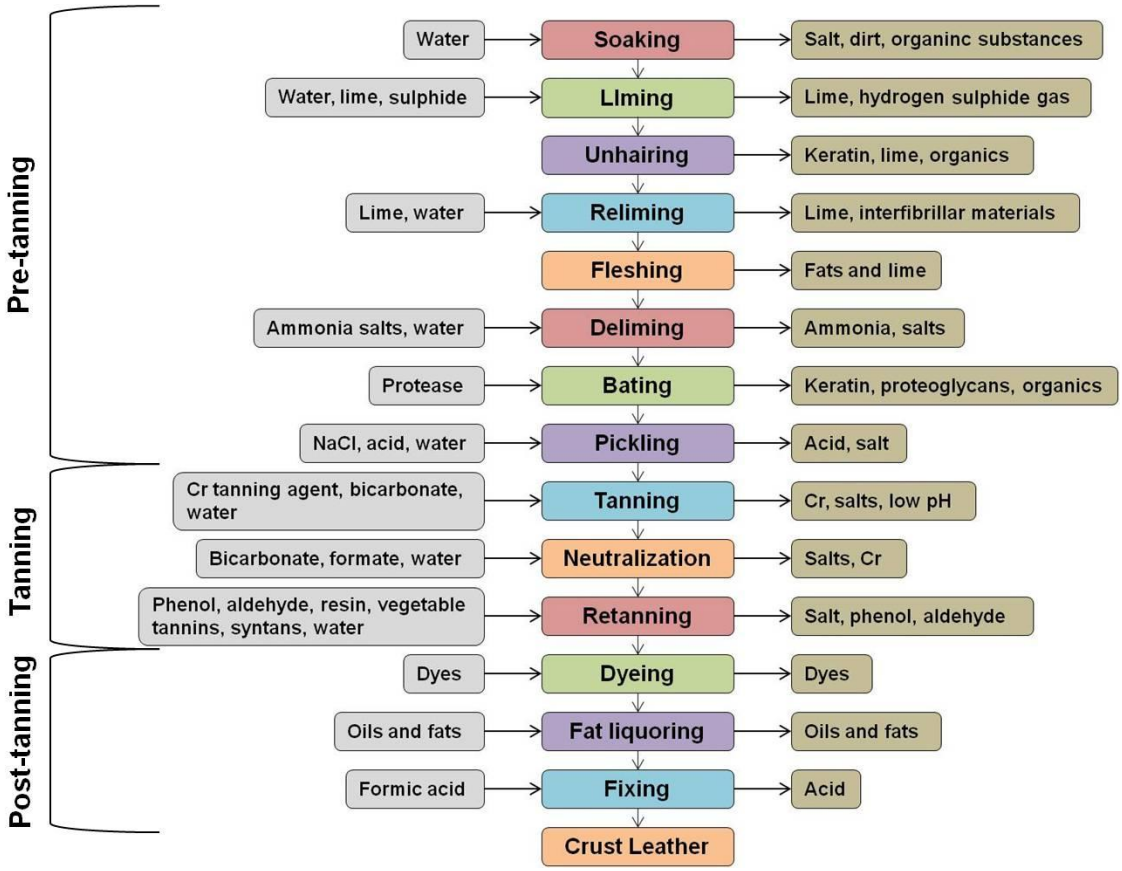


Fig. 1.1 Schematic diagram of the general tanning process (modified from Christopher et al., 2016)

1.3 Pollutants in tannery wastewaters

There are numerous by-products in leather manufacturing process which are detrimental to natural habitats. Inevitably, the environment is under increasing pressure from very hazardous solid and liquid wastes discharged indiscriminately by most leather industries. Unless treated in some ways prior to discharge, the habitat retardation and formation of "dead zones" are the end results.

The effect of excessive pollutant concentrations found in tannery wastewaters (TW) can be very severe. Liquid effluent from tanning processes contains organic matter,

chromium, sulphides, and solid waste which arise from fleshings, wet blue splits, trimmings and shavings, buffing dust etc. Tannery effluents are known to be high in organic, inorganic, dissolved and suspended solid contents accompanied by high biochemical and chemical oxygen demand and contain potentially toxic metal salt residues (Gupta et al., 2007). Obnoxious odour emanating from the decomposition of protein solid waste, presence of hydrogen sulphide gas, ammonia and volatile organic compounds are normally associated with tannery wastewaters (Mohanty, 1997). Aswathi and Rai (2005) noted that a significant part of chemicals used in the leather processing is not actually absorbed in the process but discharged into the environment.

When effluent is discharged direct into streams and rivers, it needs to be of safer quality as the environment is sensitive and highly susceptible to damage. Moreover, the greater the volume of the effluent compared to the volume of surface water, the higher the quality of the effluent demanded by the environment. On the other hand, if better housekeeping reduces the volume of water used, thus increasing the pollutant concentrations, the limits may be reasonably relaxed. Discharge limits are set with the objective of protecting the environment (UNIDO 2000). The limits imposed should always relate to the volume of effluent and the total weight of pollutants.

The substantial relocation of leather production from the industrialized countries to the developing countries that occurred in the 1960s to 1980s is known as "The Big Shift" (UNIDO, 1991). This, in effect, moved the highly polluting part of the leather-making process away from the other countries and occurred under the pressure of increasing labor, effluent treatment, and operations costs. This process was further accelerated by

the restrictions imposed on export of raw hides and skins and various incentives for higher processing levels provided in developing countries.

Low-waste generating technologies require better skilled personnel and closer technical control than conventional processing. Thus, the lack of properly trained staff at different levels remains one of the crucial constraints. The main hurdles for adopting more environmentally acceptable methods of leather processing and effluent treatment are the additional costs owing to special chemicals required to reduce/eliminate use of polluting chemicals (Gupta et al., 2007). Other factors involve installation costs of water conservation devices; wastewater collection and reuse equipment; effluent treatment and effluent monitoring equipment. Additional costs incurred for employing extra personnel and training them to maintain technical control is also a deterrent. Yet another factor is the traditional conservatism derived from hesitation over process alterations especially when satisfactory leather is being currently produced. This is particularly the case in small to medium scale semi-mechanized family owned units.

1.4 Tanning industry in India

India has about 3,000 tanneries with a total processing capacity of 700,000 tons of hides and skins per year. Over 90% of these tanneries are small or medium sized, with processing capacities of less than 2-3 tons of hides/skins per day (Kabdasli et al., 1993), capturing about 2.5% of the global market. The main reason for the rapid development and growth of the leather industry in the country is its large animal population. India holds nearly 10% of the total global market of raw hides and skins which are the basic raw material for the leather industry. Indian tanneries process hides of sheep, goats, cow

and buffalo, using both vegetable and chrome tanning. These generate toxic wastewaters in the range of 30-35 L kg^{-1} skin/hide processed with variable pH and high concentrations of suspended solids, BOD, COD, tannins, and chromium (Nandy et al., 1999). As leather processing requires large amounts of water, most of the tanneries are located near the riverbank. In India, the majority of tanneries are concentrated on the banks of the Ganga river system in North India and of the Palar river system in Tamil Nadu.

The conventional methods (physical and chemical) currently in use for treating tannery wastewaters and their disadvantages are well elucidated in literature. For example, these methods in practice consume significant amounts of energy and additional chemicals and therefore are expensive. Additionally, huge amount of sludge is generated as a by-product by these treatment methods (Tchobanoglous et al., 2003). Hence, the advantages of alternative biological treatment approaches for treatment of TW, particularly the use of bacteria and microalgae ought to be evaluated. Bacteria offer an eco-friendly means for the degradation and detoxification of tannery wastewater pollutants along with organic waste removal. Microalgae have an affinity for polyvalent metals and are very effective in removing heavy metals, nitrates and phosphates present in wastewater (Chalivendra, 2014). Hence, use of bacteria and microalgae for bioremediation of wastewater offers potential advantages over other conventional techniques in use.

1.5 Application of microorganisms in TW treatment

The role of microorganisms is gaining importance in different applications due to their versatile abilities. Single celled microorganisms are ubiquitous in our ecosystems. These organisms are mainly found in the soil systems, water, and intestinal tract of living

system. Microorganisms are classified based on the sources of energy and carbon. The major classifications are autotrophs and heterotrophs. Autotrophs are further classified into photoautotrophic and chemoautotrophic based on energy source as light and inorganic oxidation-reduction reaction, respectively (Christopher et al., 2016). Heterotrophs are further classified into chemoheterotrophic and photoheterotrophic based on their energy source, organic oxidation-reduction reaction, and light, respectively. Researchers have focused on understanding the importance and necessities of utilization of microorganisms in different technology fields for the well-being of humankind. A detailed review of earlier literature and the importance of various bacterial, microalgal strains and the microbial consortia in use for treating wastewaters is provided in Chapter 2.

This study focused on identifying some potent bacteria and microalgae capable of detoxifying tannery wastewater, either as single culture or in consortium, for eventual discharge into the environment or reuse for other processes. In order to conduct systematic and stringent experiments, the work carried out for this research was planned to address the following objectives.

1.6 Objectives of the study

1. **Isolation and taxonomic characterization of bacteria and microalgae from polluted and non-polluted regions to select organisms with bioremediation potential**

   The rationale for pursuing this objective was to isolate bacteria and microalgae from toxic tannery wastewaters as well as from a non-polluted seawater sources. The bacterial and microalgal isolates were then screened using assays for chromium tolerance, BOD and COD reduction to eventually obtain isolates with maximal potential for bio-remediating the tannery wastewater.

2. **Evaluation of tannery effluent degradation and chromium detoxification potential of microbes**

   Any evaluation of bioremediation potential of the marine bacterial and microalgal isolates enables the understanding of their suitability for environment restorations. With this principle in mind, different bacterial and microalgal isolates were grown individually and in consortium in tannery wastewater and the efficiency of detoxification was measured in terms of reduction in the concentration/levels of various toxic parameters.

3. **Biochemical and physiological characterization of strains with high tannery effluent bioremediation potential**

   The purpose of this objective was to try and elucidate the biochemical and physiological changes in the bacterial and microalgal strains as a result of growth in tannery wastewater. An understanding of changes brought about by experimental organisms in response to varying pH and salinity levels is vital for any viable bioremediation procedure. Measurements of biochemical and physiological changes (in terms of variations in metallothionein concentration, nitrate reductase activity and alkaline phosphatase activity), together with reductions in the concentration/levels of various toxic parameters were therefore undertaken.

Chapter 2

Review of literature

2.1 Preamble

Treatment of wastewater is a relatively modern practice. While the use of sewers to remove foul-smelling water were common in ancient Rome, it was not until the 19th century that large cities world over began to understand that they had to reduce the amount of pollutants in the used water they were discharging to the environment. At the end of the 19th century and in the first decade of the 20th century, wastewater was still discharged via sewers straight into the sea. In spite of the large supplies of water and the natural ability of water to cleanse itself over time, populations had become so concentrated by 1850s that outbreaks of life-threatening diseases were traced to bacteria in the polluted waters. Shore waters became increasingly polluted. Hence, in order to protect urban water courses, wastewater treatment plants were constructed for wastewater collection and treatment using physical, chemical, mechanical and biological techniques available.

Conventional wastewater treatment plants are in use today. However, it is very difficult for them to cope up with the norms stipulated by regulatory authorities. Hence, there is a dire and pragmatic need for developing new technologies for effluent treatment and making feasible modifications in the existing treatment processes. In this regard, most wastewater treatment plants employ conventional methods to reduce the contaminant concentrations and improve the quality of effluent before it is discharged to groundwater or re-enters water bodies (Carey and Migliaccio, 2009). These methods, however, have mostly been employed only for treating municipal wastewaters and are not designed/equipped for handling toxic industrial wastewaters.

The basic objectives of wastewater treatment are twofold: (1) Degrading organic wastes to levels where they do not exert a significant oxygen demand on receiving waters and (2) reducing nutrient (N and P) concentrations to levels where photosynthetic organisms in receiving waters remain limited in their growth. In brief, in the conventional treatment process, municipal and/or industrial wastewater is initially collected and subjected to two main treatment processes - primary and secondary. Pretreatment is performed during primary treatment which involves the usage of bar screens to remove large solids from the wastewater. After the primary clarifier, the wastewater still contains high amount of organic content and is sent to secondary treatment. During the secondary treatment, the organic content of the wastewater is degraded by microorganisms within the aeration tanks, and waste sludge is allowed to settle in the secondary clarifier. Sludge that settles at the bottom of the primary and secondary clarifiers is pumped to a digester via a sludge thickener to produce methane and carbon dioxide (USEPA, 1998). However, the water leaving the secondary clarifier could still contain significant amounts of nitrogen and phosphorus. Moreover, this process does not employ special methods to remove heavy metals from wastewater.

In addition to the fact that the above described nitrogen, phosphorus and heavy metal removal methods are functional, these methods have other disadvantages. They consume significant amounts of energy, use of additional chemicals and therefore, are expensive. Furthermore, toxic sludge is generated as a by-product in chemical treatment methods (Tchobanoglous et al., 2003; Hoffman, 1998) and as Mehta and Gaur (2005) point out, these techniques may be ineffective when concentration of metals in wastewater is in the range between 10–100 mg L^{-1}.

Hence, more economic and energy efficient nitrogen, phosphorus and heavy metal removal technologies are needed to overcome these problems. By introducing an alternative, biological method (bioremediation) for the treatment of metal enriched wastewaters containing high amounts of nitrates and phosphates, most of these issues can be mitigated and will provide a means for cost-effective removal of contaminants in wastewater. In view of this concept gaining practical importance, many investigations have been in place during the past 30 years or so. Pertinent literature on this aspect is reviewed in this chapter.

2.2 Bioremediation of tannery wastewaters

Tanneries are typically characterized as pollution intensive industrial complexes which generate widely varying, high-strength wastewaters. Variability of tannery wastewaters are not only from the fill and draw type operations associated with tanning processes, but also from the different procedures used for hide preparation, tanning and finishing. Khan et al. (1999) highlighted that these procedures are dictated by the types of raw hides employed and the required characteristics of the finished products. The tanning industry also has one of the highest toxic intensity per unit of output. Verheijen et al. (1996) estimated that at least about 300 kg chemicals are added for processing one ton of hides. Tannery effluent is among one of the hazardous pollutants of industry. Heavy metals, toxic chemicals, chloride, lime with high dissolved and suspended salts and other pollutant in tannery wastewaters are the major problems. Typical characteristics of tannery wastewaters (TW) are reviewed in Table 2.1. In this review, an effort has been

made to give a brief idea of alternative approaches to tannery wastewater treatment, particularly discussing and highlighting the biological methods.

Bioremediation is a process in which beneficial microbiological agents, such as yeasts, fungi and/or bacteria are used to clean up contaminated soil and water. Microbial bioremediation can cost-effectively reduce contaminants in wastewaters, protecting human health and the environment (Heitzer and Sayler, 1993; Gheewala and Annachatre, 1997; Gadd, 2000). Hassan et al. (2003) reported on the use of a number of exogenous, specialized microbes or genetically engineered microbes to optimize bioremediation. A successful, cost effective, microbial bioremediation strategy, however, relies on hydrogeologic conditions, the contaminants, microbial ecology and other spatio-temporal factors that vary widely. In any bioremediation process, the introduced microorganisms use the contaminants as nutrients or energy sources (Tang et al., 2007). Bioremediation activity through microbes, as reported by Ma et al. (2007) and Baldwin et al. (2008) are stimulated by supplementing nutrients (nitrogen and phosphorus), electron acceptor (oxygen), and substrates (methane, phenol and toluene) or by introducing microorganisms with desired catalytic capabilities.

Bacteria

Naturally occurring microorganisms, consisting of bacteria, fungi, protozoa, rotifers, and other microbes are the workhorses that can be profitably employed for wastewater treatment. Kadirvelu et al. (2003) and Manu and Chandhani (2002) have shown that microorganisms have proved to be useful in removal of pollutants from various specific industrial effluents.

Bacteria offer varied applications in the technological industry owing to its size and handling ease. Bacteria are single celled microorganisms and are small in size with different shapes. A number of chromium resistant microorganisms have been reported to detoxify hexavalent chromium, biochemical oxygen demand (BOD), chemical oxygen demand (COD) and other pollutants which include *Pseudomonas* sp., *Microbacterium* sp., *Desulfovibrio* sp., *Enterobacter* sp., *Escherichia coli*, *Shewanella algae* and *Bacillus* sp. (Camargo et al., 2003). Bacteria generate an insignificant quantity of sludge and offer potentially cost-effective remediation strategies (Sinha et al., 2011). It does not require high energy input or toxic chemical reagents and finally it offers an economical as well as eco-friendly option of metal detoxification and bioremediation.

Not long ago, Batool et al. (2012) tested an indigenous chromium-reducing bacterial strain, *Ochrobactrum intermedium*, isolated from a tannery water sample, for its Cr(VI) reducing potential. Many strains of *Bacillus* species have been found to adsorb toxic metal ions and thus may have roles in the bioremediation of contaminated soil/water systems (Hafez et al., 2002; Nourbakhsh et al., 2002). Campos et al. (2005) identified *Serratia marcescens* as a suitable bacterial species to detoxify chromate from chromium-containing industrial discharges.

Chaturvedi (2011) isolated strain *Bacillus circulans* MN1 with maximum chromate removal capacity of 71.4% at initial chromate concentration of 1110 $mg.L^{-1}$. Fakruddin et al. (2009) isolated a total of 35 isolates having Cr reducing potential. Two of the isolates, *Moraxella* and *Staphylococcus*, reduced 38% and 32% of Cr(VI), respectively.

Many studies have noted that some bacteria have the ability to reduce the toxic hexavalent chromium. These include *Shewanella oneidensis* (Daulton et al., 2007), *Pseudomonas* sp. (Desai et al., 2008), *Bacillus coagulans* (Poopal and Laxman, 2008), *Leucobacter* sp., *Exiguobacterium* sp. (Sultan and Hasnain, 2007), *Brucella* sp. (Thacker et al., 2007), and *Achromobacter* sp. (Zhu et al. 2008).

Desai et al. (2008) noted increased Cr(VI) reduction in two species of *Bacillus* when acetate was used as the carbon source. Glucose was found to enhance Cr(VI) reduction in *Agrobacterium radiobacter*, *Bacillus cereus*, *Escherichia coli* ATCC33456, and *Pseudomonas fluorescens* LB300 (Thacker et al., 2007). Recent studies on bioremediation of tannery wastewaters using bacteria are listed in Table 2.2.

Microalgae

Microalgae are important organisms for biological remediation of wastewater because they have the ability to accumulate nutrients, heavy metals, pesticides, and organic and inorganic toxic substances. The application of algae in biological wastewater treatment has gained a lot of importance in the recent years. Construction and energy costs are highly lower and the land requirement is not up to that of facultative ponds in constructed wet lands. Algae are chiefly used for the absorption of heavy metals due to their high tolerance and absorption capacities. Moreover, the large surface area/volume ratio makes it as one of the important biological materials for wastewater treatment.

During the last four decades, phycoremediation has been evaluated by numerous investigators (Oswald, 1988; Mara et al., 1996; Tadesse et al., 2003; Shi et al., 2007; Chu

et al., 2008; Craggs et al., 2012; Mustafa et al., 2012; Dixit and Singh, 2014; Posadas et al., 2014). Various techniques are in place for exploiting faster growth rates and nutrient removal efficiencies of certain microalgae for the treatment/detoxification of a variety of wastewaters. Dunn (1998) and Dunn et al. (2013) demonstrated the detoxification of tannery wastewater and nutrient removal using algal pond systems. Different microalgae such as species of *Oscillatoria*, *Phormidium*, *Ulothrix*, *Chlamydomonas, Scenedesmus* have been evaluated for their ability to grow in tannery effluent and accumulate chromium (Rai et al., 2005; Balaji et al., 2015; Ajayan et al., 2015). Notable reports on the use of *Chlorella* for bioremediation of tannery wastewater are those by Chellam and Sampathkumar (2012) and by Jaysudha and Sampathkumar (2014). Both free as well as immobilized cells of *C. salina* or *C. marina* have been examined mainly for removal of nutrients in tannery wastewater. *Chlorella vulgaris* was used by Rao et al. (2011) for removal of nutrients from tannery wastewater and by Hernandez-Zamora (2015) for removal of Congo Red, an azo-dye used for dyeing cotton, jute, leather, paper, silk and wool. Donmez and Aksu (2002) demonstrated the Cr(VI) uptake potential of *Dunaliella* sp. from saline waters for bio-removal of chromium ions from salt-bearing wastewaters. Gupta et al. (2001) indicated that the biomass of *Spirogyra* sp. is suitable for the development of efficient biosorbent for the removal and recovery of Cr(VI) from industrial wastewaters. Recent studies on bioremediation of tannery wastewaters using microalgae are listed in Table 2.3.

Microbial Consortium

Recently, there is a greater interest in the use of microbial consortia –multiple, syntrophic and interacting microbial populations– for wastewater treatment. This is because consortia can perform complicated functions that individual populations cannot. A well-built consortium more profitably can be robust and resilient to dynamic environmental fluctuations. As Fay (1992) noted, they might be able to better weather periods of nutrient limitation because of the metabolic diversity available to a mixture of species with capabilities of utilizing metabolites produced when together than in monoculture. Co-occurrence, as proposed by Burmolle et al. (2006), is thought to generate robustness to environmental fluctuations and also to promote stability through time for the members of a consortium. Hazardous waste degradation or biotransformation processes in the environment are generally carried out by consortia of many microbes rather than by one type of microbe alone (Thompson et al., 2005). Therefore, treatment systems using mixed microbial cultures are expected to provide results that are more effective than single cultures because the catabolic activities of the bacteria/algae complement each other.

Bhattacharya et al. (2015) evaluated a consortium of four naturally isolated bacterial strains as remediation tool for simultaneous removal of phenol and Cr(VI) from tannery effluent. They reported that the bacterial consortium was able to remove 100 and 78% of initial 47 mg L^{-1} phenol and 16 mg L^{-1} Cr(VI), respectively by 96 h of treatment. Gaikwad et al. (2014) treated complex wastewater using microbial consortia comprising of *Pseudomonas* sp., *Actinomycetes* sp., *Bacillus* sp., *Streptomyces* sp. and *Staphylococcus* sp. and were able to bring down the physico-chemical parameters of complex wastewater. The consortia demonstrated high COD and BOD reduction up to

90.17% and 94.02% respectively, compared to individual microbial species ranging from 42.11-59.76% for COD and 58.55-77.31% in case of BOD.

Silva-Benavides and Torzillo (2011) carried out co-culture of *Chlorella* sp. and *Plantothrix* sp. in municipal wastewater and demonstrated reduction of TN by 20% and that of PO_4-P by 25% in 4 days. Renuka et al. (2013) carried out bioremediation of sewage wastewater using consortium of filamentous strains of microalgae and reduced the initial concentrations of NO_3-N and PO_4-P by 90% and 97% after 10^{th} and 6^{th} day of treatment.

Liu et al. (2008) have used consortia of microbes containing *Bacillus* sp. and *Pseudomonas putida* for bioremediation of phenol and Cr(VI) from co-contaminated system. However, very limited studies exist in which potential bacteria were actually tested for removal of these contaminants from real industrial or in particular tannery effluents.

Table 2.1 Average pollution loads in tannery wastewater reported by various researchers

Reference	pH	Total solids	Suspended solids	Total dissolved solids	COD	BOD	TKN	Chromium	Sulphide
Apaydin et al. (2009)	7.4	–	2690	–	3700	1470	–	–	440
Ganesh et al. (2006)	7.08±0.28	10265±1460	2820±140	–	4800±350	–	225±18	95±5.5	–
Kongjao et al. (2008)	7.0-8.7	–	600-995	13,300-19,700	4100-6700	630-975	114-170	11.5-14.3	–
Koteswari and Ramanibai (2005)	–	–	20074	15,152	8000	980	–	11.2	228
Lefebvre et al. (2005)	7.7	–	5800	36,800	2200	–	270	–	–
Leta et al. (2004)	10.72	–	–	6810	11164	2908	–	32.9	508
Orhon et al. (2000)	7.79	–	915	–	2156	–	228	50.9	35.8
Ram et al. (1999)	10.5	18,884	1147	17,737	3114	1126	–	83	55
Szpyrkowicz et al. (2005)	7.7	–	–	–	2426	–	370	29.3	288
Thanigavel (2004)	8.2-8.5	19775	5025	14750	5650	–	–	–	–

All values except pH in mg L^{-1}

Table 2.2 Treatment of wastewaters with bacteria reported by various researchers

Bacteria	Isolation source	Test medium	Percent reduction					Reference
			Cr (VI)	Phenols	BOD	COD	TDS	
Acinetobacter sp. B9, *Arthrobacter* sp. B2	–	Tannery wastewater	78% (16.0)	100% (47.0)	–*	–	–	Bhattacharya et al. (2015)
Consortium: *Pseudomonas* spp., *Actinomycetes* spp., *Bacillus* spp., *Streptomyces* spp., *Staphylococcus* spp.	ETP, CETP, sludge and soil	Complex wastewater	–	–	94.02% (485)	90.17% (3286)	74.36% (7856)	Gaikwad et al. (2014)
Ochrobactrum intermedium	Tannery wastewater	Tannery wastewater	88% (100.0)	–	–	–	–	Batool et al. (2012)
Bacillus circulans MN1	Tannery wastewater	Cr-amended media	71.4% (1100)	–	–	–	–	Chaturvedi (2011)
Moxarella sp.	Tannery wastewater	Cr-amended media	38% (10)	–	–	–	–	Fakruddin et al. (2009)
Staphylococcus sp.			32% (10)					
Pseudomonas sp.	Tannery wastewater	Cr-amended media	62% (200)	–	–	–	–	Farag and Zaki (2010)
Acinetobacter sp.			53.5% (200)					
Serratia marcescens	Tannery wastewater	Cr-amended media	86% (147.1)	–	–	–	–	Campos et al. (2005)

Numbers in parentheses denote initial concentrations of the parameter in mg L^{-1}
* no data

Table 2.3 Treatment of wastewaters with microalgae reported by various researchers

Microalgae	Isolation source	Test medium	Percent reduction						Reference
			Cr (VI)	BOD	COD	TDS	NO3	PO4	
Chlorella salina	Culture collection	Tannery wastewater	–*	–	–	–	43.71% (67.59)	81.94% (8.25)	Jaysudha and Sampathkumar (2014)
Scenedesmus sp.	Tannery wastewater	Tannery wastewater	57% (12.8)	34.97% (326)	37.15% (872)	41.11% (2775)	55.71% (49.0)	97.4% (3.9)	Ajayan et al. (2015)
Chlorella vulgaris	Culture collection	Industrially polluted water					84% (0.155)	69.23% (0.44)	Domnic et al. (2009)
Synechocystis salina			–	–	–	–	82.5% (0.124)	64.52% (0.294)	
Gloeocapsa gelatinosa			–	–	–	–	80% (0.124)	74.19% (0.294)	
Dunaliella sp.	Hypersaline lake	Cr- synthetic medium	23.8% (108.6)	–	–	–	–	–	Donmez and Aksu (2002)
Spirogyra sp.		Cr- synthetic medium	96% (5.0)	–	–	–	–	–	Gupta et al. (2001)
Chlorella miniata		Cr-amended media	65% (100)	–	–	–	–	–	Han et al. (2007)

Numbers in parentheses denote initial concentrations of the parameter in mg L^{-1}
* no data

2.3 Removal of chromium from wastewater

Increased concentration of chromium is toxic to living organisms. This is in spite of the fact that chromium is an essential micronutrient for stabilization of nucleic acids, stimulation of enzyme systems, and glucose metabolism (Thacker et al., 2007; Wang and Shen, 1995). The most commonly used conventional methods for the removal of Cr(VI) are: (a) reduction to Cr(III) followed by precipitation as chromium hydroxide, (b) removal by ion exchange and (c) removal by adsorption. Francisco et al. (2010) noted that these methods are very costly owing to operational, treatment, and sludge disposal costs. Microbial reduction of Cr(VI) to Cr(III) is a potentially useful remediation strategy for Cr(VI)-contaminated environment as it is cost-effective compared to conventional methods (Chourey et al., 2008; Ohtake and Silver, 1994). The rate of chromate reduction is greatly influenced by the initial Cr(VI) concentration (Pattanapipitpaisal et al., 2001a; Wang and Xiao, 1995; Shen and Wang, 1994). However, complete reduction is done mostly at the lower concentration of metal.

Use of bacteria for chromium removal/biotransformation

Bacteria detoxify chromium mainly by reducing Cr(VI) to Cr(III), through Cr(V) and Cr(IV) as intermediates (Aguilera et al., 2004). Application of bacteria is a potentially useful process in the remediation of Cr(VI)-affected environments. Reduction of Cr(VI) to Cr(III) can be performed by a wide range of bacteria including *Pseudomonas aeruginosa* (Gopalan and Veeramani, 1994), *P. synxantha* (McLean et al., 2000), *P. putida* (Park et al., 2002), *P. ambigua* (Suzuki et al., 1992), *P. fluorescens* (Lovley, 1993), *P. dechromaticans* and *P. chromatophila* (Cheung et al., 2006). Bacteria from

other genera that have also been shown to reduce Cr(VI) include *Acinetobacter ilwoffii* (Tekerlekopoulou et al., 2010), *Bacillus megaterium* (Cheung et al., 2006), *Aeromonas dechromatica* (Lovley, 1993) and *Escherichia coli* ATCC 33456 (Shen and Wang, 1995; Fredrickson et al., 2000). Lovley (1993) reported sulfate-reducing bacteria such as *Desulfovibrio desulfuricans* and *D. vulgaris* as capable of reducing Cr(VI). Roh et al. (2002) and found extremophiles like the radiation-resistant *Deinococcus radiodurans* and Kashefi and Lovley (2000) isolated *Thermoanaerobacter ethanolicus* (from subsurface sediments) and *Pyrobaculum islandicum*, as capable of reducing Cr(VI) at high temperatures. Resistance to Cr(VI) has been investigated by Aguilera et al. (2004) using *Pseudomonas aeruginosa*, which has been attributed to the decreased uptake and/or enhanced efflux of Cr(VI) by the cell membrane. Mergeay et al. (2003), Vaneechoutte et al. (2004) and Mahdavi et al. (2013) reported a similar resistance mechanism has been reported in *Cupriavidus metallidurans* CH 34 which is resistant to eight metals including CrO_4.

Hexavalent chromium is toxic to bacteria present in contaminated soil or wastewater. The bacterial strains growing in toxic conditions are assumed to be tolerant/resistant to chromium (Vitic and Giovannetti, 2001). *Pseudomonas* sp. was the first hexavalent chromium resistant strain isolated from waste water (Romaneko and Korenkov, 1977). Metal resistance is the ability of the microorganism to survive toxic effects of metal exposure using detoxification mechanisms in direct response to the metal concerned. Metal tolerance in microorganisms is by virtue of their ability to modify intrinsic properties and/or surrounding environmental conditions (Gadd, 1992). The reduction of Cr(VI) can be identified by using diphenyl carbazide and read at 560 nm. Bacterial

strains isolated from electroplating industry showed higher reduction rate (Philip et al., 1998).

Bacillus coagulans isolated from electroplating industry was capable of reducing Cr(VI) with soluble enzyme and utilizing malate as external electron donor. The biological reduction of Cr(VI) is similar to sulphate reduction process. The ability of sulphate reducing bacterial biofilms (Smith and Gadd, 2000) to reduce Cr(VI) to insoluble Cr(III) using lactose as carbon source was reported. Almost 88% of 500 µmol L^{-1} was removed by bacteria within 48 h and 80% of Cr was precipitated by biofilm and this study proved sulphate reducing bacteria exhibiting the ability to reduce chromium in soil. Sulphate reducing bacteria reducing Cr(VI) under anaerobic conditions were isolated from metal contaminated marine sediments of Tokwawan, Hong Kong SAR (Cheung and Gu, 2003). The enrichment consortium almost completely (98.5%) reduced 0.6 mM Cr(VI) in 168 h.

The extensive analysis by Thacker et al. (2006) on the bacterium *Providencia* sp., isolated from the contaminated site of chemical industry has shown its potential to reduce Cr(VI) by 100% at concentrations ranging from 100-300 mg L^{-1} and by 99.31% at 400 mg L^{-1}, at pH 7 and temperature 37°C. Water soluble Cr(VI) decreased from the initial concentration of 383.8 to 1.7 mg kg^{-1}. Exchangeable Cr(VI) and carbonates-bound Cr (VI) were removed by 92.6 and 82.4%, respectively.

Various studies (Khanafari et al., 2008; Middleton et al., 2003; Srinath et al., 2002; Pattanapipitpaisal et al., 2001b; Campos et al., 1995; Nourbaksh et al., 1994; LLovera et al., 1993; Pun et al., 2013; Pandian et al., 2014; Devi et al., 2012) using many bacterial species like *Bacillus circulans*, *B. megaterium*, *B. coagulans*, *Agrobacterium*

radiobacter EPS-916, *Bacillus* spp., *Pseudomonas fluorescens, Bacillus* sp. QC1-2, *Microbacterium liquuefaciens*, *Zoogloea ramigera* and *Pseudomonas aeruginosa* have proven their ability of chromium removal.

Application of microalgae for chromium removal/biotransformation

In general, quite limited efforts have been made to use algal biomass for removing toxic heavy metals such as Cr from aqueous solutions. Out of thousands of algal species, only few are recognized to possess the ability to remove toxic heavy metals (Cr) from wastewaters. The commonly used species for removing Cr from wastewater are fresh water green algae like *Chlorella* spp, *Neochloris oleoabundans*, *Cladophora* spp., *Scenedesmus* spp., *Chlamydomonas reinhardtii* and blue green algae like *Microcystis aeruginosa* and *Oscillatoria*.

Microalgae require metals for their biological functions. Selected microalgae are cultivated for bio removal of certain metals. They have a potential to accumulate high concentrations of metals from the contaminated aquatic systems. Metal accumulation in algae involves two processes: an initial rapid (passive) uptake followed by a much slower (active) uptake (Bates et al., 1982). During the passive uptake, physical adsorption takes place in which the metal ions are adsorbed over the cell surface very quickly just in a few seconds or minutes and this process is metabolism-independent. Then chemisorption, a process which is metabolism-dependent takes place in which the ions are transported slowly across the cell membrane into the cytoplasm.

The algal cell wall has an anionic surface and contains many functional groups, such as hydroxyl (-OH), carboxyl (-COOH), amino ($-NH_2$), sulphydryl (-SH), etc. Since metal

ions in water are generally in the cationic form, they are adsorbed onto the cell surface (Xue et al., 1988; Crist et al., 1981). The polyphosphate bodies present in algae provide a storage pool for metals. Several researchers have established that metals such as Cr, Cd, Hg, Ni, Cu, Ti, Pb, Mg, Zn, are sequestered in polyphosphate bodies in green algae (Yu and Wang, 1983). Wood and Wang (1983) and Gadd (1990) proposed that once the metal accumulates inside the cell, its ions may be preferentially located within specific organelles and bound to proteins such as a metallothionein.

2.4 Removal of nutrients from wastewater

Bacteria

Most wastewaters are composed of organic material, i.e. proteins, carbohydrates, fats and oils; nutrients, mainly nitrogen and phosphorus; as well as trace amounts of recalcitrant organic compounds and metals (Bitton, 2005). Biodegradable organic material is biochemically oxidized by heterotrophic bacteria under aerobic conditions resulting in production of carbon dioxide, water, ammonia and new biomass. Under anaerobic conditions methanogenic archaea, partially oxidizes organic material to yield carbon dioxide, methane and new biomass.

Biological nitrogen removal is achieved by a combination of nitrification, the oxidation of ammonia to nitrate, and denitrification, the reduction of nitrate to nitrogen gas. Nitrifying bacteria are chemolithotrophs, using the inorganic nitrogen compounds as electron donors. Ammonia oxidizing bacteria (AOB), like e.g. *Nitrosomonas*, *Nitrosospira* and *Nitrosococcus*, convert ammonia to nitrite according to equation (1). According to Henze et al. (2002), the nitrite oxidizing bacteria (NOB), such as

Nitrobacter, *Nitrospira*, *Nitrococcus* and *Nitrospina* subsequently convert nitrite to nitrate consistent with the following stoichiometric formula described by equation.

$$15\ CO_2 + 13\ NH_4^+ \rightarrow 10\ NO_2^- + 3\ C_5H_7NO_2\ 23\ H^+ + 4\ H_2O \quad (1)$$

$$5\ CO_2 + NH_4^+ + 10\ NO_2^- + 2\ H_2O \rightarrow 10\ NO_3^- + C_5H_7NO_2 + H^+ \quad (2)$$

The denitrification process reduces the nitrates to nitrogen gas, thus removing nitrogen from the water phase. In the absence of molecular oxygen denitrifying organisms can respire nitrate or nitrite through a chain of enzymatic reactions coupled to the bacterial inner membrane. Synthesis of the enzymes involved in denitrification is induced under anoxic conditions. In the presence of molecular oxygen, the aerobic electron transport system is employed since the redox potential of oxygen is higher than for nitrate (Henze et al., 2002). The stoichiometric formula for the overall process (Mateju et al., 1992), here with acetate as electron donor, is presented below:

$$5CH_3COOH + 8NO_3^- \rightarrow 8HCO_3^- + 2CO_2 + 6H_2O + 4N_2 \quad (3)$$

The ability to denitrify is widespread among heterotrophic bacteria and archaea making it difficult to determine which microorganisms are most important for in situ denitrification in wastewater treatment plants (Wagner and Loy, 2002). Bitton (2005), Mateju et al. (1992) and Wagner et al. (2002) reported that species *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, *Paracoccus*, *Methylobacterium*, *Bacillus* and *Hyphomicrobium* are commonly identified as part of the denitrifying microbial flora in wastewaters.

Biological phosphorus removal is achieved by intracellular accumulation of polyphosphates in combination with cell uptake for growth. The most efficient phosphate

removal bacteria are called polyphosphate accumulating organisms (POAs). PAOs require alternating anaerobic and aerobic environments to obtain a high net uptake of phosphorus. The phosphorus content in bacterial cells is usually around 1-3 % of the dry weight while the corresponding percentage for PAOs can reach 10% (Bitton, 2005; Srivastava and Srivastava, 2005). By removing biomass after the aerobic step, the phosphorus is removed from the wastewater. Traditional isolation procedures have failed to identify bacteria possessing the characteristics ascribed to PAOs. However, as Oehmen et al. (2007) observed, cultivation-independent molecular techniques have identified a group of Rhodocyclus related bacteria, named "Candidatus Accumulibacter Phosphatis", as PAOs. Some bacterial strains have been found to take up enhanced amounts of phosphorus under solely aerobic conditions. The possibility to by-pass the anaerobic step is advantageous from a process design point of view. Further, *Acinetobacter calcoaceticus* (Srivastava and Srivastava, 2005), *Acinetobacter iwoffi* (Ghigliazza et al., 1998) and *Aeromonas hydrophila* (Mbwele, 2006) are known to possess enhanced phosphorus uptake ability.

Microalgae

Nutrient removal by algae involves various metabolic pathways of the algal cell. Salts/ions of carbon, nitrogen, phosphorous and sulfur sustain, stimulate and support algal growth. Metals such as potassium, sodium, calcium, iron and magnesium serve as micronutrients for their growth.

Organic nitrogen is the key element in biological substances like enzymes, peptides, proteins, chlorophylls and energy transfer molecules such as ADP (adenosine

diphosphate) and ATP (adenosine-5'-triphosphate) (Barsanti and Gualtieri, 2006). Organic nitrogen is derived from inorganic sources including nitrite (NO_2^-), nitrate (NO_3^-), nitric acid (HNO_3), ammonia (NH_3), ammonium (NH_4^+), and nitrogen gas (N_2). Microalgae can convert inorganic nitrite, nitrate and ammonium to organic nitrogen forms through a process called assimilation. Only eukaryotic algae can perform assimilation of inorganic nitrogen (Cai et al., 2013). As shown in Fig. 2.1, translocation of inorganic nitrogen takes place across the plasma membrane where reduction of nitrate takes place followed by the incorporation of ammonium into amino acids and glutamine.

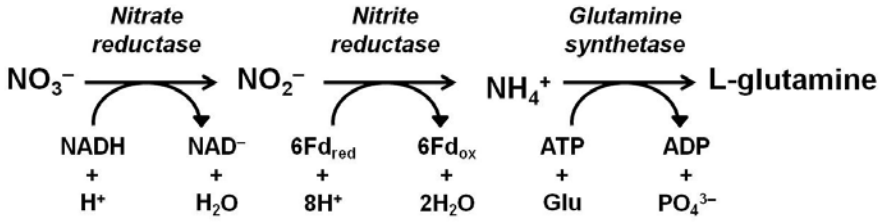


Fig 2.1 Conversion of inorganic nitrogen to organic nitrogen (Chalivendra, 2014)

Phosphorus is essential for synthesis of lipids, proteins and nucleic acids. It plays a crucial role in cell growth and metabolism of algae. During metabolism, phosphorus, mainly in the forms of $H_2PO_4^-$ and HPO_4^{2-}, is incorporated into organic compounds through a process called phosphorylation. Phosphorylation is an active process and it requires energy. This energy comes from the oxidation of respiratory substrates, the electron transport system of the mitochondria, or from light. Generation of ATP from ADP takes place during phosphorylation and microalgae is able to assimilate and store phosphorus in excess within the cell in the form of volutin granules. These reserves will be available during the growth cycle in the absence of phosphorus in the media. The algal growth rate, according to Larsdotter (2006), may not respond at once to changes in the

external concentration of phosphorus, as opposed to the immediate responses to temperature and light.

For removal of excess phosphorus/nutrients Renuka et al. (2013) used *Calthrix* sp from sewage wastewater. They found that 44–91% PO_4–P and 57–58% NO_3-N can be removed and a highest dry cell weight of 0.97 mg L^{-1} can be attained in sewage wastewater using this species. Kim et al. (1998) treated swine wastewater with *Chlorella vulgaris* and reported 96 and 95.3% removal of phosphorus and nitrogen, respectively. Travieso et al. (2008) reported removal of 90.2, 84.1 and 85.5% organic nitrogen, ammonia, and total phosphorus, respectively from distillery wastewater with an anaerobic fixed-bed reactor in a microalgae pond. Colak and Kaya (1988) reported removal of nitrogen (50.2%) and phosphorus (85.7%) in industrial wastewater treatment and elimination of phosphorus (97.8%) in domestic wastewater treated by algae. Ji et al. (1999) used *Scenedesmus obiliquus* to remove nitrogen and phosphorus from piggery wastewater. The authors reported 23 to 58% removal of TN and 48 to 69% removal of TP. Bhatnagar et al. (2010) identified a species *Chlorella minutissima* in wastewater treatment oxidation ponds in India. On characterizing the species, it was found that it was a eukaryotic species belonging to family *Chlorellaceae*. They cultivated *Chlorella minutissima* in high concentrations of raw sewage (municipal wastewater) and standard BG 11 medium. The results showed that growth of *Chlorella minutissima* in municipal wastewater is 146% more than that in standard BG 11 medium. Further, it was found that this species can grow well under heterotrophic and mixotrophic conditions over a wide pH range of 4-10.

From the foregoing, it is amply evident that large volumes of literature are available from the copious efforts of researchers with the sole purpose of decontaminating the hazardous wastes from wastewaters so as to safeguard natural characteristics of terrestrial, atmospheric and aquatic ecosystems. While “idea-novelty” is obscure owing to burgeoning global efforts on bioremediation, any new life-form/s evaluated and accepted by the community of environmentalists needs to be taken note of and, adopted to improve the ecosystem health.

Chapter 3

Collection and characterization of bacteria and microalgae with tannery effluent detoxification potential

3.1 Introduction

Tannery wastewater contains large amount of chemical compounds including toxic substances. As highlighted in the previous chapters, it is among the most hazardous wastewaters discharged by industries. Uberoi (2003) listed that tannery wastewaters (TW) contain heavy metals, toxic chemicals, chlorides, lime, with high dissolved and suspended salt and other pollutants.

Such types of wastewaters with high organic load and inorganic pollutants cause many ecological problems and their adverse effects on both flora and fauna are well known. Their discharge alters physical and chemical properties of the soil, mostly reducing the fertility of land and more vitally, the crop production. Their continuous discharge into natural water bodies does lead to eutrophication, affecting the aquatic life besides making the aquatic bodies unfit for various uses including the loss of potable quality (Manu et al., 2011; Gaikar et al., 2010). Thus, the challenges associated with treatment and, safe disposal of wastewaters cannot be ignored. Environmentalists and most governments are looking for cheap, efficient, effective and long lasting solutions for wastewater treatment and recycling. In developing countries like India, the physico-chemical methods of wastewater treatment being tried are inevitably cost intensive. Therefore they cannot be employed by all industries. In recent years however, biological treatment options are becoming popular (Vishakha et al., 2013). Some of these attempts have helped in developing relatively efficient, low cost waste treatment systems.

In order to design an efficient biological waste water treatment, it is important to know the composition of native flora from the wastewater and to identify the strains which can

metabolize/detoxify/biotransform hazardous compounds. Highly diverse and specialized microbial communities are present in the environment which can efficiently detoxify many pollutants. While this detoxification process is usually slow, because of many other advantages the biological treatment option offers, one needs to evaluate the potential of microflora from natural environments. Hence, a practical approach is to collect, identify, evaluate the populations of microorganisms capable of pollutant reduction/detoxification. This can be achieved by addition of exogenous microorganisms in addition to the indigenous populations already present in the wastewaters.

The use of exogenous marine and salt-tolerant microbes for treating tannery wastewaters ought to offer many advantages for eco-sustainable bioremediation of marine environments. For evaluating eco-friendly approaches of bioremediation of salt-laden TW, the use of marine microorganisms such as bacteria and microalgae is preferable to that of non-marine origin mainly owing to the salt tolerance characteristic of marine microflora besides their resilient adaptations to some of the ever-dynamic marine ecosystems. It is likely that such alternative, cost-effective bioremediation approaches will be further useful for clean-up of hazardous wastes in natural habitats.

3.2 Materials and methods

3.2.1 Sample collection

The tannery wastewater used for this study was collected from the CETP Pallavaram Tanners Industrial Effluent Treatment (PTIET) Co. Ltd., Pallavaram, Chennai and from Upper India Tannery (UIT) Pvt. Ltd., Kanpur, Uttar Pradesh.

The CETP at Pallavaram treats around 3000 m^3 day^{-1} of wastewater from the cluster of tanneries processing mostly semi-finish to finished leather in the surrounding. There are nearly 200 tanneries operating in and around Pallavaram area. About 122 tannery units in the Pallavaram cluster discharge their wastewaters to this CETP for treatment.

The tannery in Kanpur is involved in production of finished leathers, like vegetable tanned sole and harness leathers, safety shoe leather (for safety and occupational footwear), furniture and automobile upholstery leathers. The tannery has the capacity of producing more than 1.2 million square meters of finished leathers annually.

The TW samples were collected randomly in July 2013, September 2014 and September 2015 since the wastewater quality is not subject to seasonal variation as it is directly transported from the industrial operation sites to the CETP via closed pipelines. Wastewater samples were mixed well and processed soon after arriving in the lab and analyzed for various physico-chemical properties.

Seawater samples were collected from coast off Anjuna, Goa (15°35'04.1"N 73°44'12.7″E). This sampling site was chosen because it is quite cut off from the main beach spots and is relatively inaccessible to tourist and other anthropogenic activities. Samples were stored in polyethylene bottles in a cool box with plenty of synthetic ice during their 36 h transport to the laboratory.

3.2.2 Isolation of bacteria

Bacteria were isolated from the TW and seawater by following the general procedure of dilution plating method. In brief, an aliquot of 0.1 to 0.3 mL from 10^{-1} to 10^{-3} dilutions of

tannery wastewater and seawater samples were spread plated onto seawater-nutrient agar (NA prepared in 1:1 seawater: deionized water) plates (HiMedia, Mumbai) amended with 50 mg L^{-1} $K_2Cr_4O_7$ and incubated at 28±2°C for 24−48 hours. Distinct colonies on each plate were counted at the end of incubation. Randomly chosen bacterial colonies were sub-cultured by streaking onto fresh SWNA plates to obtain pure cultures for purity and transferred to agar slants and stored in the refrigerator at 4°C for future use.

3.2.3 Isolation of microalgae

Seawater and TW samples (1 L) were filtered through 0.7µm GF/C glass microfiber filter paper (Whatman, USA). The filter paper was placed in 250 mL flasks containing 100 mL algal culture broth (ACB, HiMedia, Mumbai) amended with 50 mg L^{-1} $K_2Cr_4O_7$. These flasks were kept at a constant temperature of 28±2°C under fluorescent illumination of 150-300 µmol photons m^{-2} s^{-1} (with 12:12 h light:dark photoperiod) till visible growth of microalgae was seen. One mL aliquots of the algal culture broth was spread onto 50 mg L^{-1} Cr(VI)-amended algal culture agar (ACA, HiMedia, Mumbai) plates and incubated at a constant temperature of 28±2°C under fluorescent illumination of 150-300 µmol photons m^{-2} s^{-1} (with 12:12 h light:dark photoperiod) till visible growth was observed on the plates. The clumps of microalgal cells growing on these plates were re-streaked several times onto fresh Cr(VI)-ACA plates until distinct, well isolated colonies were obtained. Isolated microalgal colonies were picked and inoculated into 100 mL Cr(VI)-ACB and grown under same conditions mentioned above. The acclimatized monoalgal cultures were used in all the following experiments.

3.2.4 Characteristics of tannery wastewater

The physico-chemical parameters BOD, COD, nitrates (NO_3-N), phosphates (PO_4-P), sulphates (SO_4-S), total dissolved solids (TDS), total chromium (Cr) and hexavalent chromium [Cr(VI)] were measured from the TW samples. Measurements of BOD and COD were made following standard methods described in APHA (2005). The BOD was determined using the 5-day BOD test and the COD, using open reflux oxidation method. TDS was determined by gravimetric method as per APHA (2005). TDS was calculated by measuring the residual weight after drying known sample volumes filtered through 0.7µm glass microfiber filters at 180°C.

Nitrate concentrations were colorimetrically measured following the Cd column reduction method (APHA, 2005). Phosphates were measured using the Ascorbic acid method (APHA, 2005). Sulphates were measured following the barium chloride precipitation method (APHA, 2005).

For measuring total chromium concentrations, the samples were digested with concentrated nitric acid followed by filtration through 0.45µm filter paper, while for hexavalent chromium, 0.45µm filtered samples without any pre-digestion were analyzed. The chromium contents were measured using the colorimetric Diphenylcarbazide (DPC) method (APHA, 2005). H_2S was determined by the purge-and-trap method as described in APHA (2005), in which HCl was used to volatilize acid-volatile sulphide at room temperature. The H_2S gas produced was trapped in zinc-acetate solution and measured by iodometric titration of the ZnS precipitate formed. Phenols were analyzed using the 4-aminoantipyrine colorimetric method (APHA, 2005). NH_3 was measured using the Nesslerisation method, total nitrogen (TN) following the Kjeldahl method and chlorides

using the argentometric method (APHA, 2005). Bacterial counts in the tannery wastewater were determined using standard dilution plate method.

3.2.5 Screening of bacteria and microalgae to select potent isolates

Many isolates of bacteria and microalgae were screened based on their ability to grow on Tannery Wastewater Agar (TWA). For this purpose, Tannery Wastewater Agar (TWA) was used as the sole carbon source. The method of Prakasam and Dondero (1978) was followed for preparing TWA. Briefly, the tannery wastewater (1L) was autoclaved at 121°C for 30 minutes and then filtered through glass wool. The filtrate was made up to 1L with deionized water incorporated with mineral salts [g L^{-1}: K_2HPO_4 (0.5), $MgSO_4.7H_2O$ (0.01), $(NH_4)_2SO_4$ (1.0), K_2HPO_4 (1.3), KCl (0.05), $FeSO_4.7H_2O$ (0.01)]. To this solution, 15 g of agar powder (HiMedia, Mumbai) was added for solidification. This was autoclaved again for 30 minutes. On cooling, the TWA plates were inoculated with the test isolates and incubated for growth.

The bacterial test isolates were incubated at 28±2°C for 24–48 hours while the microalgal isolates were incubated at 28±2°C under fluorescent illumination of 150-300 µmol photons m^{-2} s^{-1} (with 12:12 h light:dark photoperiod) for 2-3 weeks. Appearance of colonies on the plates indicated that the isolates were capable of growth in tannery wastewater. The isolates that grew on TWA were further screened based on tolerance to different chromium concentrations. Nutrient agar and algae culture agar plates were amended with various concentrations (50-1000 mg L^{-1}) of Cr(VI) and a loop full of bacterial and microalgal culture streaked and plates incubated at 28±2°C for 5-7 days for visible growth.

3.2.6 Identification of isolates

Bacteria

The bacterial isolates grown on tannery wastewater and selected for further experiments were maintained on NA with 50 mg L^{-1} Cr(VI). All these isolates were tested for their biochemical characteristics according to Holt et al. (2000). The following are the major morphological and biochemical tests carried out.

Gram staining was done on 24 h old cultures and observed microscopically.

Shape was made out microscopically while checking out for Gram character.

Motility was also examined microscopically by hanging drop method.

Enzyme profiles: Production or elaboration of various enzymes like urease, nitrate reductase, oxidase, catalase, gelatinase, caseinase, amylase by these isolates was carried out as per Holt et al. (2000).

Other tests: Reduction of NO_3^-, oxidation-fermentation reactions, production of methyl red, Voges-Proskauer reaction, and H_2S production were tested following Holt et al. (2000).

Microalgae

The microalgal isolates were identified based on cultural behaviour and morphological examination of cells. The strains were viewed under a light microscope (Nikon Eclipse 80i, Japan); the nature of filaments, shape and size of cells, heterocysts and akinetes, were observed and identified using the keys provided by Desikachary (1959).

3.2.7 Molecular identification of isolates

The genomic DNA from the pure cultures was extracted using Genomic DNA isolation kit (Sigma-Aldrich, USA), as per manufacturer's instructions. Bacterial 16S rRNA gene was amplified by following standard polymerase chain reaction (PCR) method using universal primer set, 27F (5′–AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5'-GGTTACCTTGTTACGACTT-3′) developed by Weisburg et al. (1991). The microalgal 18S rRNA gene was amplified by following standard polymerase chain reaction (PCR) method using universal primer set, CYA359F (5′–GGGGAATYTTCCGCAATGGG-3′) and CYA781R (5′-GACTACWGGGGTATCTAATCCCWTT-3′). The PCR mixture (50 µL) contained 1µL of extracted DNA (5–50 ng μL^{-1}), 1 µL of each primer at a concentration of 0.5 µM, 25 µL of Ready MixTaq PCR mix (Sigma-Aldrich, USA) [1.5 U Taq DNA polymerase; 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate (dNTP)], and 22µL of milliQ water. Then the PCR was carried out with the temperature profile as follows: Initial denaturation step of 3 min at 95°C, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. The final extension step was for 10 min at 72°C. The PCR was performed using Thermocycler (Applied Biosystems, USA). PCR products thus obtained were checked on 1% agarose gel. The purified PCR products were sequenced using ABI 31310XL (Applied Biosystems, USA) genetic analyzer housed in our laboratory.

The sequences obtained were aligned properly, checked for chimeras and edited using Vecscreen software (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). They were then compared with NCBI database through BLAST searches (http://blast.ncbi.nlm.nih.gov). In this comparison, sequences of type strains most closely related to the sequences of the

isolates were searched. The analysis was performed on the Phylogeny.fr platform and comprised the following steps. Sequences were aligned with MUSCLE (v3.8.31) configured for highest accuracy (MUSCLE with default settings). After alignment, ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b). The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aLRT). Reliability for internal branch was assessed using the aLRT test (SH-Like). Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3).

3.3 Results

3.3.1 Tannery wastewater characteristics

The physico-chemical characteristics of the tannery wastewaters are listed in Table 3.1. The tannery effluents were greyish-brown in color. The pH of both effluents ranged between 7.4-7.6. The concentration of solids in the Chennai TW was twice that of Kanpur TW. The Chennai TW had higher concentration of total solids (9500 mg L^{-1}) which were largely contributed by dissolved solids (8166 mg L^{-1}) in the effluent. The Kanpur TW had higher BOD (1350 mg L^{-1}) and COD (4000 mg L^{-1}) levels compared to Chennai effluent , however, the oxygen demand in both effluents was exceedingly beyond the BIS (1994) permissible limits. Sulphide concentrations were also found to be above the permissible limit in both effluents. Sulphates were excessive in Chennai TW (2075 mg L^{-1}) while that in Kanpur TW was within the permissible limit of 1000 mg L^{-1}. The Chennai TW had high amount of nitrates (1.53 mg L^{-1}) while the total nitrogen (14336 mg L^{-1}) and ammonia (2734 mg L^{-1}) contents were far higher in the Kanpur TW. Higher concentrations of

phosphates were measured in the Chennai TW. The phenol concentrations in both effluents fell within the permissible limit. The chloride concentrations in the effluents ranged from 1300 – 1500 mg L^{-1}. Higher bacterial counts ($1.49x10^8$) were found in the Kanpur effluent compared to that ($3.82x10^4$) of Chennai TW. The chromium concentrations in the Chennai and Kanpur effluents were 9.57 and 8.2 mg L^{-1}, respectively.

3.3.2 Characteristics of bacterial and microalgal isolates

A total of 27 seawater bacteria (SW) and 19 TW bacteria were obtained on the 50 mg L^{-1} Cr(VI) amended media plates. On further screening based on Cr tolerance tests and growth on TWA five bacterial isolates were selected for detailed study (Fig. 3.4). Among the bacterial isolates, three SW isolates - SWI46, SWI49 and SWI53, able to tolerate 400, 100 and 300 mg L^{-1} of Cr were obtained as the highly potent ones. Among the TW bacteria, two isolates TWI25 and TWI61 were able to tolerate high concentrations of 200 and 500 mg L^{-1} of Cr, respectively (Table 3.2).

The bacterial isolates were identified by an array of biochemical tests. Their morphological and biochemical characteristics are listed in Table 3.3. All five bacterial isolates were gram positive. The isolates TWI25, TWI61 and SWI46 were short rods (<2 µm), SWI53 was large rods (2-5 µm) while SWI49 was coccoid. TWI25 and SWI53 were not motile. Taxonomic identity of these isolates is mentioned in the legend of the Table 3.3. Isolates SWI46 and SWI49 were positive for methyl red and DNase tests while TWI61 and SWI53 were positive for Voges Proskauer tests. All tested isolates were negative for Indole. Isolates TWI25 and TWI61 utilized citrate. Urease was positive for SWI49 and SWI53 while nitrate reduction was exhibited by TWI61, SWI49 and SWI53. None of the isolates

were positive for Oxidase test. All except SWI46 produced catalase. Gelatinase was produced only by SWI49. Caseinase was produced by TWI61 and SWI49 while amylase by TWI25, TWI61 and SWI49. The bacteria were identified using 16S rRNA gene sequencing as belonging to *Gordonia* sp. (TWI25), *Bacillus cereus* (TWI61), *Exiguobacterium mexicanum* (SWI46), *E. aurantiacum* (SWI49), and *Aeromicrobium* sp. (SWI53) (Table 3.4). The accession numbers obtained for the sequences are KY196512 to KY196516 (Table 3.4).

3.3.3 Characteristics of microalgal isolates

Following screening based on Cr tolerance tests and growth on TWA two microalgal isolates were selected for detailed study (Fig. 3.4), one from tannery wastewater (NIOCCV) and one from seawater (NIOCP) that were efficiently able to tolerate upto 100 mg L^{-1} of Cr.

Light microscopic images of the microalgal species isolated in this study are shown in Fig. 3.5. The isolated microalgae were classified the as belonging to the genus *Chlorella* (NIOCCV) and *Phormidium* (NIOCP), respectively, based on morphological analysis and comparison to algae identification keys (Desikachary, 1959) available in literature as well as molecular identification techniques (Table 3.4).

Cells in Fig. 3.5a show the morphology of NIOCCV observed under a light microscope. According to taxonomical grouping based on morphology and physiological properties, NIOCCV belonged to genus *Chlorella*. Cells were microscopic, unicellular, green color, spherical in shape; with 2-10 μm diameter and were similar to *Chlorella* sp. cells as

described by Safi et al. (2014). The NIOCP cells/filaments were solitary or formed clusters, straight or slightly coiled (Fig. 3.5b). The trichomes were cylindrical, long, with rounded ends, 0.5-1.0 μm wide, 2-4 μm long and pale blue-green in colour. Apical cells were rounded. The trichomes were benthic and formed sheath like colonies. The cells were classified the as belonging to the genus *Phormidium* based on morphological characteristics and comparison to algae identification keys (Desikachary, 1959). The microalgae were identified using 18S rRNA gene sequencing as belonging to *Chlorella vulgaris* (NIOCCV) and *Phormidium* sp. (NIOCP). The accession numbers obtained for the sequences are MF196918 and MF196919 (Table 3.4).

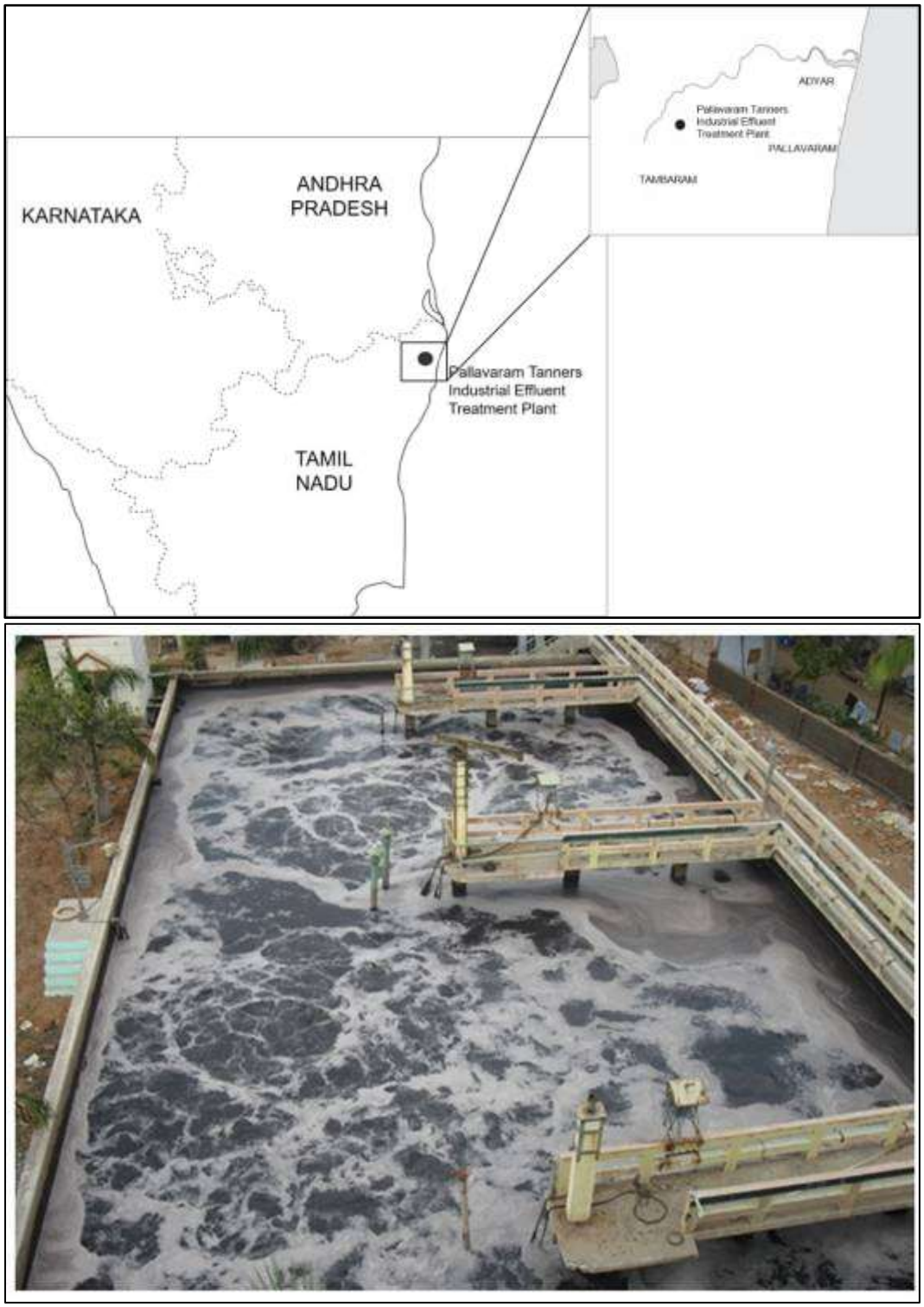


Fig. 3.1 Collection of tannery wastewater from Pallavaram Tanners Industrial Effluent Treatment Plant, Chennai, India during July 2013 and September 2015

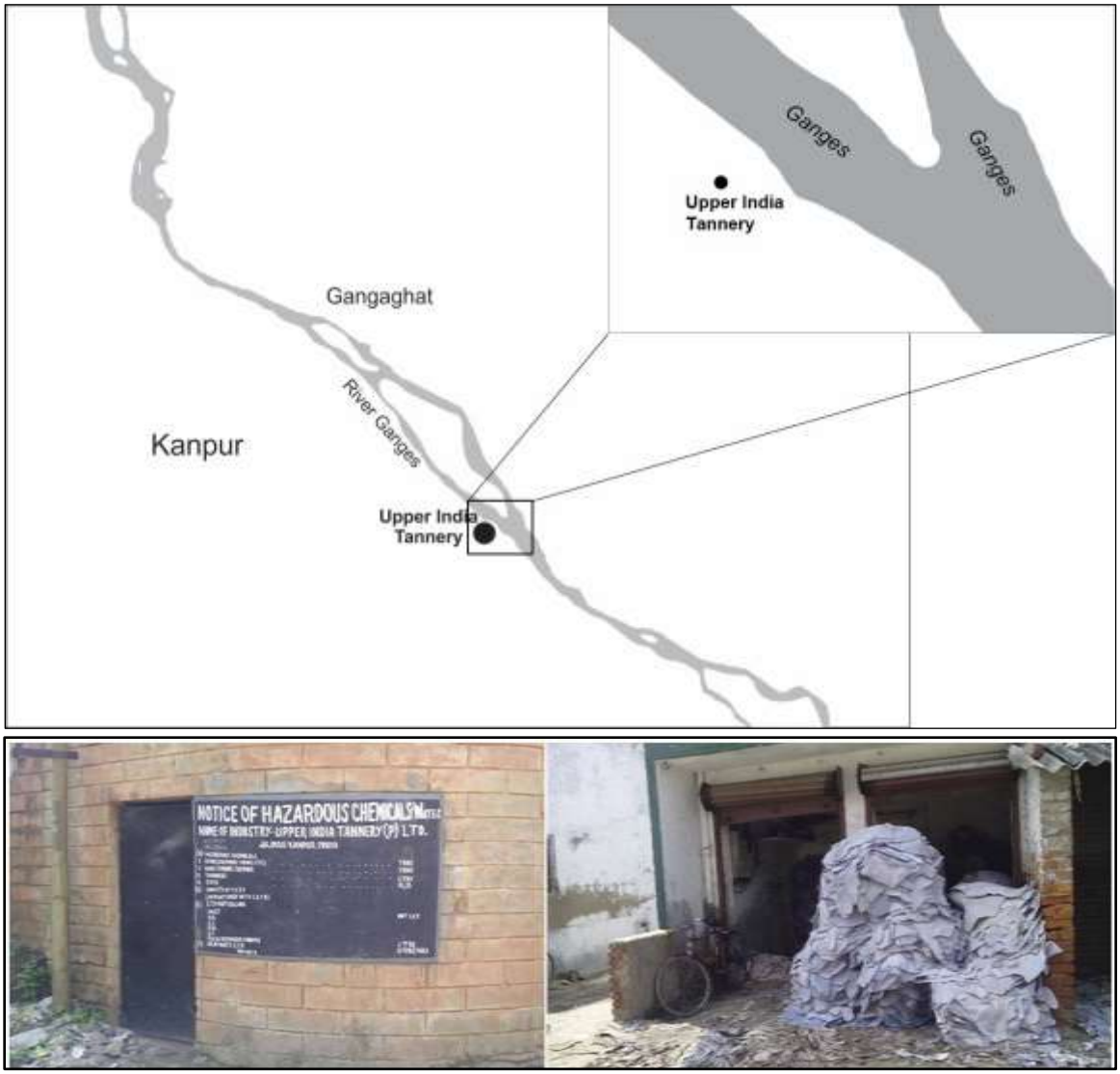


Fig. 3.2 Collection of tannery wastewater from Upper India Tannery Pvt. Ltd., Kanpur, India during September 2014

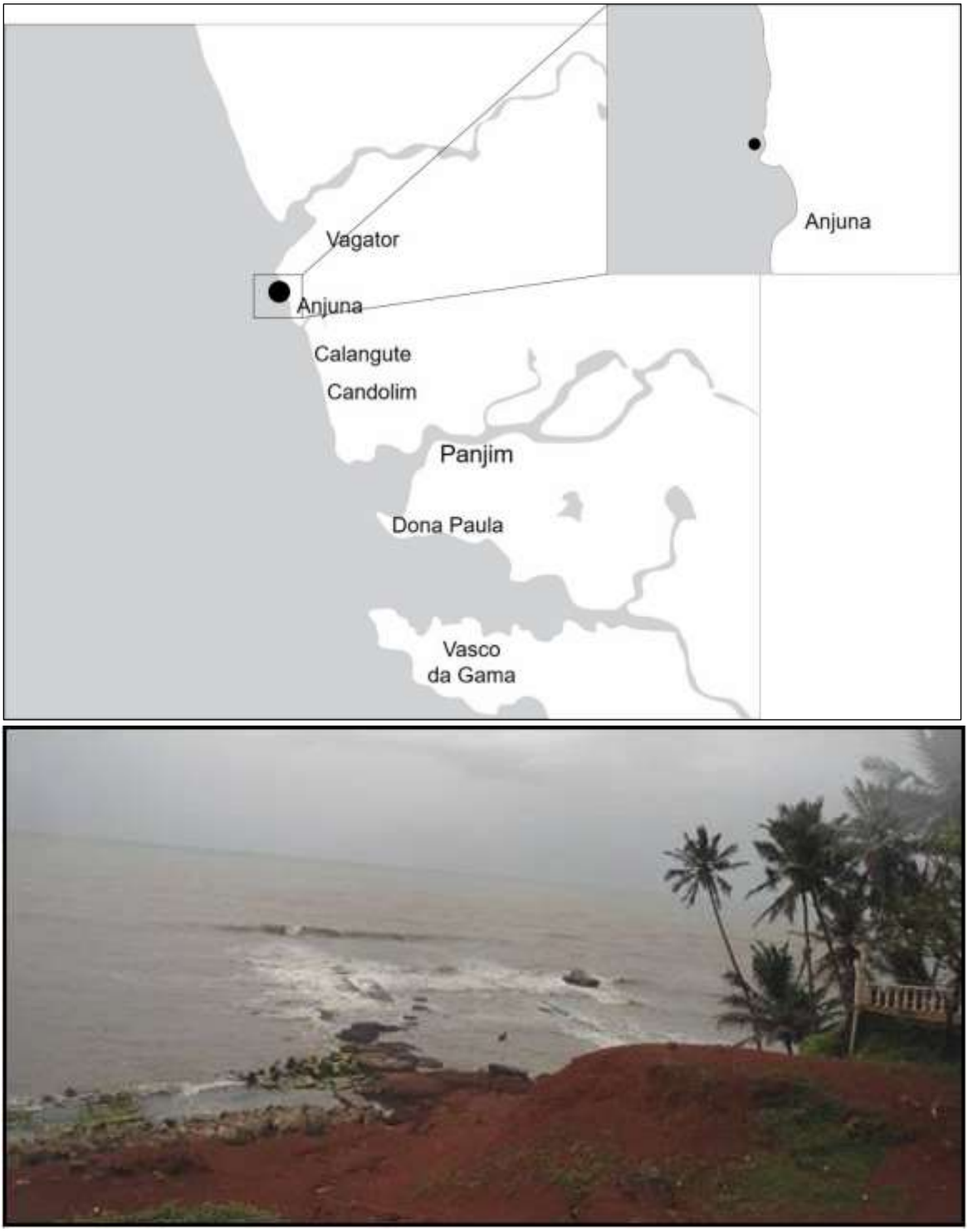


Fig. 3.3 Collection of seawater samples from Anjuna, Goa, India during July 2013

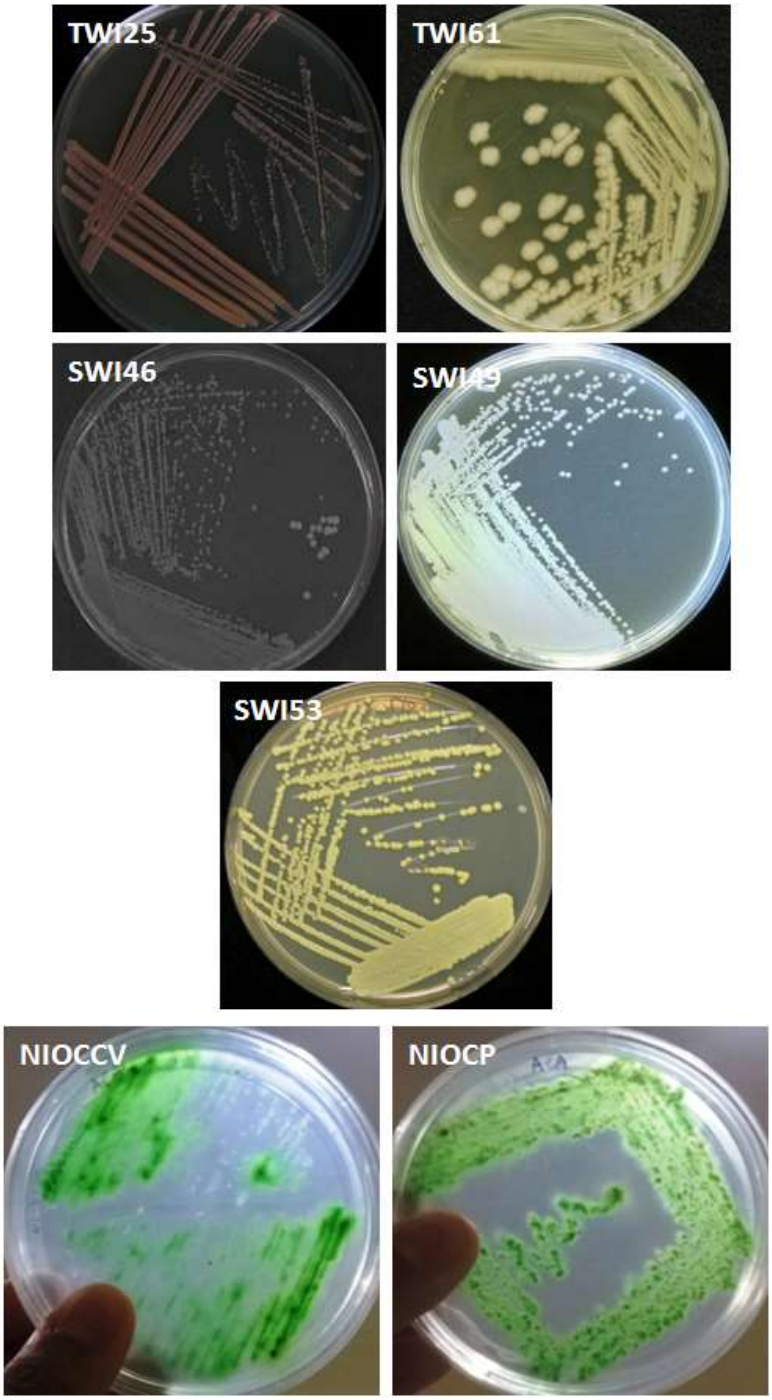
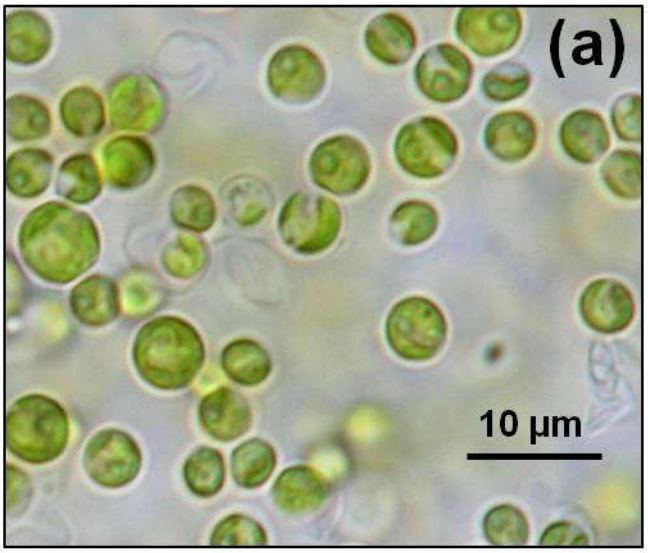
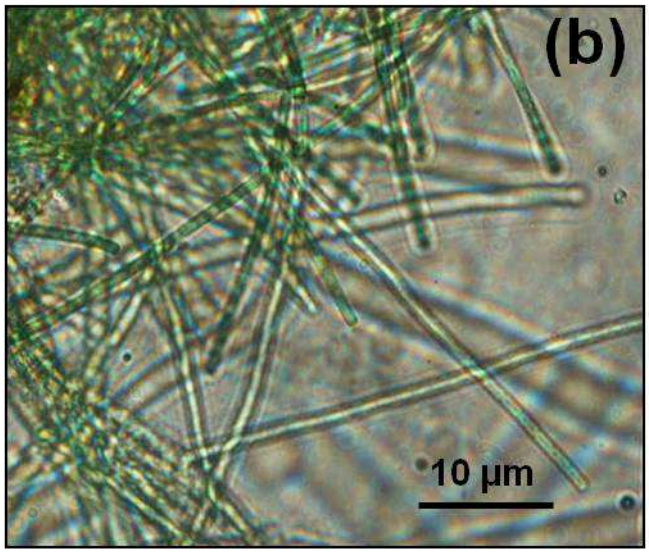


Fig. 3.4 Bacterial and microalgal isolates from tannery wastewater and seawater after screening on 50 mg L^{-1} Cr-amended media and TWA.
TWI25=*Gordonia* sp.; TWI61=*Bacillus cereus*; SWI46=*Exiguobacterium mexicanum*; SWI49=*Exiguobacterium aurantiacum*; SWI53=*Aeromicrobium* sp.; NIOCCV=*Chlorella vulgaris*, NIOCP=*Phormidium* sp.



Chlorella vulgaris (NIOCCV)



Phormidium sp. (NIOCP)

Fig. 3.5 Microphotographs of microalgal isolates (a) *Chlorella vulgaris* (NIOCCV) and (b) *Phormidium* sp. (NIOCP)

Table 3.1 Physico-chemical characteristics of tannery wastewaters collected from Chennai and Kanpur

Parameter	**Chennai TW**	**Kanpur TW**	**Permissible limits (BIS, 1994)**
pH	7.6	7.45	5.5-9.0
TS (mg L^{-1})	9500	5000	2200
TSS (mg L^{-1})	1595	500	100
TDS (mg L^{-1})	8166.67	4333.33	2100
BOD (mg L^{-1})	1096	1350	30
COD (mg L^{-1})	3520	4000	250
H_2S (S^{2-}) (mg L^{-1})	60.8	11.75	2
SO_4-S (mg L^{-1})	2075.15	178.69	1000
NO_3-N (mg L^{-1})	1.53	0.93	-
TKN (mg L^{-1})	1522.12	14336.28	-
NH_3-N (mg L^{-1})	749.03	2734.16	50
PO_4-P (mg L^{-1})	2.79	0.43	-
Phenols (mg L^{-1})	10.1	3.8	5-50
Cl^- (mg L^{-1})	1481.81	1364.83	-
Cr (mg L^{-1})	9.57	8.2	2
Bacterial counts (cfu mL^{-1})	3.82×10^4	1.49×10^8	-

*no set limit

Table 3.2 Chromium tolerance efficiencies of bacterial and microalgal isolates cultured on growth media amended with 50-1000 mg L^{-1} $K_2Cr_4O_7$.

Isolate	Growth on TWA	Source	Cr tolerance (mg L^{-1})
Bacteria			
TWI25	+	Tannery wastewater	200
TWI61	+++	Tannery wastewater	500
SWI46	+	Sea water	100
SWI49	++	Sea water	400
SWI53	++	Sea water	300
Microalgae			
NIOCCV	+	Sea water	100
NIOCP	+	Sea water	100

Table 3.3 Various characteristics (morphological and biochemical) of isolates grown in nutrient medium with 50 mg L^{-1} Cr.

TWI25=*Gordonia* sp.; TWI61=*Bacillus cereus*; SWI46=*Exiguobacterium mexicanum*; SWI49=*Exiguobacterium aurantiacum*; SWI53=*Aeromicrobium* sp.; NIOCCV=*Chlorella vulgaris*, NIOCP=*Phormidium* sp.

Characteristic	**Isolate #**				
	TWI25	**TWI61**	**SWI46**	**SWI49**	**SWI53**
Colour	Pink	Cream	White	Orange	Yellow
Shape	Rods	Rods	Rods	coccoid	Rods
Gram stain	+	+	+	+	+
Motility	-	+	+	+	-
Indole	-	-	-	-	-
MR	-	-	+	+	-
VP	-	+	-	-	+
SC	+	+	-	-	-
Urease	-	-	-	+	+
Nitrate reductase	-	+	-	+	+
Oxidase	-	-	-	-	-
Catalase	+	+	-	+	+
Gelatinase	-	-	-	+	-
Caseinase	-	+	-	+	-
DNase	-	-	+	+	-
Starch	+	+	-	+	-

Table 3.4 Accession numbers for the identified bacterial and microalgal strains

Isolate	Strain	GenBank Acc. No.
TWI25	*Gordonia* sp.	KY196512
TWI61	*Bacillus cereus*	KY196516
SWI46	*Exiguobacterium mexicanum*	KY196513
SWI49	*Exiguobacterium aurantiacum*	KY196514
SWI53	*Aeromicrobium* sp.	KY196515
NIOCCV	*Chlorella vulgaris*	MF196918
NIOCP	*Phormidium* sp.	MF196919

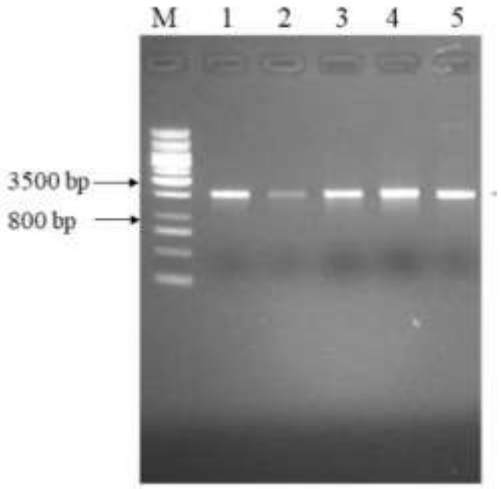


Fig. 3.6a Agarose gel electrophoresis (1%) of PCR products of 16S rRNA gene from TWI25 (Lane 1), TWI61 (Lane 2), SWI46 (Lane 3), SWI49 (Lane 4) and SWI53 (Lane 5). Lane M, 1 Kb DNA ladder (Marker).
TWI25=*Gordonia* sp.; TWI61=*Bacillus cereus*; SWI46=*Exiguobacterium mexicanum*; SWI49=*Exiguobacterium aurantiacum*; SWI53=*Aeromicrobium* sp.

Fig. 3.6b Agarose gel electrophoresis (1%) of PCR products of 18S rRNA gene from NIOCCV (Lane 1) and NIOCP (Lane 2). Lane M, 1 Kb DNA ladder (Marker).
NIOCCV=*Chlorella vulgaris*, NIOCP=*Phormidium* sp.

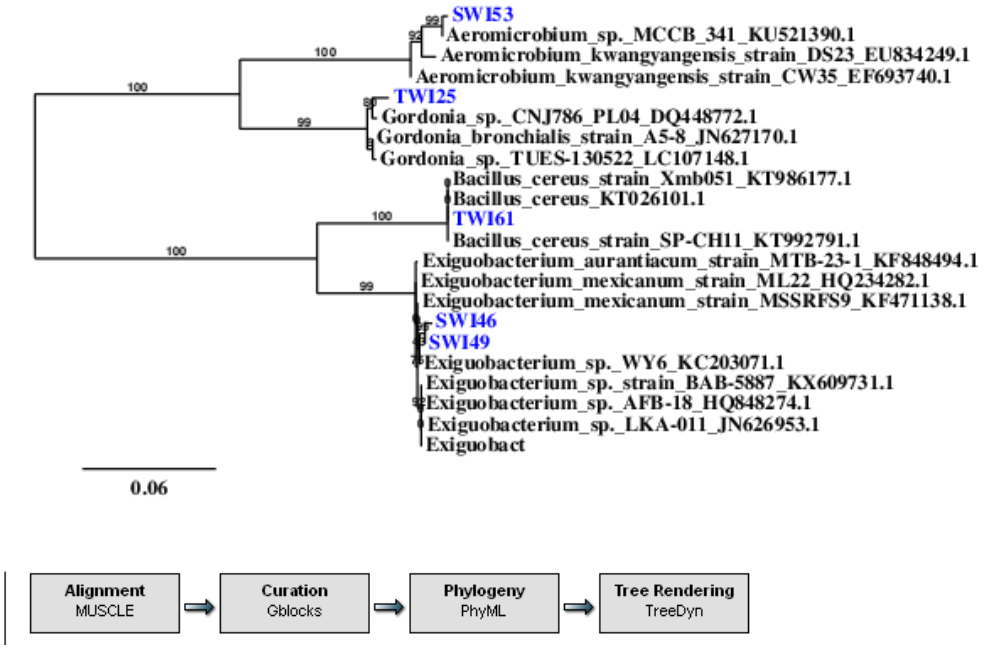


Fig. 3.7a Phylogenetic tree of bacterial isolates identified by 16S rRNA gene sequencing. Sequences in blue boldface are from this study. The percentage of boot strap support are shown on the branches. The scale bar 0.06 indicates 6% nucleotide sequence substitution.

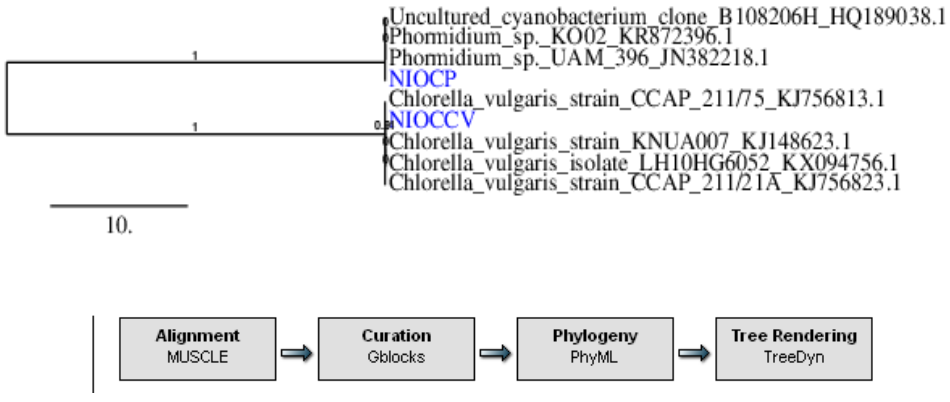


Fig. 3.7b Phylogenetic tree of microalgal isolates identified by 16S rRNA gene sequencing. Sequences in blue boldface are from this study. The percentage of boot strap support are shown on the branches.

3.4 Discussion

The most hazardous tannery wastewater is difficult to treat and quite difficult to achieve complete attainment of safe discharge limits through physico-chemical treatment methods. Furthermore, tannery effluent is quite variable in nature depending upon different production schemes and the size of the tanneries. The physico-chemical analyses of the two tannery wastewaters bring to the fore that the high concentrations of pollutants in the complex and hazardous wastewater which include high BOD, COD, TDS, Cr and nutrients among others. The effluent characteristics are representative of leather tanning wastewater and agree well with the values reported in the literature by Carucci et al. (1999), Kabdasli et al. (1999) and Zengin et al. (2002).

The isolation of Cr(VI) tolerant bacteria was carried out in tannery effluent and coastal marine waters. Five isolates comprising *Gordonia* sp., *Bacillus cereus, Exiguobacterium mexicanum, E. auratiacum* and *Aeromicrobium* sp. which were tolerant to ≥100 mg L^{-1} chromate level were selected for detailed study. Three of the isolates were resistant to high levels of chromate (200 mg L^{-1}) whereas two isolates were able to grow in higher Cr concentrations of >400 mg L^{-1}. The capacity for hexavalent chromium reduction is widespread and is reported by Flores and Perez (1999) from disparate bacterial genera such as *Bacillus cereus, Bacillus subtilis, Pseudomonas aeruginosa, Achromobacter eurydice, Micrococcus roseus* and *Escherichia coli*

Various studies (Khanafari et al., 2008; Middleton et al., 2003; Pattanapipitpaisal et al., 2001b; Campos et al., 1995; Nourbaksh et al., 1994; LLovera et al., 1993; Pandian et al., 2014; Devi et al., 2012) using many bacterial species like *Bacillus circulans, B. megaterium, B. coagulans, Agrobacterium radiobacter* EPS-916, *Bacillus* spp.,

Pseudomonas fluorescens, Bacillus sp. QC1-2, *Microbacterium liquuefaciens*, *Zoogloea ramigera* and *Pseudomonas aeruginosa* have proven their ability of chromium removal.

The bacterial isolate TWI61 (*Bacillus cereus*) was able to tolerate the highest concentration of Cr(VI) of 500 mg L^{-1}. Megharaj et al. (2003) isolated chromium -resistant bacterial strain *Bacillus cereus* S-6, tolerant to chromium concentration of 100 mg L^{-1}, from tannery effluents, which was used for the reduction of toxic hexavalent chromium into less toxic trivalent chromium. Previous works by Kamala-Kannan et al. (2007) and Rehman et al. (2008) reported the dominance of *Bacillus* spp. in chromium contaminated environments. The isolates identified as *Gordonia* sp. and *Aeromicrobium* sp. were able to tolerate Cr(VI) concentrations of 200 and 300 mg L^{-1}, respectively. None of these species have been reported to be Cr(VI)-resistant or Cr(VI)-reducing strains. Their luxuriant growth in Cr-amended media, by culturable methods may be due either to the Cr(VI) resistance and Cr(VI)- reducing ability of the strains and/or to the various TW contaminants.

Microalgae appear to vary considerably in their tolerance to chromium. The mechanism for the differences in algal sensitivity to chromium is not well known. However, for other heavy metals, the differences in sensitivity are apparently related to the size of the algal species (Munawar and Munawar, 1982), rate of uptake of the metal (Conway and Williams, 1979), sites of metal-binding (Hart, 1975), or genetic determinant (Singh et al., 1978). Rai et al. (2005) who reported the occurrence of the blue-green algae *Aphanocapsa grevillea, Oscillatoria animalis, O. tenuis, Phormidium bohneri,* and green algae *Chlamydomonas angulosa, Chlorococcum humicolo, C. vitiosum,* and *Ulothrix* sp. in tannery wastewater suggest that their metal accumulation potential is enhanced when exposed to contaminated habitats. Filip et al. (1979) isolated algae from wastewater lagoons and exposed them to 1-

40 mg L^{-1} Cr(VI). Only one alga (*Oscillatoria* sp.) grew while other algal species (*Scenedesmus, Chlorella, Microcystis* sp.) were virtually absent in the Cr-amended media. Hollibaugh et al. (1980) tested for Cr-tolerance on the species composition of natural marine assemblages of phytoplankton and observed *Skeletonema costatum* to tolerate Cr better than other species tested. Petria (1978) reported that chromium was more inhibitory to photosynthesis than growth in *Chlorella* species. Addition of 50-100 µg Cr L^{-1} to the medium greatly reduced the photosynthesis while 10 mg L^{-1} was required to inhibit the growth. In contrast, the *Chlorella* sp. strain isolated in this study, as well as the *Phormidium* sp. were able to tolerate and grew well in medium with100 mg L^{-1} Cr(VI).

The relative abundance of some of the microorganisms isolated in this study from crude effluent suggests that they could be the most competent bacteria for treating Cr(VI)-contaminated effluents. All the five species isolated and characterized in this study being capable of tolerating and growing in TWA and in media amended with Cr(VI) are potential candidates for exploring their potential for decontaminating the hazardous TW. Similarly the case with the microalgae with potential of not only Cr(VI) tolerance but also of their ability to grow well, reduces many other pollutants. These aspects are detailed in chapters 4 and 5. In the overall such tedious but essential selection steps of bioremediating organisms from natural and contaminated effluents would lead to evolve protocols for faster, reliable methods for metal pollution clean-up.

Chapter 4

Detoxification of tannery effluent by bacteria and their consortium

4.1 Introduction

Biological treatment of wastewaters is much sought after these days. It is being applied in many process industries to detoxify or bioremediate a variety of effluents. Low cost, energy savings and environmental safety are the advantages associated with wastewater treatment using biota. Use of microorganisms in this regard has proved to be useful in removal of pollutants from various specific industrial effluents (Kadirvelu et al., 2003; Manu and Chandhani, 2002). Naturally occurring microorganisms, consisting of bacteria, fungi, protozoa, rotifers, and other microbes, are the workhorses of wastewater treatment. These organisms thrive on many of the complex substances contained in wastewater. Ramakrishnan (2012) suggested smaller sizes, large surface area-to volume ratio and contact interfaces with their surrounding environment are some of the ideal features of microorganisms qualifying them as candidates for bioremediation.

As documented in Chapter 2, several studies (for eg., Batool et al., 2012; Fakruddin et al., 2009; Desai et al., 2008; Zhu et al., 2008; Daulton et al., 2007; Thacker et al., 2007; Sultan and Hasnain, 2007) have evaluated biological reduction of Cr(VI), BOD, COD and other pollutants by aerobic and anaerobic bacteria. Metal detoxification and/or bioremediation using Cr(VI) reducing microflora would prove to be of practical significance in particular in tannery wastewater treatment.

The interest in the use of single cultures and microbial consortia – multiple interacting microbial populations – for wastewater treatment has ecological advantages. In that, microbial consortia, in particular, can perform complex functions that individual species cannot. Since a carefully evolved/developed consortium on the basis of several trials can

be more robust to environmental fluctuations it is worthwhile to investigate on them. As Burmolle et al. (2006) propose, living in a community is thought both to generate robustness to environmental fluctuations and to promote stability through time for the members in a consortium. Furthermore, when together they might be able to better cope with periods of nutrient limitation.

It is important to note that though conventional wastewater treatment plants are running, it is very difficult for them to cope up with the norms devised by regulatory authorities. Accordingly, there is a dire need to develop new technologies for effluent treatment or to make appropriate modifications in existing treatment processes. In view of this, one of the objectives in the present study was to screen chromium-resistant bacterial isolates from tannery wastewater and from open marine waters as to test their ability, individually and in consortium, to degrade toxic wastes from tannery effluents.

4.2 Materials and Methods

4.2.1 Tannery wastewater

Wastewater samples collected from tannery CETP located in Pallavaram, Chennai, India were used for this study. Physico-chemical characteristics of wastewater *viz.*, pH, total solid (TS), total dissolved solids (TDS), total suspended solids (TSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), nutrients and chromium were measured using standard methods (APHA, 2005) as described in Chapter 3, section 3.2.4.

4.2.2 Isolation of bacteria

Bacteria were isolated from the TW and seawater samples by general procedure of dilution plate method (Chapter 3, section 3.2.3) unto nutrient agar plates amended with 50 mg L^{-1} Cr(VI). Distinct colonies were selected and further tested for their growth on Tannery Wastewater Agar (TWA), prepared according to the method of Prakasam and Dondero (1978). The isolates capable of growing on TWA were selected for further experiments.

4.2.3 Growth of bacterial cultures in tannery wastewater

Growth studies were carried out with the five selected bacterial isolates to compare their growth in Nutrient Broth (NB) versus Tannery Wastewater (TW). Tannery wastewater used was absolute, amended with mineral salts [g L^{-1}: K_2HPO_4 (0.5), Mg $SO_4.7H_2O$ (0.01), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (1.3), KCl (0.05), $FeSO_4.7H_2O$ (0.01)]. The experiment was set up in 500 mL flasks containing 100 mL of nutrient broth and filter sterilized absolute tannery effluent. The primary inoculums (starter culture) were prepared by inoculating a loop of bacteria in 50 mL of NB in 250 mL flasks at 28±2°C (room temperature) at 200 rpm for 24 h. Each flask was inoculated with 0.1% (v/v) of primary inoculums of each bacterium separately. Cultures were incubated at 200 rpm for 72 h and optical density was observed at six hour intervals for growth study.

4.2.4 Evaluation of Cr(VI) reduction

The overnight grown cultures of test bacteria were inoculated (2% v/v) in 100 mL nutrient broth medium (HiMedia, Mumbai) amended with 100 mg L^{-1} Cr(VI) and

incubated at 28±2°C for up to 72 h. The decrease in Cr (VI) concentration in the experimental flasks was measured after 12 h time intervals by 1,5-diphenyl carbazide method (APHA, 2005). The test samples were acidified (pH 1-2) and 1,5-diphenyl carbazide was added and Cr(VI) concentration was detected using spectrophotometer (Shimadzu, Japan) at 540 nm.

4.2.5 Bioremediation studies

Preparation of bacterial inocula

Five Cr(VI) tolerant bacterial cultures namely *Gordonia* sp. (Accn. No. KY196512), *Bacillus cereus* (Accn. No. KY196516), *Exiguobacterium mexicanum* (Accn. No. KY196513), *E. aurantiacum* (Accn. No. KY196514), and *Aeromicrobium* sp. (Accn. No. KY196515) were used in the experiments that follow. They were grown in liquid nutrient broth at pH 7.0 on an orbital shaker at 150 rpm at 28±2°C for 24 h in 100 mL nutrient broth. Well-grown culture suspensions with uniform optical density (OD_{600}) were used for the bioremediation experiments.

Experimental design

The acclimatized isolates were cultured in a BOD incubator shaker at 28±2°C and pH 7.0. Ten mL of culture sample were taken and centrifuged at 12,000 rpm to pellet the cells. The cell pellets were then transferred to 100 mL of wastewater in 250 mL conical flasks. For the consortium set-up, 2 mL from each of the five bacterial cultures were added to 100 mL of wastewater in a 250 mL conical flask. The set-up was kept at 28±2°C on rotatory shaker with 120 rpm for 72 h. The treated samples were collected at six

hourly intervals, allowed to stand for 10–15 min, and centrifuged. Supernatant was taken for analyzing BOD, COD, TDS, and Cr(VI) concentrations. Two controls (one of TW without any added bacterial culture and, another of standard nutrient broth (HiMedia, Mumbai) with bacterial cells) were included to confirm and check the effect of TW on the growth of algae during the experiment. The pH of the treated samples were checked and found unchanged. All the results are the average of three experimental sets.

4.2.6 Toxicity bioassay

Brine shrimp (*Artemia salina*) bioassay (Kiviranta et al., 1991) was used to assess the reduction in toxicity in the remediated tannery effluent. Briefly, one day old *Artemia* hatchlings were placed in multi-well plates (10 nos. well^{-1}). Survival was monitored from 1 mL treated and untreated wastewater samples and seawater (positive control) for 3 days. The assay was conducted in five replicates. The number of dead nauplii/hatchlings after 24, 48 and 72 h were counted and percent survival calculated.

4.3 Results

4.3.1 Physiochemical characteristics of effluent

The initial concentrations of physico-chemical parameters of TW used in this experiment are presented in Table 4.1. The TW was slightly basic in nature (pH 7.6), greyish-brown in colour, contained large amounts of TDS (8166.67 ± 288.7 mg L^{-1}), and had high values of BOD (1096 ± 42.5 mg L^{-1}) and COD (3520 ± 51.2 mg L^{-1}) (Table 4.1). The effluent from the tanneries also had high concentrations of nitrates (1.53 ± 0.002 mg L^{-1}), phosphates (2.29 ± 0.05 mg L^{-1}), and chromium (8.7 ± 0.85 mg L^{-1}).

4.3.2 Cr (VI) reduction by bacterial cultures

As described in Chapter 3, section 3.3, two isolates (TWI25, TWI61) were obtained from the tannery wastewater and three (SWI46, SWI49, SWI53) from coastal waters off Goa. The bacteria were identified by 16S rRNA gene sequencing as described earlier in Chapter 3 as belonging to *Gordonia* sp. (Accn. No. KY196512), *Bacillus cereus* (Accn. No. KY196516), *Exiguobacterium mexicanum* (Accn. No. KY196513), *E. aurantiacum* (Accn. No. KY196514), and *Aeromicrobium* sp. (Accn. No. KY196515). All tested isolates were tolerant to hexavalent chromium concentration of ≥100 mg L^{-1} (Chapter 3, section 3.3). Cr(VI) reduction by the selected five bacterial isolates ranged from 32.64 to 96.35% (Fig. 4.1) when grown in media containing 100 mg Cr(VI) L^{-1}. Maximum reduction (96.1%) of Cr(VI), within 72 h of incubation at 28±2°C, was observed for *Bacillus cereus* followed by *Aeromicrobium* sp. (95.78%) and *Exiguobacterium mexicanum* (88.38%). *Gordonia* sp. reduced Cr(VI) by 77.4% while *Exiguobacterium aurantiacum* was able to reduce only 32.6% after 72 h.

4.3.3 Reduction of other pollutants

Growth of bacterial isolates, individually and in consortium in TW was about 4.0 times lower than that in the nutrient broth (Fig. 4.2). Between the isolates, the growth in TW was the highest for *Bacillus cereus* (OD_{600} 0.698±0.01) by 72 h, followed individually by *Aeromicrobium* sp. (OD_{600} 0.501±0.02) and consortium. (OD_{600} 0.498±0.11). From the results of the bioremediation experiment (Fig. 4.3), it could be inferred that all the tested bacterial isolates played an effective role in reducing BOD, COD, NO_3-N, PO_4-P, Cr(VI) and TDS levels of tannery wastewater compared to the control sets. Compared to the

control set, after 72 h of treatment, *Aeromicrobium* sp., *Bacillus cereus* and the consortium respectively reduced the BOD by 85.3%, 78.3% and 75.3%. Maximum reduction of the initial COD levels of TW was observed for *Aeromicrobium* sp. (89.6%), followed by *Bacillus cereus* (82.4%) and *Exiguobacterium mexicanum* (71.96%). Concentrations of nitrates and phosphates were also efficiently reduced by the bacterial isolates after 72 h of incubation. Treatment of the TW with *B. cereus* and *Aeromicrobium* sp. accorded maximum reduction efficiencies for nitrates (89.8%) and phosphates (80.81%), respectively. Cr(VI) levels in the TW were reduced by above 99% when treated with *B. cereus* and by 96.7% with *Aeromicrobium* sp. Reduction efficiencies for TDS levels in the TW ranged from 33.36 – 46.6% with marked decrease after 72 h, compared to the control set.

4.3.4 Toxicity assay

Percent survival of brine shrimp (*Artemia salina*) nauplii/hatchlings subjected to treated and untreated effluent for 24, 48 and 72 h varied rather remarkably (Fig. 4.4). Notably, in the consortium-treated effluent, the survival of *Artemia* nauplii was over 95.8±1.6 % at 48 h and 81.53±2.1% at 72 h. This was comparable to the survival of nauplii in seawater (maintained as positive control) which was upto 100% for 48 h and 90% by 72 h. After 48 h, as much as 87.5% of nauplii survived in *B. cereus* treated effluent and 81.4% in *Aeromicrobium* sp. treated effluent. Generally, treatment by bacteria improved the quality of the effluent further enhancing the survival of *Artemia* nauplii. Survival was significantly poorer in the untreated effluent with only 20% survival that too only upto 24

h. Overall, the survival of *Artemia* hatchlings in the bacteria-treated, especially consortium treated effluent was far higher.

Table 4.1 Physico-chemical characteristics of tannery wastewater

Parameter	**Raw TW**	**Maximum permissible limits (BIS, 1994)**
pH	7.6± 0.00	5.5-9.0
Salinity (PSU)	15.00 ± 0.30	-*
Total Solids (mg L^{-1})	9500 ± 0.50	2200
Total Suspended Solids (mg L^{-1})	1595 ± 30.4	100
Total Dissolved Solids (mg L^{-1})	8166.67 ± 288.7	2100
BOD (mg L^{-1})	1096 ± 42.5	30
COD (mg L^{-1})	3520 ± 51.2	250
Nitrates (mg L^{-1})	1.53 ± 0.002	-
Phosphates (mg L^{-1})	2.29 ± 0.05	-
Cr (mg L^{-1})	8.7 ± 0.85	2

* no set limit

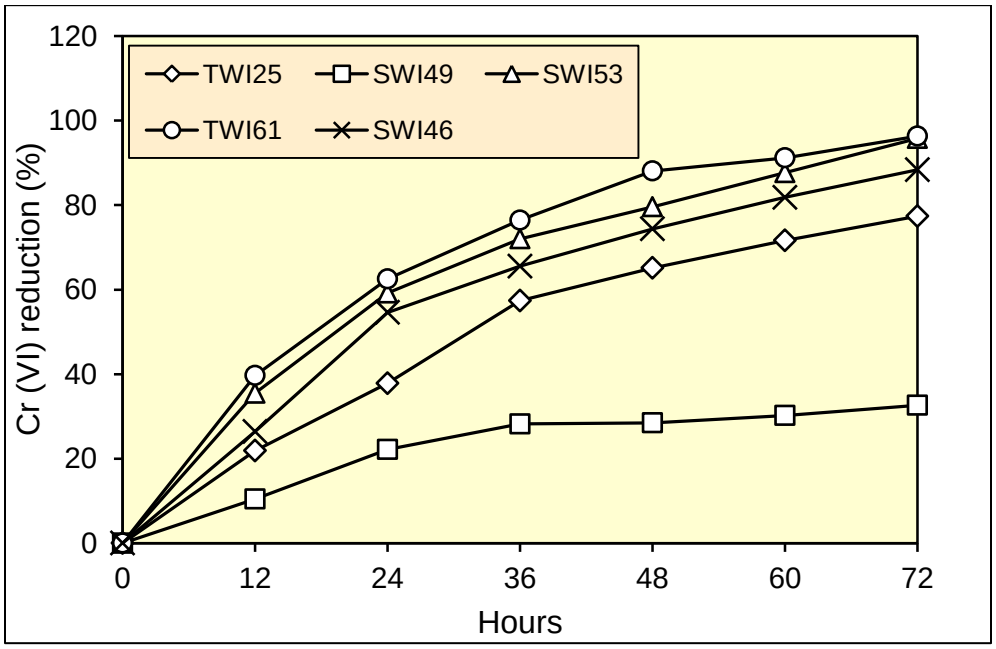


Fig. 4.1 Reduction of hexavalent Cr (100 mg L^{-1}) by bacterial isolates.

TWI25=*Gordonia* sp.; TWI61=*Bacillus cereus*; SWI46=*Exiguobacterium mexicanum*; SWI49=*Exiguobacterium aurantiacum*; SWI53=*Aeromicrobium* sp.

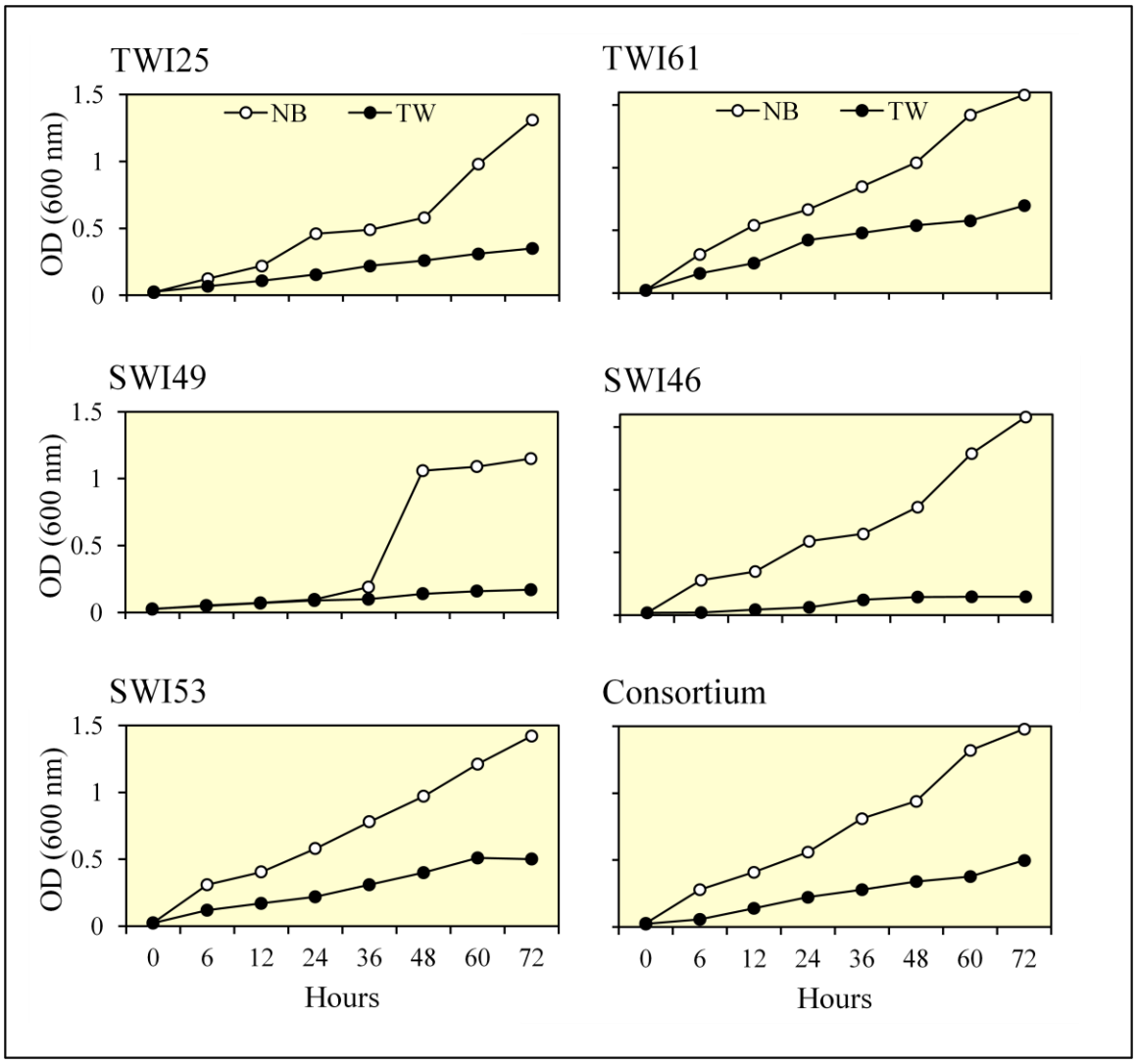


Fig. 4.2 Growth of bacterial isolates in tannery wastewater compared against standard nutrient broth. TWI25=*Gordonia* sp.; TWI61=*Bacillus cereus*; SWI46=*Exiguobacterium mexicanum*; SWI49=*Exiguobacterium aurantiacum*; SWI53=*Aeromicrobium* sp. NB=Nutrient broth; TW=Tannery wastewater

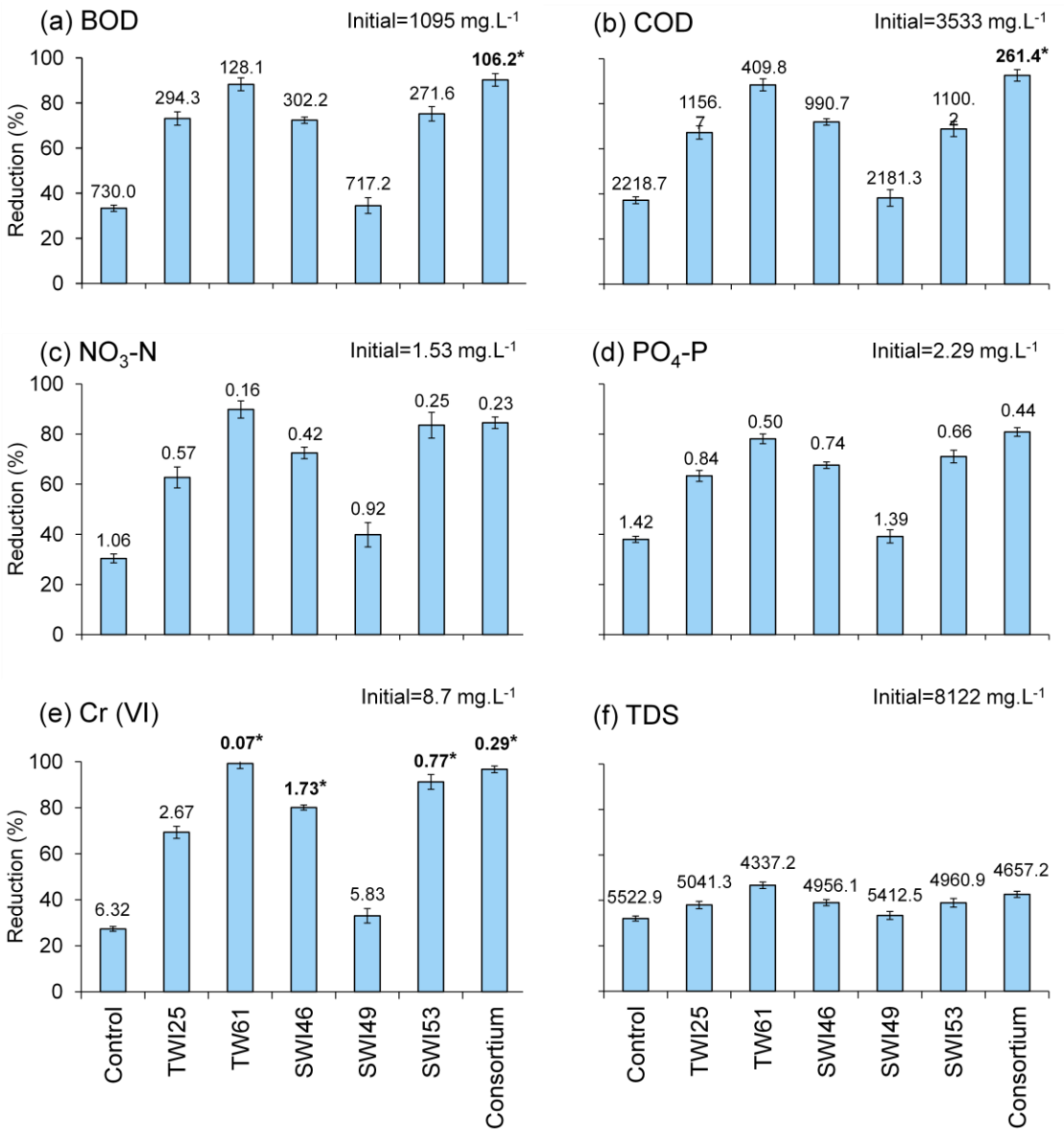


Fig. 4.3 Reduction in various parameters of tannery wastewater after 72 h treatment with bacterial isolates. Numbers above the bars equal final concentrations of the parameters after 72 h. Numbers in boldface and (*) denote final concentrations below/near the BIS permissible limit for safe discharge. TWI25=*Gordonia* sp.; TWI61=*Bacillus cereus*; SWI46=*Exiguobacterium mexicanum*; SWI49=*Exiguobacterium aurantiacum*; SWI53=*Aeromicrobium* sp.

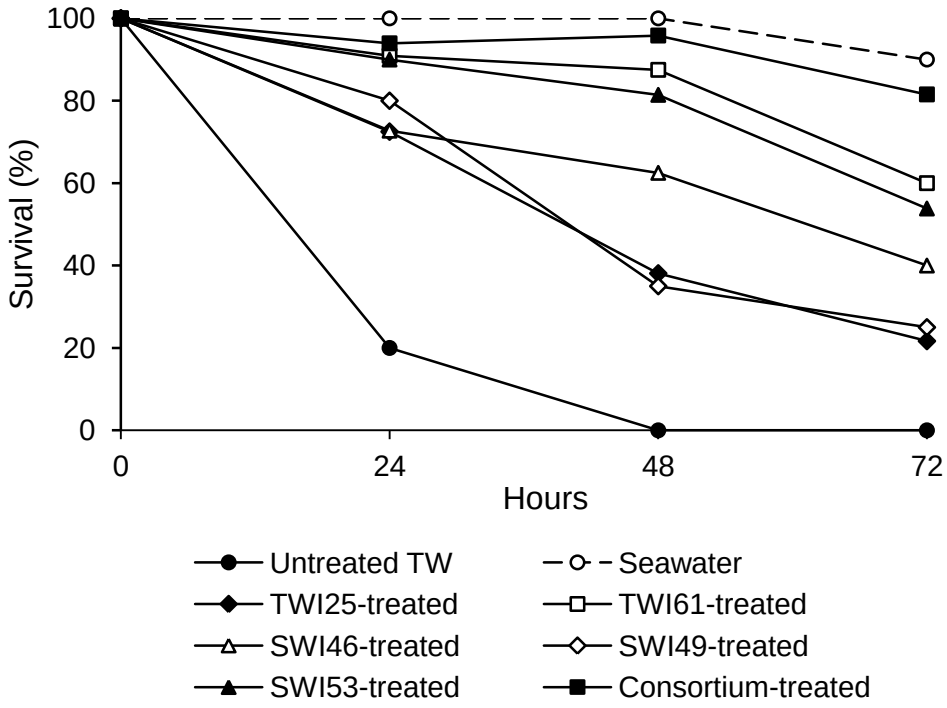


Fig. 4.4 Toxicity of untreated and treated TW measured as percent survival of *Artemia* nauplii for 72 h.

4.4 Discussion

Metabolic modes possible by a mix of species combined with the ability to share metabolites within the community syntropically are the key factors in a well constructed microbial consortium. Hence, the consortial approach is an effective alternative for wastewater treatment. In addition, synergistic interactions among the species in a consortium have greater potential in treating different types of wastewater, than what can be achieved through the use of monoculture/s.

Tannery effluent is one of the most difficult wastewaters in terms of treatability. Furthermore, it is quite variable in its composition depending upon different production schemes in the industry. Results of physico-chemical analyses of the TW examined in this study (Table 4.1) indicated all the striking features of a complex and deleterious wastewater. It had very high BOD, COD, TDS, Cr and nutrient concentrations. The effluent characteristics are representative of typical tanning wastewater and are in the ranges reported earlier by Carucci et al. (1999), Kabdasli et al. (1999), Zengin et al. (2002).

The isolation of Cr(VI) tolerant bacteria was carried out in tannery effluent and coastal marine waters. *Gordonia* sp., *Bacillus cereus, Exiguobacterium mexicanum, E. auratiacum* and *Aeromicribium* sp., tolerant to ≥100 mg Cr(VI) L^{-1}, were selected for detailed study (Chapter 3). Flores and Perez (1999) noted that the capacity for hexavalent chromium reduction is widespread and is reported in organisms such as *Bacillus cereus, Bacillus subtilis, Pseudomonas aeruginosa, Achromobacter eurydice, Micrococcus roseus* and *Escherichia coli*.

Monocultures of different bacterial strains have been used in most Cr(VI) bioremediation studies (Zahoor and Rehman, 2009; He et al., 2011; Farag and Zaki, 2010). However, in nature, single species seldom survive in a complex environment. Therefore, using pure cultures under controlled lab conditions may not emulate actual environmental conditions, particularly in highly contaminated areas that have more than a single metal present. This notwithstanding, the bacterial isolate TWI61 *Bacillus cereus* was able to tolerate the highest concentration of 500 mg Cr (VI) L^{-1} and reduced 96.35% of 100 mg Cr(VI) L^{-1} in 72 h. Megharaj et al. (2003) isolated chromium resistant bacterial strain *Bacillus cereus* S-6 from tannery effluents which was used for the reduction of toxic hexavalent chromium to less toxic trivalent chromium. At an initial hexavalent chromium concentration of 100 mg L^{-1}, their strain was able to reduce almost 67% of hexavalent chromium within 24 h incubation period. Previous works by Kamala-Kannan et al. (2007); Rehman et al. (2008) reported the dominance of *Bacillus* spp. in chromium contaminated environments.

According to Sannasi et al. (2006), bacteria are more stable and survive better when they exist in mixed cultures. In addition, most consortia are metabolically superior for removing metals and are more suitable for field application (Kader et al., 2007). Considering these advantages, consortia developed from natural environments offer more efficient Cr(VI) reduction.

The consortium of five strains of bacteria developed in this study was found to be more effective in the faster reduction of BOD, COD and Cr(VI), among other parameters. This is true on comparison with recent studies. For instance, Mandal et al. (2010) treated

tannery wastewater using three isolated bacteria (halophiles) separately and in combination. Their combination was reported to have additive effect in waste degradation by reducing COD by 80% and BOD by 87% in 5 days. Mongkolthanaruk and Dharmsthiti (2002) formulated bacterial consortium including *Pseudomonas, Bacillus* and *Acinetobacter* using molasses for treating lipid rich wastewater and the consortium reduced BOD by 83.9%. Kumar et al. (2007) used the bacterial consortium of *Pseudomonas aeruginosa*, *Bacillus megaterium* and *Stenotrophomonas maltophilia* for treating paper and pulp mill effluent and observed BOD reduction from 87 to 89% and COD reduction from 67% to 71%.

The magnitude of metal ion removal or detoxification by microorganisms from the effluent differs from strain to strain due to the properties of the metal as well as the biochemical traits of the microorganisms themselves. The cell wall capsules and slime layers of various microorganisms contain polysaccharides as basic building blocks, which have an ion exchange property helping in removal of specific metals (Mandal et al. 2010). They also contain proteins and lipids and therefore offer a host of functional groups capable of binding to heavy metals.

Most studies on tannery wastewater treatment using bacteria have only dealt with Cr(VI) reduction. Bhattacharya et al. (2015) reported 78% reduction of Cr(VI) in TW by *Acinetobacter* sp. Batool et al. (2012) demonstrated 88% Cr(VI) reduction using *Ochrobactrum intermedium*. The bacterial strains used in this study were far superior in remediating Cr(VI) from full strength TW. *B. cereus* efficiently reduced 99.2% of Cr(VI) in 72 h while *Aeromicrobium* sp. of the bacterial consortium reduced 91.2% and 96.7%

of Cr(VI), respectively. Several researchers (Chaturvedi, 2011; Farag and Zaki, 2010; Fakruddin et al., 2009; Campos et al., 2005) isolated Cr(VI) reducing bacteria from tannery wastewaters and tested their reduction potential in Cr-amended nutrient media. They reported reduction efficiencies in the range 38-88%.

Toxicity tests are indispensable tools for evaluating the quality and pollutant levels in an effluent. The brine shrimp, *Artemia salina* is the preferred choice animal for toxicity studies. The ease of its culture, short generation time, low commercial cost of its dormant eggs (cysts), are the advantages with *Artemia* toxicity assays (Vanhaecke et al., 1981). Hasegawa et al. (2014) determined the toxicity of tannery wastewater, before and after zinc oxide-assisted photocatalytic treatment, using dry shrimp eggs. Islam et al. (2014) studied the toxic effects of varying dilutions of tannery wastewater on brine shrimp nauplii.

Toxicity testing of tannery wastewater in this study, ascertained that the untreated wastewater is deleterious to the hatchlings of *Artemia salina*. The treatment of tannery wastewater with bacterial strains, individually and in consortium, largely improved the wastewater quality as is evidenced by the higher percent survival of the *A. salina* nauplii. Substantial reduction in the concentrations of the toxicants that were of far higher concentrations than BIS (1994) permitted limits is also indicated by higher survival percentage. Further, the biotransformation of toxic metals, in general, hexavalent chromium from the wastewater by the marine bacterial isolates ought to be facilitating the prolonged survival. Therefore, the toxicity results of this work showed that survival

of *A. salina* was an important tool to evaluate the efficacy of TW bioremediation by bacteria and their consortium.

Being ecologically versatile, highly diverse microbial communities present in the environment can efficiently reduce/detoxify many pollutants. This natural process however, is quite slow, leading to rapid accumulation of pollutants in the environment. Hence, a practical approach as Perpetuo et al. (2011) suggest, is to enhance the clear up process by the addition of exogenous microorganisms either singly or in combination in order to augment the bioremediation potential of indigenous populations. The use of exogenous marine, salt-tolerant microbes for the removal of toxicants from tannery wastewaters replete with innumerable salts offers many advantages for eco-sustainable bioremediation of effluent-polluted marine environments. As evidenced by the toxicity assay in this study, the wastewater quality improved substantially as well.

Chapter 5

Detoxification of tannery effluent by microalgae and their consortium

5.1 Introduction

Microalgae are among the fastest growing, photosynthesizing organisms and can complete an entire growth cycle every half-a-day if adequate amounts of sunlight, water, carbon dioxide, and nutrients are available. The eukaryotic microalgae and cyanobacteria are ideal for economical and eco-friendly removal of nutrients from wastewaters because of their high N and P requirement for growth (Mata et al., 2012). For most microalgae, wastewater serves as suitable medium as it supplies most of the necessary nutrients for their growth. This attribute can be used to significantly reduce the cost associated with wastewater treatment, as well as to mitigate greenhouse gas emissions (Pittman et al., 2011). Compared to physicochemical treatment technologies treatment of wastewaters using microalgae is proving to be far more efficient and safe. Christenson et al. (2011) and Ding et al. (2014) propose that microalgae remove toxicants including heavy metals at less cost. As also suggested by Rawat et al. (2011), microalgal biomass generated from wastewater treatment plants, as opposed to the accumulation of hazardous sludge in conventional treatment methods, can be utilized for production of bioenergy, animal feed, pharmaceuticals, and fertilizers.

Various reports are available on phycoremediation which employ microalgae for the treatment/detoxification of a variety of wastewaters (Oswald, 1988; Mara et al., 1996; Tadesse et al., 2003; Shi et al., 2007; Chu et al., 2008; Craggs et al., 2012; Mustafa et al., 2012; Dixit and Singh, 2014; Posadas et al., 2014). Studies of Silva-Benavides and Torzillo (2011), Singh et al. (2011), Renuka et al. (2013), Chinnasamy et al. (2010), Riano et al. (2011) emphasize the advantages of wastewater treatment and biomass production by consortia of microalgae. The importance of consortia, as against single

organisms, has been illustrated by the studies of Bhatnagar et al. (2010) and Silva-Benavides and Torzillo (2011), in terms of survival, biomass production, as well as nutrient removal. Also, the algal consortia can be of practical value as some of them may grow far better together in wastewaters, or the loss of one alga from the consortium may be compensated by the continued/luxuriant growth of other alga/e (Chinnasamy et al., 2010). Hence, there is urgent need to screen promising native microalgae from wastewaters and other water sources that not only have the potential to sequester excessive nutrients but also can form consortia, with extensive biomass applications after harvesting from the contaminated sites.

Tannery wastewater treatment by most recently reported studies, for example by Ajayan et al. (2015), Ajayan and Selvaraju (2012), Rehman (2011) and Chandra et al. (2004) have either focused on diluting the wastewater, or on examining Cr(VI) reduction only. Moreover, the use of *marine* microalgal strains for examining their potential in this regard is lacking and needs focus. In order to recognize the bioremediation potential of marine microbes already possessing the ionic adjustments in their marine milieu with close to 95 of the 109 named elements and their compounds, it is useful to evaluate their potential, among other heavy metals, to reduce/biotransform Cr(VI) as well as their ability to deal with other toxic components found in hazardous TW. Hence, this study aimed at examining the phycoremediation potential of marine strains of *Chlorella* sp. and *Phormidium* sp., individually and in consortium. Through this approach, it was intended to check if it is possible to improve the tannery wastewater for safe discharge into the open or for various other uses listed in Das et al. (2016). This study indeed focussed on coming up with an uncomplicated but effective method for treating 100% raw tannery

wastewater and to assess the advantages of consortium versus monoalgal culture for bioremediation.

5.2 Materials and Methods

5.2.1 Characteristics of tannery wastewater

Tannery wastewater (TW) sample collected from tannery CETP at Pallavaram, Chennai, Tamil Nadu was used for this study. Various physical and chemical parameters of the wastewater like BOD, COD, TN, TP, Cr, TS, TSS, TDS were analysed using standard methods listed in APHA (2005) as described in Chapter 3, section 3.2.4.

5.2.2 Isolation of microalgae

Marine green alga *Chlorella* sp. and marine cyanobacterium *Phormidium* sp. used in this study were isolated from coastal waters off Goa and cultured in algal culture medium (ACM, HiMedia, Mumbai) grown at a constant temperature of 28±0.5^{o}C under fluorescent illumination of 150-300 µmol photons m^{-2} s^{-1} (with 12:12 h light:dark photoperiod) as described in Chapter 3, section 3.2.3.

5.2.3 Experimental design

The phycoremediation experiments were carried out in triplicates in 15 L polypropylene tanks. These tanks were washed with diluted HNO_3 and then rinsed with deionized water. Ten litre aliquots of 100% strength TW were dispensed into the tanks. Three sets of treatments were set up; the first two sets were inoculated with single alga, *Chlorella* sp. or *Phormidium* sp., and one set with these two microalgae together, as a consortium. To

each tank, 8.0 g of the wet algal cells were added on day 0. Both algae were added at 4.0 g per tank in the consortium set. The experiment was carried out for 20 days at constant temperature of 28±0.5^{o}C under fluorescent illumination of sufficiently adequate light intensity 225 µmol photons m^{-2} s^{-1} (12:12 h light:dark photoperiod). This light intensity was chosen to ensure optimum illumination throughout the 10 L medium (fluid depth 15 cm). Two controls (one of TW without any algal inoculum and another of standard algae culture medium with algal cells) were included to compare the effect of TW on the growth and phycoremediation potential of algae. Sub-samples of 100 mL aliquots from all experimental tanks were drawn on day 0, 5, 10, 15, 20 and analyzed for algal growth and different physico-chemical parameters.

5.2.4 Analytical procedures

Measurements of BOD, COD, TN, TP, total Cr and TDS from the experimental setup were done as described above for TW characteristics. The TS and TSS were not analysed because the algal biomass adds to the total/suspended solids, interfering with accurate measurements. To avoid underestimation of *Phormidium* sp. cells, measuring Chl *a* was sought as a reliable option since the filaments of *Phormidium* cells clumped both in the ACM and TW. Hence, growth analyses of both *Chlorella* sp. and *Phormidium* sp. were done using Chl *a* measurements. On all sampling days, samples were stirred to maintain homogeneity, 10 mL aliquots drawn from all treatments, filtered through GF/C (0.7 µm, Whatman, USA) and the filter paper transferred into a vial with 10 mL 90% (v/v) methanol and held overnight for extraction. Chl *a* in this extract was measured spectrophotometrically following Parsons et al. (1984).

5.2.5 Reduction efficiency

The reduction/removal efficiencies (%) of the various parameters were calculated by following Ji et al. (2011).

$$R = \frac{Ci - Ce}{Ci} \times 100$$

Where,

R = the removal/reduction percentage at each measurement time,

Ci = the initial concentration of a given parameter, and

Ce = the remainder concentration on a given sampling day

5.2.6 Statistical analyses

The growth of algae individually or in consortium, measured as increase in Chl *a*, was correlated with reduction of various pollutant concentrations in the wastewater during the experimental period. Results are expressed as Pearson correlation coefficients of which values of ≥ 0.4, ≥ 0.6 and ≥ 0.8 are statistically significant with $p < 0.05$, < 0.01 and < 0.001, respectively. Two-way analysis of variance (ANOVA) was done to check the efficiency of microalgae, individually and in consortium. This was to check if the reduction of measured toxicants were statistically significant. Subsequently, pair-wise comparisons were performed using post hoc Tukey's HSD (Honestly Significant Difference) tests to evaluate at which time period of the experiment, the reduction of measured toxicants was statistically significant at $p < 0.05$.

5.3 Results

5.3.1 Characteristics of tannery wastewater

The initial concentrations of physico-chemical parameters of the tannery wastewater are presented in Table 5.1. The wastewater collected from the tannery CETP before its treatment process, had dark brown colour, salinity of 15 g L^{-1}, high BOD, COD, TN, TP, Cr and TDS concentrations. The BOD and COD concentrations were as high as 1520 (±42.5) mg L^{-1} and 3070 (±51.2) mg L^{-1}, respectively. This untreated TW had high concentrations of TN (822±51.2 mg L^{-1}), TP (1.89±0.05 mg L^{-1}) and total chromium (9.57±0.28 mg L^{-1}). Notably, the TDS (5166.67±288.7 mg L^{-1}) were higher than TSS (1595±30.4 mg L^{-1}), indicating high contents of dissolved salts in the wastewater.

5.3.2 Growth of algae in tannery wastewater

Growth of the microalgae, individually and as consortium, in TW was about 1.5 times lower than that in standard algal culture media. Chlorophyll *a* concentrations in the TW were observed to be the highest in the consortium (12.5±0.49 mg L^{-1}) by day 20, followed individually by *Chlorella* sp. (11.8±0.26 mg L^{-1}) and *Phormidium* sp. (9.21±0.84 mg L^{-1}) (Fig. 5.1). In the consortium, the microalgal growth in TW was 1.34 times the growth of *Chlorella* and 1.5 times the growth of *Phormidium* strains in unialgal culture setups.

5.3.3 Reduction of different physico-chemical parameters following algal growth

There was a significant reduction in all measured parameters in TW after treatment with algae (Table 5.2). Percent reduction of all examined parameters was significantly higher

($p<0.001$) in the monoalgal and consortium sets than in the control set. Maximum reduction of BOD from 1520±261 mg L^{-1} to 100.25±8.5 mg L^{-1} was observed in the consortium (Fig. 5.2). As much as 93.4% of BOD was reduced by the algal consortium which was statistically highly significant ($p<0.001$). The level of BOD was brought down to near the BIS (1994) permissible limit of 100 mg L^{-1} by day 20 in the consortium. Individually, *Chlorella* sp. and *Phormidium* sp. also reduced the BOD by 89.87% and 86.97%, respectively.

Similarly, the COD reduction was the highest in the consortium (92.60±11.11%), followed by *Chlorella* sp. (86.43±13.82%) and *Phormidium* sp. (79.13±3.95%). Algal consortium effectively brought down COD levels in TW from 3048±407 mg L^{-1} to 225.46±12.9 mg L^{-1} by day 20, which is within permissible limit of 250 mg L^{-1} for safe discharge. Initial concentrations of TN (822±182 mg L^{-1}) and TP (1.89±0.27 mg L^{-1}) were reduced to 72.66±19.76 mg L^{-1} and 0.21 mg L^{-1}, respectively by algal consortium, thereby meeting the BIS safe discharge standards by day 15.

Removal of total chromium concentrations was as high as 94.45% with algal consortium followed individually by *Phormidium* sp. (93.18±4.65%) and *Chlorella* sp. (90.17±14.42%) (Table 5.2). In the consortium set up, the initial Cr content (9.57±1.6 mg L^{-1}) was quite efficiently reduced, leaving behind about 0.52─0.98 mg L^{-1}, which is far below the safe permissible discharge limit of 2 mg L^{-1}. The high concentrations of TDS (>5000 mg L^{-1}) in untreated wastewater were reduced to 2155─2546 mg L^{-1} by day 20 (Fig. 5.2) with an overall TDS removal of 53.46%. Highest TDS reduction of 58.28%

was observed in the consortium. Notably, only the consortium brought down TDS levels close to the safe discharge limit of 2100 mg L^{-1}.

Statistically significant reduction ($p<0.001$) of all measured parameters was observed for the three treatments compared to the control set (Table 5.3), though these reduction efficiencies were observed to be significant on different days of treatments (Table 5.4). For instance, statistically significant reduction (post-hoc Tukey's HSD, $p<0.01$) of BOD occurred upto day 5 of treatment in the *Chlorella* and consortium sets; and upto day 10 in the *Phormidium* set. However, reduction of BOD to permissible limits by consortium was achieved by day 20. Similarly, the algal consortium aided significant ($p<0.01$) reduction of COD upto day 15, while the BIS permissible discharge limit was attained by day 20. Reduction of TN levels by the consortium was statistically significant ($p<0.01$) upto day 10 and safe discharge limit were attained by day 20. Significant reductions of TP and Cr were achieved within 5-10 days by *Chlorella* sp., *Phormidium* sp. and their consortium and permissible discharge limits by day 15. The permissible discharge limit for TDS was reached by day 20 in the consortium set with significant reductions occurring upto day 10.

Treatment of the tannery effluent with the consortium of *Chlorella* sp. and *Phormidium* sp. brought down the levels of BOD, COD and total chromium to within the permissible limits for safe discharge of effluents (Fig. 5.2). The reduction of BOD, COD, TN, TP, Cr and TDS in the TW correlated positively with the growth (measured as increase in Chl *a*) of *Chlorella* sp. and *Phormidium* sp., individually and in consortium. The positive trend

in correlation of Chl *a* with different parameters during the experimental period (Fig. 5.4) can be ascribed to the growth related reduction of these toxicants.

Table 5.1 Physico-chemical characteristics of raw untreated tannery wastewater (TW)

Parameter	**Raw TW**	**Max. permissible limits (BIS, 1994)**
pH	7.76 ± 0.00	5.5-9.0
Salinity (PSU)	15.00 ± 0.30	–
TS ($mg.L^{-1}$)	6761.00 ± 50.20	2200
TSS ($mg.L^{-1}$)	1595.00 ± 30.40	100
TDS ($mg.L^{-1}$)	5166.67 ± 288.70	2100
BOD ($mg.L^{-1}$)	1520.00 ± 42.50	100
COD ($mg.L^{-1}$)	3070.00 ± 51.20	250
TN (mg.L-1)	822.00 ± 51.20	100
TP ($mg.L^{-1}$)	1.89 ± 0.05	1.0
Cr ($mg.L^{-1}$)	9.57 ± 0.28	2.0

(Standard deviation, n=3)

Table 5.2 Percent reductions of different parameters in tannery wastewater after treatment with *Chlorella* sp., *Phormidium sp.* and their consortium

	Day	BOD	COD	TN	TP	Cr	TDS
Control	5	10.52±2.10	1.09±0.21	20.33±4.07	17.46±3.49	0.51±0.10	6.44±1.29
	10	18.42±3.68	10.47±2.09	27.26±5.45	31.22±6.24	1.73±0.35	7.73±1.55
	15	35.52±7.10	25.11±5.02	52.89±10.58	46.03±9.20	18.90±3.78	39.67±7.93
	20	43.81±8.76	39.35±7.87	65.75±13.15	55.55±11.11	26.05±5.20	42.35±8.47
***Chlorella* sp.**	5	51.25±8.20	55.46±8.87	14.02±2.24	39.01±6.24	32.84±5.25	4.50±0.72
	10	63.52±10.16	65.81±10.53	69.49±11.12	45.97±7.36	70.05±11.21	43.86±7.02
	15	81.26±13.00	79.84±12.78	86.85±13.89	72.94±11.67	81.38±13.02	46.44±7.43
	20	89.86±14.37	86.43±13.82	89.75±14.36	83.07±13.29	90.17±14.42	51.37±8.21
***Phormidium* sp.**	5	49.58±2.47	35.56±1.78	26.79±1.34	38.73±1.94	45.76±2.29	21.92±1.10
	10	72.41±3.62	58.27±2.91	63.65±3.18	42.33±2.12	76.20±3.81	43.22±2.16
	15	79.53±3.97	70.61±3.53	81.74±4.09	56.82±2.84	90.45±4.52	43.86±2.19
	20	86.97±4.34	79.13±3.95	87.55±4.38	76.19±3.80	93.18±4.66	50.71±2.54
Consortium	5	62.29±7.47	40.98±4.92	32.49±3.89	12.17±1.46	58.75±7.05	35.48±4.26
	10	75.29±9.03	64.25±7.71	74.69±8.96	50.79±6.09	69.86±8.38	56.77±6.81
	15	84.94±10.19	83.40±10.00	88.90±10.67	83.07±9.97	92.51±11.10	54.19±6.50
	20	93.40±11.21	92.60±11.11	91.16±10.93	88.88±10.66	94.45±11.33	58.28±6.99

(Standard deviation, n=3)

Table 5.3 Significant differences in the removal efficiencies between treatments and days for each parameter (Two-way ANOVA)

	Source of variance	Sum of squares	Degree/s of freedom	Mean of squares	F value*
BOD	Treatment	3769403	3	1256468	89.455
n=60	Day	10610668	4	2652667	188.858
	Treatment*Day	1010264	12	84189	5.994
	Error	561832	40	14046	–
COD	Treatment	16044669	3	5348223	71.263
n=60	Day	38953965	4	9738491	129.761
	Treatment*Day	4754580	12	396215	5.279
	Error	3001983	40	75050	–
TN	Treatment	341605	3	113868	25.489
n=60	Day	4187391	4	1046848	234.335
	Treatment*Day	235655	12	19638	4.396
	Error	178693	40	4467	–
TP	Treatment	1.10735	3	0.36912	7.699
n=60	Day	15.69348	4	3.92337	81.837
	Treatment*Day	1.30472	12	0.10873	2.268
	Error	1.91766	40	0.04794	–
Cr	Treatment	265.290	3	88.430	62.283
n=60	Day	421.180	4	105.295	74.162
	Treatment*Day	80.993	12	6.749	4.754
	Error	56.792	40	1.420	–
TDS	Treatment	9586023	3	3195341	94.33
n=60	Day	58106037	4	14526509	428.85
	Treatment*Day	8050715	12	670893	19.81
	Error	1354920	40	33873	–

*All F values are highly significant at $p<0.05$

Table 5.4 Statistically significant differences in the reduction of different parameter (post-hoc Tukey's HSD, $p < 0.05$). Numbers in parenthesis indicate the days in which permissible limits for safe discharge were reached.

		No. of days*	Tukey HSD p-value	Tukey HSD inference
BOD	*Chlorella* sp.	5	0.0010053	** p<0.01
	Phormidium sp.	10	0.0010053	** p<0.01
	Consortium	5 (20)	0.0010053	** p<0.01
COD	*Chlorella* sp.	5	0.0010053	** p<0.01
	Phormidium sp.	10	0.0010053	** p<0.01
	Consortium	15 (20)	0.0014486	** p<0.01
TN	*Chlorella* sp.	10	0.0010053	** p<0.01
	Phormidium sp.	15	0.019637	* p<0.05
	Consortium	10 (20)	0.0010053	** p<0.01
TP	*Chlorella* sp.	5 (15)	0.0150289	* p<0.05
	Phormidium sp.	5 (15)	0.0451089	* p<0.05
	Consortium	15 (10)	0.0036347	** p<0.01
Cr	*Chlorella* sp.	10 (15)	0.0184453	* p<0.05
	Phormidium sp.	5 (15)	0.0083061	** p<0.01
	Consortium	5 (15)	0.0011342	** p<0.01
TDS	*Chlorella* sp.	20	0.0315698	* p<0.05
	Phormidium sp.	20	0.0192649	* p<0.05
	Consortium	10 (20)	0.0078187	** p<0.01

*****Statistically significant reduction ($p<0.05$) in pollutant concentration

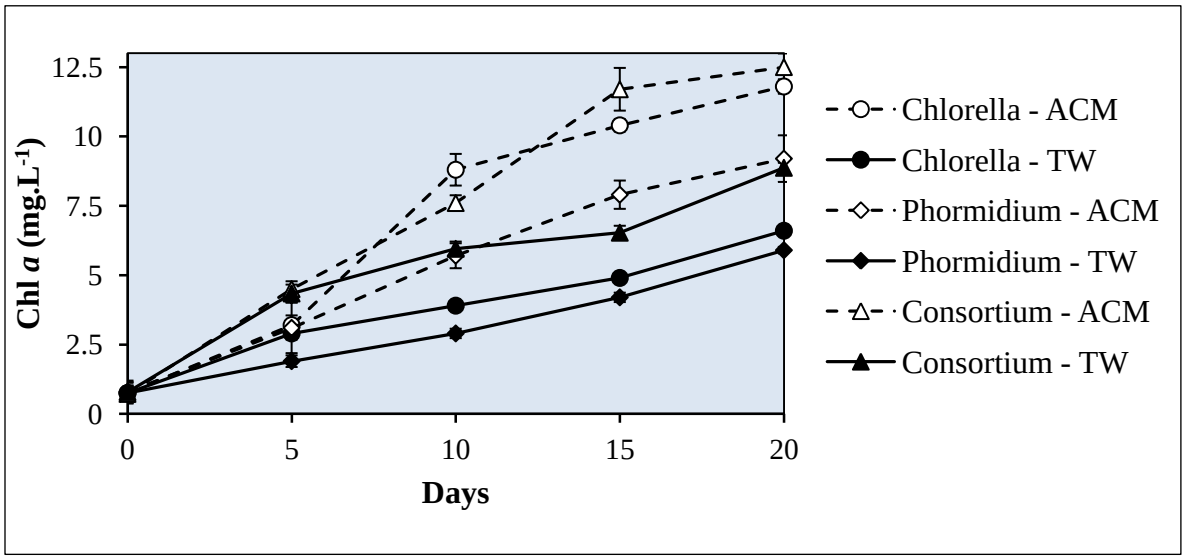


Fig. 5.1 Growth of *Chlorella* sp., *Phormidium* sp. and their consortium in tannery wastewater. ACM=Algal culture medium; TW=Tannery wastewater.

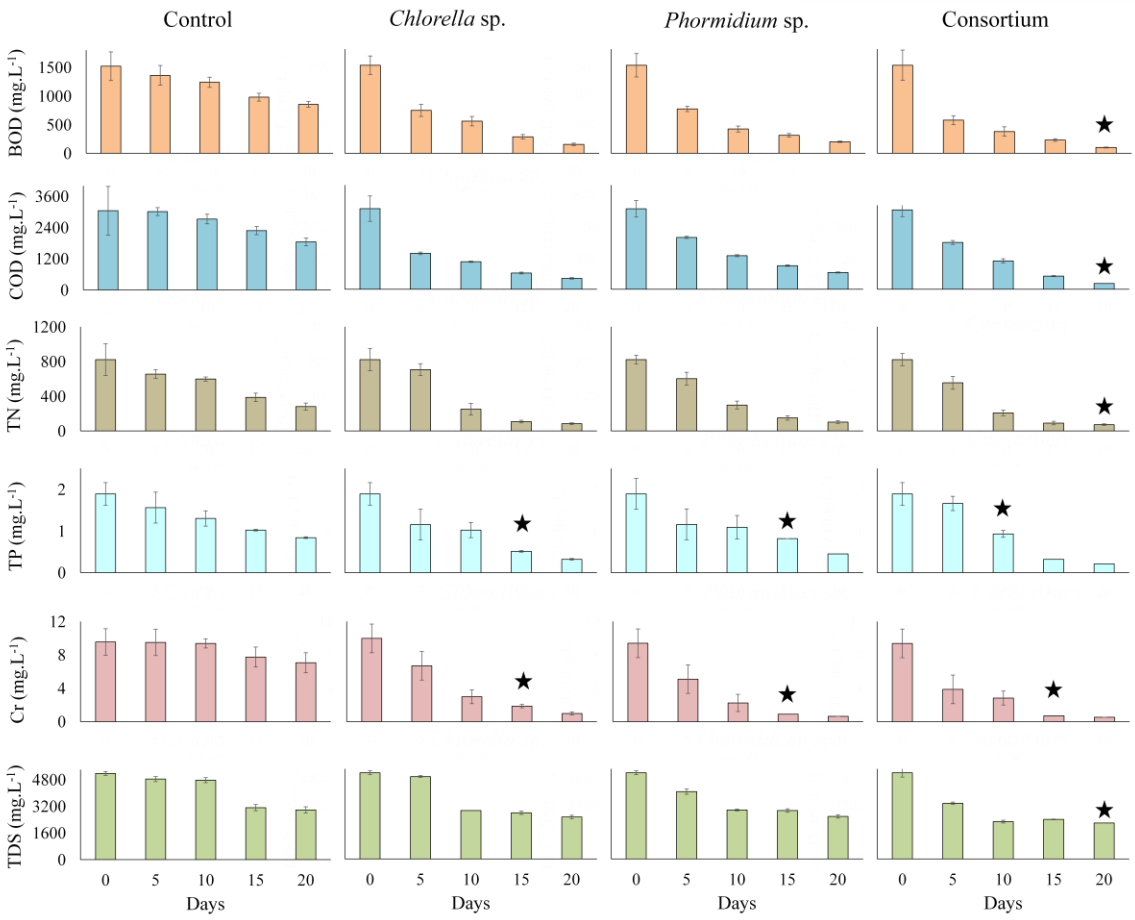


Fig. 5.2 Reduction of pollutant concentrations from TW by *Chlorella* sp., *Phormidium* sp. and their consortium. (★) indicates concentrations that fall below the BIS permissible limit for safe discharge.

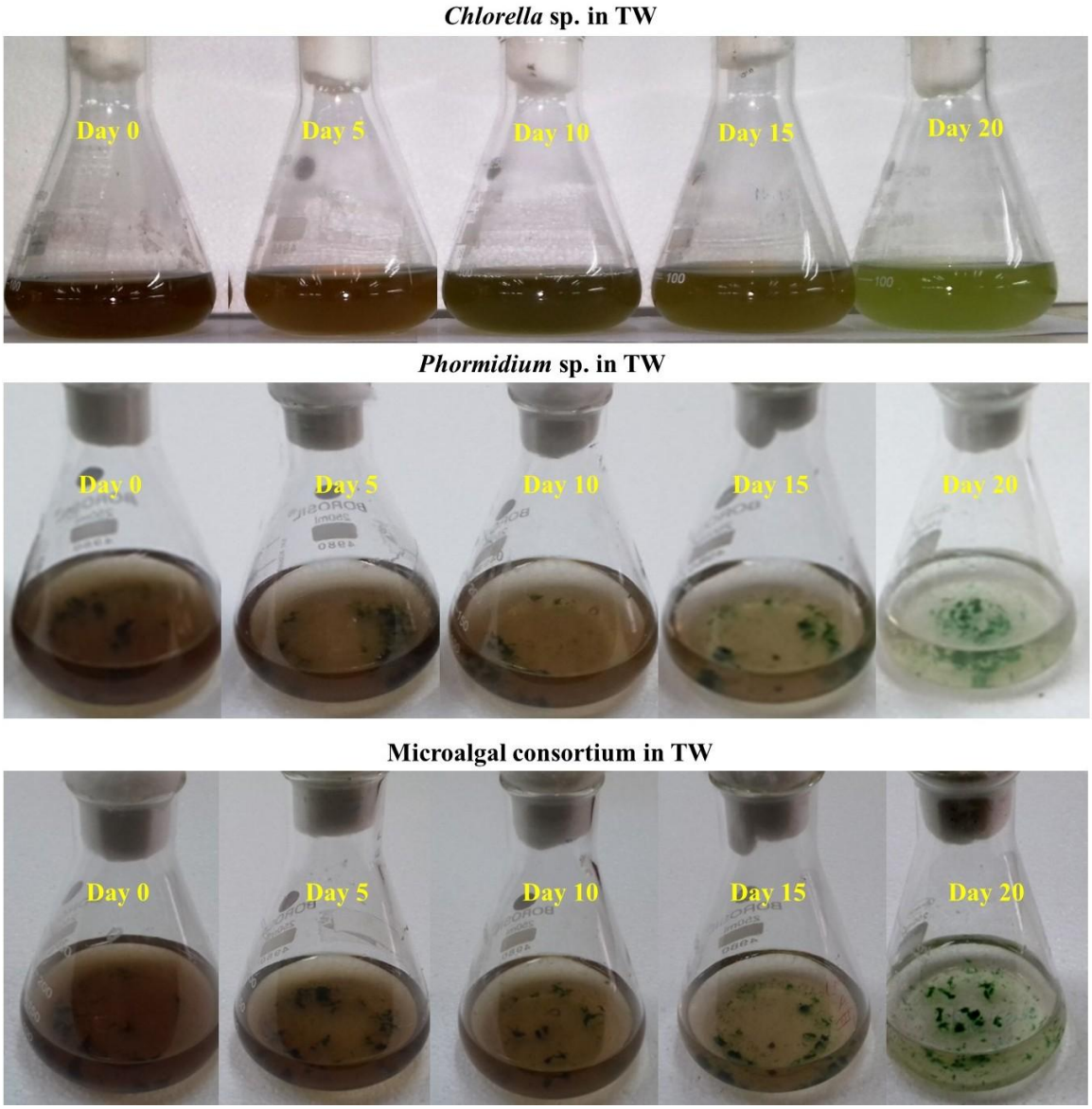


Fig. 5.3 Photograph showing growth of *Chlorella* sp., *Phormidium* sp. and their consortium in tannery wastewater (TW).

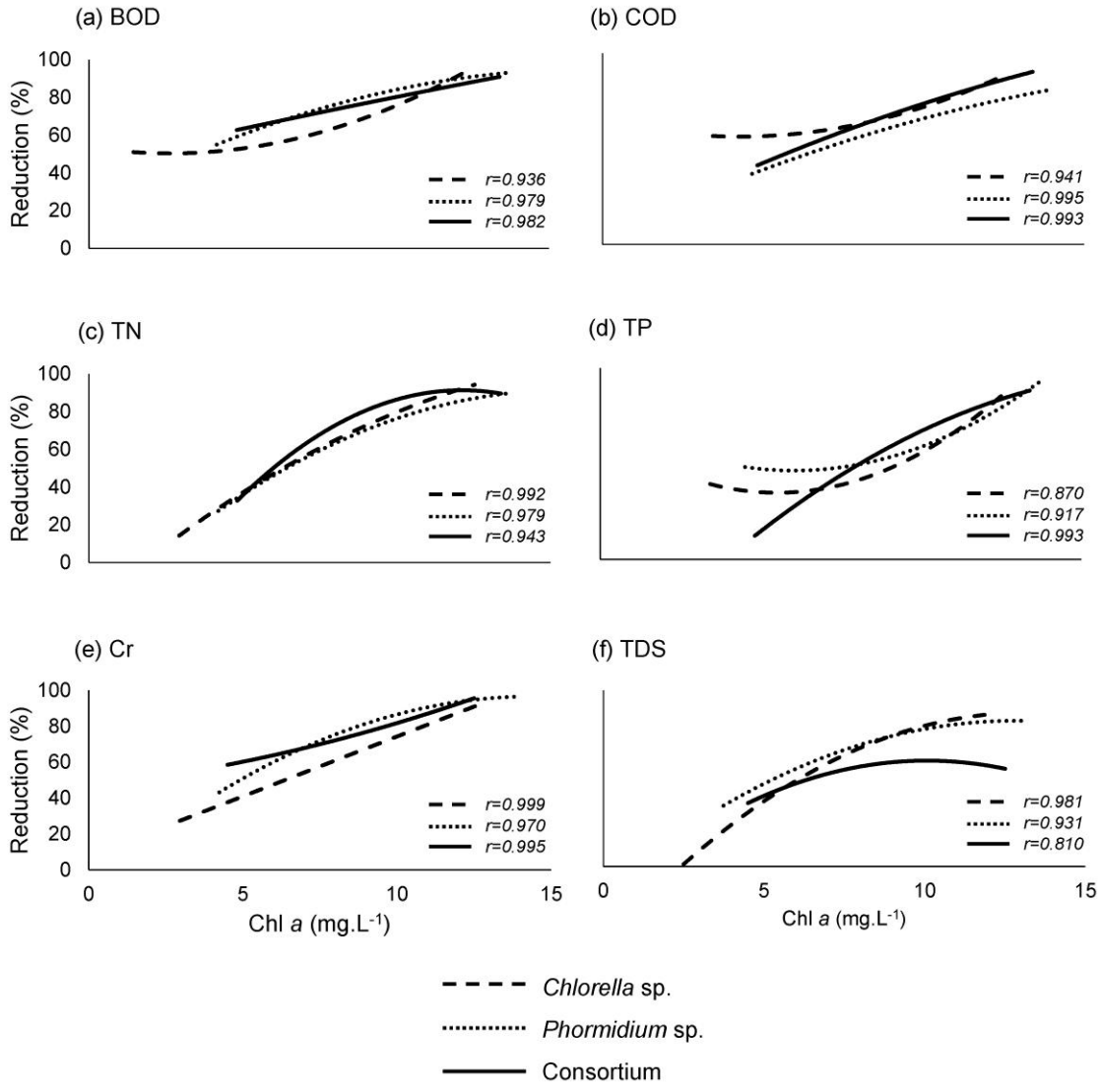


Fig. 5.4 Correlation plots of the various pollutant removal efficiencies with the Chl *a* concentrations of *Chlorella* sp., *Phormidium* sp. and their consortium.

5.4 Discussion

Studies on the use of various microalgae for bioremediation of wastewaters are many. The isolation, screening and characterization of environmental isolates for evaluating their bioremediation potential are essential to develop improved bioremediation strategies. Further, development of microalgal consortia through new searches needs to continue so as to obtain sets of microalgae that can effectively remediate hazardous wastewaters.

A general apprehension about employing certain unicellular microalgae in wastewater treatment is their microscopic dimensions, which – as indicated by Grima et al. (2003) – makes biomass harvesting cumbersome and time-consuming. In this context, filamentous microalgae with their larger dimensions (aiding harvest with filtration/flocculation) and aggregate/mat forming properties, can be a viable option, as they help to significantly reduce the harvesting cost as has been suggested by Chen et al. (2011) and Hori et al. (2002). Moreover, as demonstrated in this study, using the fast growing unicellular microalgae along with filamentous cyanobacteria in consortium appears to be a more advantageous approach for TW treatment.

Biochemical oxygen demand (BOD) and chemical oxygen demand (COD) are important parameters for assessing the water quality. The effectiveness of any treatment process is judged chiefly by the reduction of BOD and COD from the wastewater. These are measures of the amount of oxygen needed by microorganisms and/or chemical oxidants to oxidize the organics in the wastewater. High BOD and COD lead to depletion of oxygen from water causing deleterious effects on *in situ* flora and fauna. The microalgal consortium set examined in this study brought down the BOD levels of TW remarkably

to safe/acceptable discharge limits. The highest reduction of BOD and COD was by consortium treatment than by any of the single alga. The concentration of BOD and COD in all three treatments were significantly ($p<0.05$) reduced by day 20.

Growth of both algae examined in consortium strongly correlated with the reduction of BOD (r=0.982) and COD (r=0.993). These substantial reduction efficiencies of BOD (93.40%) and COD (92.60%) by day 20 are way higher than those reported by Nanda et al. (2010) for *Nostoc* sp. where BOD and COD in dilute TW (~15% strength) were reduced by 57.5% and 37.8%, respectively, in a longer treatment period of 28 days.

A major problem in wastewater treatment lies in the removal of nitrogenous and phosphorus compounds. Total nitrogen (TN) and total phosphorous (TP) are essential nutrients for growth of microalgae. Excess amounts of these in receiving waters may lead to low levels of dissolved oxygen and negatively alter various plant life and other native organisms. The approach of co-culturing *Chlorella* sp. and *Phormidium* sp. seems useful, in that, these algae in consortium reduced the TN concentration of TW to below the permissible limit of 100 mg L^{-1} by day 20. Similarly, TP concentrations were also efficiently reduced by day 15. These results are superior to those reported earlier by Silva-Benavides and Torzillo (2011) for co-culture of *Chlorella* sp. and *Plantothrix* sp. in municipal wastewater which is far more benign than the noxious full strength TW. They demonstrated reduction of TN by 20% and that of PO_4-P by 25% in 4 days. Renuka et al. (2013) carried out bioremediation of sewage wastewater using consortium of filamentous strains of microalgae and reduced the initial concentrations of NO_3-N and PO_4-P by 90% and 97% after 10^{th} and 6^{th} day of treatment. Although we did not quantify the protein

content in the biomass produced in TW, it is likely that the TN taken up from the TW is assimilated to build the protein content and for other related metabolic functions.

The tolerance limit for total Cr for discharge into inland surface waters is 2 mg L^{-1} (BIS, 1994). In order to comply with this limit, it is essential that industries treat their effluents to reduce its chromium concentration. A number of methods for the removal of metal ions from contaminated solutions have been reported including reduction, ion exchange, chemical and electrochemical precipitation, evaporation, solvent extraction, reverse osmosis and adsorption (Tiwari et al., 1989). Most of these methods suffer from drawbacks such as high capital and operational costs or the disposal of the residual and recalcitrant metal sludge. Algae, on the other hand, efficiently biotransform/detoxify heavy metal ions usually through the process of biosorption, adsorption and bioaccumulation (Gin et al., 2002; Rehman et al., 2007). As Orandi and Lewis (2013) proposed, the recovery of accumulated metals from the algal biomass can be achieved through washes with dilute acid or via adsorption/desorption cycles of alga itself. Our marine strains of *Chlorella* sp. and *Phormidium* sp. substantially reduced chromium concentrations in TW by 81.38% and 90.45%, respectively. Furthermore, algal consortium reduced 92.51% of Cr by day 15. Rehman (2011) reported 68% reduction in Cr concentrations from TW in 20 days using *Euglena proxima*. Apparently, the chromium transformation potential of *Chlorella* and *Phormidium* sp. consortium is far greater.

The TDS content of wastewater is indicative of the presence of various inorganic solutes, minerals, chlorides, metallic ions, alkalis and acids in both colloidal and dissolved forms

(Kabir et al., 2002; Rahman et al., 2012). These inorganic dissolved solids are difficult to remove/ameliorate in the natural receiving waters and their high concentration can be detrimental to living organisms. Therefore, it is important that the TDS of industrial effluent be necessarily reduced to its safe discharge levels before the effluent is let into open water bodies (Misha et al., 2004). The only feasible and economical way to remove TDS from wastewaters is by using microorganisms, which either adsorb or metabolically utilize the dissolved solids so that they become part of the microbial cells, i.e. particulate matter. Phycoremediation is a promising alternative method for TDS removal in treatment of industrial wastewaters, as it is well-known to be a sustainable, cost-effective method. However, the efficiency of TDS removal by algae mainly depends on the tolerance to the wastewater and their TDS uptake capability. Treatment of the TW with consortium of *Chlorella* and *Phormidium* sp. effectively enhanced the removal of TDS to 58.28% compared to that with individual culture of *Chlorella* sp. (51.37%) and *Phormidium* sp. (50.71%). TDS reduction strongly correlated (r=0.810) with the growth of consortium in the TW. Notably, only the consortium of the two algae could achieve TDS reductions close to the permissible limit set by BIS (1994). Furthermore, results of this study are useful to highlight that the use of marine and salt-tolerant microalgae offer advantage of treating TW replete with high amount of salts.

Notably, some reduction in concentrations of most examined parameters was observed in the control set without added microalga. The reduction in this case might be due to activity of indigenous microorganisms of the wastewater. Zahoor and Rehman (2009) observed about 52-62% reduction of Cr(VI) in control set due to native microflora of wastewater. However, treating the wastewaters with added microalgae is more practical

and would enhance/ hasten the remediation process (Perpetuo et al., 2011). As seen in our study, the concentrations of hazardous parameters in particular, reduced rather more quickly in the sets with added microalgae, individually or as consortium.

Post-hoc Tukey's HSD test is useful to elucidate the number of days required to achieve significant reduction of the hazardous parameters. Greater percent reduction of most pollutants occurred within 5-10 days. It is apparent that maximum utilization/transformation of toxicants occurs during the logarithmic growth phase. Although significant reductions of the parameters are achieved within 5-10 day period, our analysis clearly shows that reduction of all hazardous parameters to safe limits occurs by day 20. From our first attempts of employing marine microalgal strains for TW bioremediation, it is clear that there is a greater ecological advantage. In that, the hazard levels of TW are brought down drastically within 5-10 days. Additional trials of employing more marine microalgae and/or suitable modifications in the treatment process might prove not only far eco-friendlier but also economical and in the long run prove very sustainable.

Experimental results describing the use of two or more microalgal species to treat TW are scarce. In this regard, results from this study strongly suggest that a pragmatic approach would be to adopt the consortium based phycoremediation strategies to detoxify and/or bring down the pollutant loads from such hazardous effluents as TW. The growth response of different types of microalgae in wastewater also vary, because they differ in their inherent abilities, like nutrient uptake; tolerance to harsh/extreme environmental conditions and competition with indigenous organisms. Further, the difficulties that arise

with the use of monostrains of microalgae, such as growth in diverse environments and harvesting problems, highlight that the consortial approach may be a more effective alternative for wastewater treatment. Such consortia show synergistic interactions and have wider potential to treat different types of wastewater, than monostrains.

Chapter 6

Biochemical and physiological characterization of tannery detoxifying bacteria and microalgae

6.1 Introduction

Microbes mutate. This mutation is to adapt to changing milieu and to physico-chemical adversities. Physiological, biochemical, molecular and structural changes are integral to the life-forms in order for their continued existence. Modifications of (1) physiological (osmotic, metabolic, cytosolic, respiratory), (2) biochemical (enzymatic, proteinaceous, lipid and energy-related), (3) molecular (ionic, nucleic acid, ribosomal, cell surface, electrochemical) functions are essential for most species to resiliently adapt to their surrounding milieu.

Metallothioneins (MTs) are low molecular mass (6–8000 daltons) and cysteine-rich (20–30%), metal-binding proteins. Their synthesis, as illustrated by Kägi and Schäffer (1988), represents a specific response of the organisms to pollution by heavy metals such as chromium, zinc, cadmium, and mercury. MTs bind toxic metals, providing cell protection against metal toxicity (Isart and Vasak, 2002). As a general model, MT synthesis is induced in conditions of elevated metal concentration, providing more binding sites for metal ions and limiting latent damage (Nordberg, 1998). The main functions of MTs are related to metal metabolism (detoxification and storage of heavy metals) and to the regulation of cellular copper and zinc metabolism in response to physiological and environmental changes (Linde et al., 2001; Langton et al., 2002). Regulation of MT biosynthesis by metals is considered a biological mechanism used to maintain homeostatic concentrations of essential and non-essential free metal ions by chelation. Thus, an increase of MT levels in a cell can be interpreted as a previous exposure to heavy metals.

As noted by several studies (Lobban and Harrison, 1994, Oliveira and Plastino, 1994), nitrogen is the most important abiotic factor which is proven to limit the algal growth in marine environment and its main available source is in the form of nitrate (NO_3^-) (Chapman and Harrison, 1988). The assimilation of nitrate involves its cytoplasmic reduction to nitrite (NO_2^-), catalyzed by the enzyme nitrate reductase (NR) using NAD(P)H as electron donor. In the chloroplasts, nitrite is quickly reduced to ammonium (NH_4^+) by the enzyme nitrite reductase (NiR) that uses ferredoxine as electron donor (Lea and Leegood, 1995), and ammonium is then incorporated to nitrogen molecules as amino acids, purines, pirimidines and amines.

Phosphorus is another frequently limiting nutrient for phytoplankton growth in aquatic ecosystems (Schindler, 1977; Hecky and Kilham, 1988; Hudson et al., 2000), and its availability is an important factor to control phytoplankton productivity and species composition (Cotner and Wetzel, 1992). Eutrophication is one of the most widespread environmental problems of inland waters. Concomitant with the process of eutrophication, the proportion of dissolved organic phosphorus in the total phosphorus pool has been dramatically increased (Hudson et al., 2000). Therefore, when external inorganic phosphorus concentration is very low in the water column during summer algal blooms, the extent to which algae could benefit from organic phosphorus compounds may be one of key factors to determine the algal interspecific competition. Some algae are capable of obtaining phosphate from dissolved organic phosphorus (DOP) in the absence of dissolved inorganic phosphorus (DIP) to sustain their growth (Whitton et al., 1991; Oh et al., 2002). The utilization of DOP is associated with a number of enzymes, among which alkaline phosphatase was regarded as the most important one in

hydrolyzing a variety of forms of DOP sources (Shan et al., 1994). Alkaline phosphatase activity (APA) is often a common marker of phosphate stress in many phytoplankton (Dyhrman and Palenik, 1999), and the phosphorus status of phytoplankton communities can be evaluated by measurement of total APA in enzyme assays.

As noted in chapters 4 and 5, many native bacterial and microalgal strains were found to be efficient detoxifiers of tannery wastewater even at its full strength. From the laboratory experiments, it was confirmed that these microbes have great potential to bioremediate the TW. In this regard, analysis comprising biochemical and physiological parameters would help demonstrate the possible ways the hazardous wastes are dealt with by the microbial strains. In addition to examining growth (detailed in chapters 4 and 5), it was hypothesized that quantifying the rate and/or amounts of metallothionein (MT), alkaline phosphatase (AP) and nitrate reductase (NR) will provide information on the possible role of these factors in bioremediation. Accordingly, different experiments were planned and conducted.

6.2 Materials and methods

Based on results of studies detailed in chapters 4 and 5, the highly potent bacterial strains *B. cereus*, *Aeromicrobium* sp. and the microalgal strains *C. vulgaris*, *Phormidium* sp. were used for the experiments described below. These strains were examined individually and in consortium to study the effect of pH and salinity on their biochemical and physiological characteristics.

6.2.1 Experimental setup

To understand the effect of pH and salinity on the physiological characteristics of bacterial and microalgal isolates, the pH of the tannery wastewater (TW) was adjusted to 5, 7 and 10 separately. Aliquots of 100 mL aliquots were dispensed in triplicate sets into 250 mL flasks. Similarly, the TW (with an initial salinity of 15 psu) was diluted with deinonized water to salinity of 5 psu. This solution was adjusted to 15 and 30 psu separately by addition of NaCl. Aliquots of 100 mL of the 5, 15 and 30 psu TW were dispensed into triplicate sets in 250 mL flasks. The exponential cultures of the bacterial and microalgal strains were inoculated into the flasks individually and in consortium. The inocula of the bacterial and microalgal cultures were prepared as described in chapters 4 and 5 respectively.

Individual bacterial cultures were inoculated at 1% (v/v) into the above described TW sets and were grown for a period of 72 h. Similarly, individual microalgal cultures were inoculated at 2% (v/v) and were grown in the TW for a period of 15 days. For the consortium set, both bacterial cultures and algal cultures were inoculated into the TW media to build the consortium which was allowed to grow for 15 days.

For bacterial sets, the samples were harvested during the log phase (12h), late log phase (36h) and stationary phase (60h). Similarly, for the microalgal and consortium sets, the samples were harvested during the log phase (day 5), late log phase (day 10) and stationary phase (day 15). The bacterial and microalgal cells were harvested by centrifuging the TW media cultures at 10000 rpm for 10 min and the ensuing cell pellets were washed with physiological saline/phosphate buffer. The cells were examined for

their nitrate reductase (NR), alkaline phosphatase (AP) and metallothioneins (MT) contents as described below. The supernatants of TW were analyzed for their residual biochemical oxygen demand (BOD), chemical oxygen demand (COD), chromium (Cr) nitrates (NO_3-N), phosphates (PO_4-P). Measurements of BOD, COD, Cr(VI), NO_3-N and PO_4-P were made following standard methods described in APHA (2005) as detailed in previous chapters.

6.2.2 Metallothionein assay

The method of Linde and Garcia-Vazquez (2006) was slightly modified for metallothionein estimation from the cell preparations of bacteria and microalgae. For this, 0.1 g of bacterial or microalgal cells were added to 10 mL of the homogenization buffer (0.5 M sucrose, 20 mM Tris-HCl buffer, pH 8.6, containing 0.01% β-mercaptoethanol) in 15 mL glass or plastic tubes. The suspension was homogenized by employing a tissue homogenizer. The homogenate was distributes into aliquots (3 mL).

Homogenates were centrifuged at 30,000x*g* for 20 min to obtain a supernatant containing metallothionein. To this supernatant, 1.05 mL of ice cold absolute ethanol and 80 µL of chloroform were added per 1 mL of the resulting supernatant. This preparation was then centrifuged (at 4ºC) at 6000x*g* for 10 min. Three volumes of cold ethanol were added to the resulting supernatant and stored at –20°C for 1 h. This was again centrifuged at 6000x*g* for 10 min. The resulting pellets were washed with ethanol:chloroform:homogenization buffer (87:1:12) and then centrifuged again at 6000x*g* for 10 min and later dried under vacuum to complete evaporation. The dried pellet was resuspended in 300 µL of 5 mM Tris-HCl, 1 mM EDTA, pH 7. The

resuspended metallothionein fraction was added to 4.2 mL of 0.43 mM 5,5´-dithiobis (nitrobenzoic acid) in 0.2 M phosphate buffer, pH 8 and allowed to stand for 30 min at room temperature. Then the concentration of reduced sulfhydryl was evaluated by reading the absorbance at 412 nm in a spectrophotometer (Shimadzu, Japan).

A standard curve with different concentrations of GSH was prepared as a standard reference for a correct quantification of MT in the samples. GSH contains one cysteine per molecule; thus, it is a standard for quantifying cysteines in protein analyses. The amount of metallothionein in the samples was estimated using the GSH standard, assuming that 1 mol of MT contains 20 mol of cysteine.

6.2.3 Alkaline phosphatase activity assay

Alkaline phosphatase was assayed colorimetrically in three replicate samples following Reichardt et al. (1967). Their procedure uses p-nitrophenyl phosphate (pNPP) as substrate, as modified by Hernández et al. (1992). Pellets of 0.1 g of bacterial or microalgal cells were incubated in 10 mL of a reaction mixture consisting of 700 µM pNPP (Sigma, USA), 50mM Tris-HC1 buffer, pH 8.3. The initial substrate concentration was kept sufficiently high to ensure that no more than 10% was hydrolyzed during assay. Continuously air bubbling was done during the assay to oxygenate and supply CO_2. After 45 min of incubation at 25°C, the absorbance was read at 410nm against a blank (buffer and substrate solution without cells) in a spectrophotometer. The enzyme activity was reported as µmol paranitrophenol (pNP) released g wt^{-1} h^{-1}, formed by the hydrolysis of pNPP to phosphorus (P) and pNP.

6.2.4 Nitrate reductase assay

The nitrate reductase assay was performed according to the method of Chapman and Harrison (1988). The samples were homogenized in standard extraction buffer (0.2 M phosphate buffer, pH 8.0; 5 mM EDTA; 1 mM DTT and 0.3% w/v BSA) at a concentration of 0.1 g wet weight per 1 mL of buffer. Cell debris was removed by centrifugation at 12,000 *g* for 15 minutes at 4°C. The supernatant (crude extract) was recovered and kept on ice until the NR activity was assayed for an hour. Nitrate reductase activity was determined by pre-incubating the crude extracts in the reaction mixture (0.2 M phosphate buffer, pH 8.0; 6 mM KNO_3 and 0.5 mM $MgSO_4$) at 20°C for 10 minutes. The mixture was then incubated for another 5 minutes after addition of 0.02 mM NADH. Controls without NADH were prepared for each experiment. The enzymatic reaction was interrupted by adding 1.4 mM $ZnSO_4$ and 43% v/v ethanol. Precipitates were removed by centrifugation at 12,000 g for 10 minutes at 20°C. Nitrite concentrations in the supernatants were determined by measuring absorption at 543 nm after the addition of 1 mL 9.6 mM sulphanilamide and 1 mL 0.7 mM *n*-(1-naphtyl)ethylenediamine dihydrochloride. One unit of NR (U) is the enzymatic activity producing 1 μmol of nitrite per minute at 20°C.

6.2.5 Statistical analysis

Principal component analysis (PCA) was carried out using Primer 6 (PRIMER-E, Plymouth, UK) to check if there are significant differences in the activities of individual strains in terms of reducing the BOD, COD, Cr, NO_3-N, PO_4-P and in their activities of NR, AP and MT production in relation to changes to pH and salinity of the TW.

6.3 Results

6.3.1 Effect of pH on production of MT and activities of NR and AP

Bacteria

The highest reduction of BOD (52.8%) by *B. cereus* was at pH 10. The isolate optimally reduced COD, NO_3-N and PO_4-P concentrations by 68.26%, 74.21% and 74.48%, respectively at pH 7. The NR (3.20 U g^{-1}) and AP (0.78 µmol pNP g^{-1} h^{-1}) concentrations were also observed to be highest at pH 7 during the stationary phase (Table 6.1). The highest Cr(VI) reduction was also at pH 10, with correspondingly high concentrations of MT. As for *Aeromicrobium* sp., the highest reductions of BOD (68.53%), COD (81.33%) and Cr(VI) (79.79%) occurred at pH 10, with increased MT levels at pH 10 and 30 psu during log phase. The optimum pH and salinity for reduction of nutrients was pH 7 and 15 psu (Tables 6.1 and 6.2). Higher levels of NR and AP were measured at pH 7 and 15 psu.

Microalgae

The highest reduction of BOD (67.97%) and COD (82.72%) for TW treated with *C. vulgaris* was at pH 10 (Table 6.1). *C. vulgaris* was tolerant to wider range of pH values. The MT concentration was the highest (4.32 µmol GSH) at pH 5 along with maximum removal of Cr(VI) at pH 5 (Fig. 6.3a). In *Phormidium* sp., maximum reduction of BOD, COD, NO_3-N and PO_4-P occurred at pH 7 and at 15 psu. Highest reduction/transformation efficiencies of Cr(VI) were measured at pH 5 (93.46%) and at 5 psu (91.02%) (Table 6.1 & 6.2). Maximum production of MT (4.2 µmol GSH) was seen

at pH 5 during the late log phase. Maximum production of NR and AP was influenced by pH 7 and 10 (Fig. 6.4a).

Higher reduction efficiencies were achieved with the bacterial-microalgal consortium compared to individual cultures (Table 6.1 & 6.2). In fact, the combination of pH 7 and 15 psu was optimum for maximal reduction of BOD, COD, NO_3-N and PO_4-P. However, the Cr(VI) reduction (94.68%) was the highest at pH 10 and 30 psu.

6.3.2 Effect of salinity on production of MT and activities of NR and AP

Overall, the isolates *B. cereus* and *C. vulgaris* were tolerant to wider range of salinities. *B. cereus* was tolerant to wide range of salinities (Fig. 6.1b) and higher pollutant reduction efficiencies were observed at 5 and 15 psu. No distinct influence of salinities was observed on production of MT, NR and AP. In the overall, reduction of all parameters by *B. cereus* was the highest at 15 psu (Table 6.2). In comparison, the *Aeromicrobium* sp brought down the highest reductions of the tested parameters at 30 psu during log phase. Maximum reductions of pollutants by *Aeromicrobium* sp. were observed at 30 psu. Increased synthesis of MT, NR and AP were observed either at 15 and/or at 30 psu (Fig. 6.2b). In general, the optimum pH and salinity for reduction of nutrients were pH 7 and 15 psu (Tables 6.1 and 6.2). Maximum concentrations of metallothioneins (MTs) and chromium reduction efficiencies in the bacterial isolates were in the alkaline pH ranges.

Among microalgae, high reduction efficiencies were observed in sets grown with *C. vulgaris* across all three salinities. Concentrations of MT (4.7 µmol GSH) were higher at

30 psu (Fig. 6.3b). In the case of *Phormidium*, the activities of NR and AP were maximum at 15 and 30 psu. Synthesis of MT increased (2.45 µmol GSH) at 5 psu during the late log phase (Fig. 6.4b)

In the consortium, the highest concentrations of MT were observed at alkaline pH. Maximum concentrations of nitrate reductase and alkaline phosphatase were observed at pH 7 and 10. Increased concentrations of nitrate reductase and alkaline phosphatase were observed at 15 and 30 psu. Thus, the consortium consisting of both bacteria and microalgae was more efficient in reducing the TW pollutants and was more resilient to wide range of pH and salinities.

6.3.4 Principal Component Analysis

The PCA plot revealed that chromium removal efficiency in *B. cereus* was highest at alkaline pH 10 with overall high pollutant reductions at pH 7 and 10. Significantly higher concentrations of NR and AP activity were observed during late log phase (Fig. 6.1a). The PCA plot affirmed that *Aeromicrobium* sp. was tolerant to a wider range of pH than *B. cereus*. The highest concentrations of MT were in the set grown in pH 10. Maximum removal of Cr was observed at pH 10 (Fig. 6.2a). In the case of *Phormidium*, pollutant reduction efficiencies significantly varied at 5 and 15 psu while synthesis of NR and AP was maximum at 15 and 30 psu (Fig. 6.4b). Synthesis of MT increased at 5 psu during the late log phase as was clear from the PCA. The PCA plots of the consortium sets explain increased synthesis of NR and AP at pH 5 and 7 during the late log phase. Increased synthesis of MT was at pH 10 (Fig. 6.5a) and increased synthesis of MT, NR and AP were observed in all 3 salinities during late log phase (Fig. 6.5b). It is to be noted

that the highest concentrations of MTs and chromium reduction efficiencies were observed in acidic pH range in the TW experimental sets with microalgal isolates.

Table 6.1 Effect of different pH on various parameters in tannery wastewater treated with bacteria, microalgae and their consortium

	pH 5			pH 7			pH 10		
	Log	LLog	Sphase	Log	LLog	Sphase	Log	LLog	Sphase
Bacillus cereus									
Metallothionein	2.10	2.30	2.42	3.10	3.80	3.40	3.20	4.50	4.78
Nitrate reductase	1.80	2.45	2.50	1.98	2.49	3.20	2.05	2.33	2.98
Alkaline phosphatase	0.16	0.31	0.45	0.32	0.55	0.78	0.36	0.52	0.66
BOD	863.86	740.00	595.00	863.86	640.00	495.00	863.00	695.00	420.00
COD	2879.53	1850.00	1081.82	2879.53	1600.00	951.92	2696.88	1523.81	990.00
NO_3-N	1.53	1.12	0.98	1.53	0.90	0.41	1.53	0.85	0.56
PO_4-P	2.70	2.20	1.22	2.70	1.98	0.74	2.70	1.75	0.91
Cr	9.50	7.51	5.20	9.50	6.20	2.70	9.50	4.32	1.58
***Aeromicrobium* sp.**									
Metallothionein	2.10	2.30	2.20	2.10	3.80	2.90	2.20	4.10	3.90
Nitrate reductase	1.20	1.45	2.10	1.48	2.89	3.57	2.05	2.20	2.48
Alkaline phosphatase	0.26	0.41	0.45	0.32	0.55	0.78	0.36	0.52	0.66
BOD	812.20	780.00	680.00	812.20	680.00	480.00	812.00	580.00	280.00
COD	2707.33	1950.00	1236.36	2707.33	1700.00	923.08	2537.50	1380.95	560.00
NO_3-N	1.53	1.32	1.21	1.53	1.23	0.56	1.53	1.11	0.78
PO_4-P	2.70	2.26	1.78	2.70	1.64	0.97	2.70	1.97	1.45
Cr	9.50	6.56	5.25	9.50	5.35	2.89	9.50	4.32	1.98
Chlorella vulgaris									
Metallothionein	3.10	4.32	3.89	3.10	3.80	2.90	1.98	2.10	2.20
Nitrate reductase	1.20	1.45	1.60	1.48	2.46	3.57	2.05	1.89	2.48
Alkaline phosphatase	0.26	0.31	0.35	0.32	0.42	0.68	0.36	0.52	0.56
BOD	840.00	695.00	285.00	840.00	595.00	305.00	840.00	695.00	385.00
COD	2800.00	1737.50	518.18	2800.00	1487.50	586.54	2625.00	1654.76	770.00

(Continued)

NO_3-N	2.70	2.26	1.54	2.70	1.64	0.87	2.70	1.97	1.65
PO_4-P	9.98	2.99	0.68	9.50	5.35	1.89	9.98	4.98	3.98
Cr	3.20	4.09	4.20	3.10	3.10	2.90	2.10	2.50	2.20
***Phormidium* sp.**									
Metallothionein	1.20	1.45	2.10	1.48	2.89	2.57	1.97	2.20	2.48
Nitrate reductase	0.26	0.41	0.35	0.32	0.45	0.58	0.36	0.52	0.46
Alkaline phosphatase	817.20	700.00	580.00	817.20	500.00	380.00	817.20	650.00	380.00
BOD	2724.00	1750.00	1054.55	2724.00	1250.00	730.77	2553.75	1547.62	760.00
COD	1.53	1.42	1.40	1.53	1.38	0.88	1.53	1.11	0.98
NO_3-N	2.70	2.26	1.99	2.70	1.64	1.38	2.70	1.97	1.45
PO_4-P	9.39	2.24	0.64	9.50	5.35	2.79	9.39	2.04	1.64
Cr									
Consortium	2.10	2.98	3.20	3.10	3.80	2.90	3.20	4.50	4.20
Metallothionein	1.20	1.45	2.10	1.48	2.89	3.57	2.05	2.20	2.48
Nitrate reductase	0.26	0.41	0.45	0.32	0.55	0.78	0.36	0.52	0.66
Alkaline phosphatase	882.40	450.00	185.00	782.40	371.00	120.00	782.40	671.00	385.00
BOD	2941.33	1125.00	636.36	2608.00	927.50	210.00	2445.00	1597.62	770.00
COD	1.53	1.32	1.05	1.53	1.13	0.36	1.53	1.11	0.67
NO_3-N	2.70	2.26	1.56	2.70	1.64	0.77	2.70	1.97	1.45
PO_4-P	9.50	2.24	0.86	9.50	5.35	1.79	9.37	2.82	0.52
Cr	2.10	2.30	2.42	3.10	3.80	3.40	3.20	4.50	4.78

Log=logarithmic phase; LLog=late logarithmic phase; Sphase=stationary phase; Units of metallothionein= µmol GSH; nitrate reductase=NR U g^{-1}; Alkaline phosphatase= µmol pNP g^{-1} h^{-1}; Units of BOD, COD, NO_3-N, PO_4-P, Cr (VI) are mg L^{-1}

Table 6.2 Effect of different salinities on various parameters in tannery wastewater treated with bacteria, microalgae and their consortium

	5 PSU			**15 PSU**			**30 PSU**		
	Log	**Llog**	**Sphase**	**Log**	**Llog**	**Sphase**	**Log**	**Llog**	**Sphase**
Bacillus cereus									
Metallothionein	3.10	3.50	3.20	3.10	3.80	4.20	3.20	3.10	2.80
Nitrate reductase	1.80	2.45	2.50	1.98	2.49	3.20	2.05	2.20	2.06
Alkaline phosphatase	0.26	0.41	0.45	0.32	0.55	0.78	0.36	0.52	0.66
BOD	863.86	740.00	415.00	863.86	640.00	312.00	863.00	695.00	650.00
COD	2879.53	1850.00	1081.82	2879.53	1600.00	951.92	2696.88	2265.00	1970.00
NO_3-N	1.53	1.12	0.48	1.53	0.90	0.41	1.53	1.35	1.05
PO_4-P	2.70	2.20	0.84	2.70	1.98	0.74	2.70	2.40	1.98
Cr	9.50	7.51	2.20	9.50	6.20	1.70	9.50	7.56	5.23
***Aeromicrobium* sp.**									
Metallothionein	2.10	2.30	2.20	2.10	3.80	2.90	2.20	4.10	3.20
Nitrate reductase	1.20	1.45	2.10	1.48	2.89	3.57	2.05	2.20	2.48
Alkaline phosphatase	0.26	0.41	0.45	0.32	0.55	0.78	0.36	0.52	0.66
BOD	812.20	780.00	680.00	812.20	680.00	480.00	812.00	580.00	280.00
COD	2707.33	1950.00	1236.36	2707.33	1700.00	923.08	2537.50	1380.95	560.00
NO_3-N	1.53	1.32	1.21	1.53	1.23	0.56	1.53	1.11	0.78
PO_4-P	2.70	2.26	1.78	2.70	1.64	0.97	2.70	1.97	1.45
Cr	9.50	6.56	4.25	9.50	5.35	2.79	9.50	4.32	1.98
Chlorella vulgaris									
Metallothionein	2.10	2.50	3.20	3.10	3.80	3.90	3.20	4.50	4.70
Nitrate reductase	1.20	1.45	2.10	1.48	2.89	3.17	1.87	2.20	2.18
Alkaline phosphatase	0.26	0.41	0.45	0.32	0.55	0.58	0.36	0.52	0.66
BOD	840.00	556.00	185.00	840.00	633.00	205.00	840.00	590.00	255.00
COD	2800.00	1737.50	318.00	2800.00	1487.50	286.00	2625.00	1654.76	470.00
NO_3-N	1.53	1.32	0.55	1.53	1.11	0.36	1.53	1.25	0.38

(Continued)

PO_4-P	9.98	3.90	0.98	9.50	5.22	1.79	9.98	2.99	0.98
Cr	3.20	4.50	4.20	3.10	3.80	2.90	2.10	3.10	2.90
***Phormidium* sp.**									
Metallothionein	1.20	1.45	2.45	1.48	1.89	3.27	2.05	2.20	2.48
Nitrate reductase	0.26	0.41	0.45	0.32	0.55	0.78	0.36	0.52	0.66
Alkaline phosphatase	817.20	700.00	580.00	817.20	500.00	380.00	817.20	650.00	620.00
BOD	2724.00	1750.00	1054.55	2724.00	1250.00	730.77	2553.75	2156.00	1985.00
COD	1.53	0.97	0.65	1.53	0.78	0.41	1.53	1.35	1.08
NO_3-N	2.70	2.26	1.78	2.70	1.64	0.97	2.70	1.97	1.80
PO_4-P	9.39	3.25	0.88	9.50	4.56	2.19	9.39	7.25	4.64
Cr									
Consortium	2.10	2.50	2.20	3.10	3.80	2.90	3.20	4.50	4.20
Metallothionein	2.20	2.45	3.10	1.48	2.89	3.57	2.05	2.20	3.48
Nitrate reductase	0.26	0.41	0.45	0.32	0.55	0.78	0.36	0.52	0.66
Alkaline phosphatase	882.40	450.00	185.00	782.40	371.00	120.00	782.40	671.00	385.00
BOD	2941.33	1125.00	636.36	2608.00	927.50	210.00	2445.00	1597.62	770.00
COD	1.53	1.32	0.78	1.53	1.23	0.56	1.53	1.11	0.68
NO_3-N	2.70	2.26	0.78	2.70	1.64	0.67	2.70	1.97	0.65
PO_4-P	9.50	2.24	0.86	9.50	5.35	2.79	9.37	2.82	0.52
Cr	3.10	3.50	3.20	3.10	3.80	4.20	3.20	3.10	2.80

Log=logarithmic phase; LLog=late logarithmic phase; Sphase=stationary phase; Units of metallothionein= µmol GSH; nitrate reductase=NR U g^{-1}; Alkaline phosphatase= µmol pNP g^{-1} h^{-1}; Units of BOD, COD, NO_3-N, PO_4-P, Cr (VI) are in mg L^{-1}

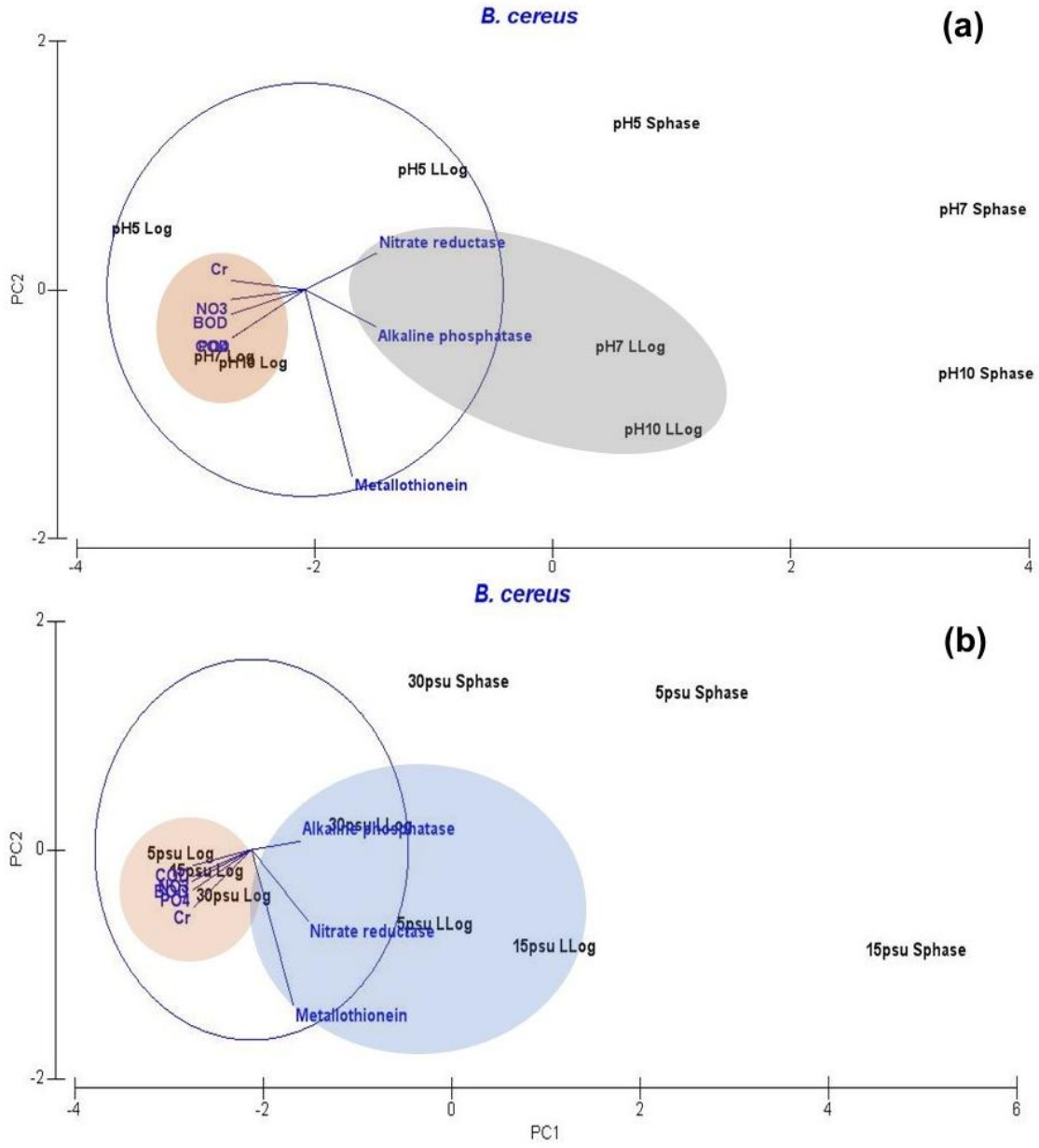


Fig. 6.1 Effect of (a) pH and (b) salinity on the pollutant reduction efficiencies and MT, NR and AP concentrations of *Bacillus cereus*

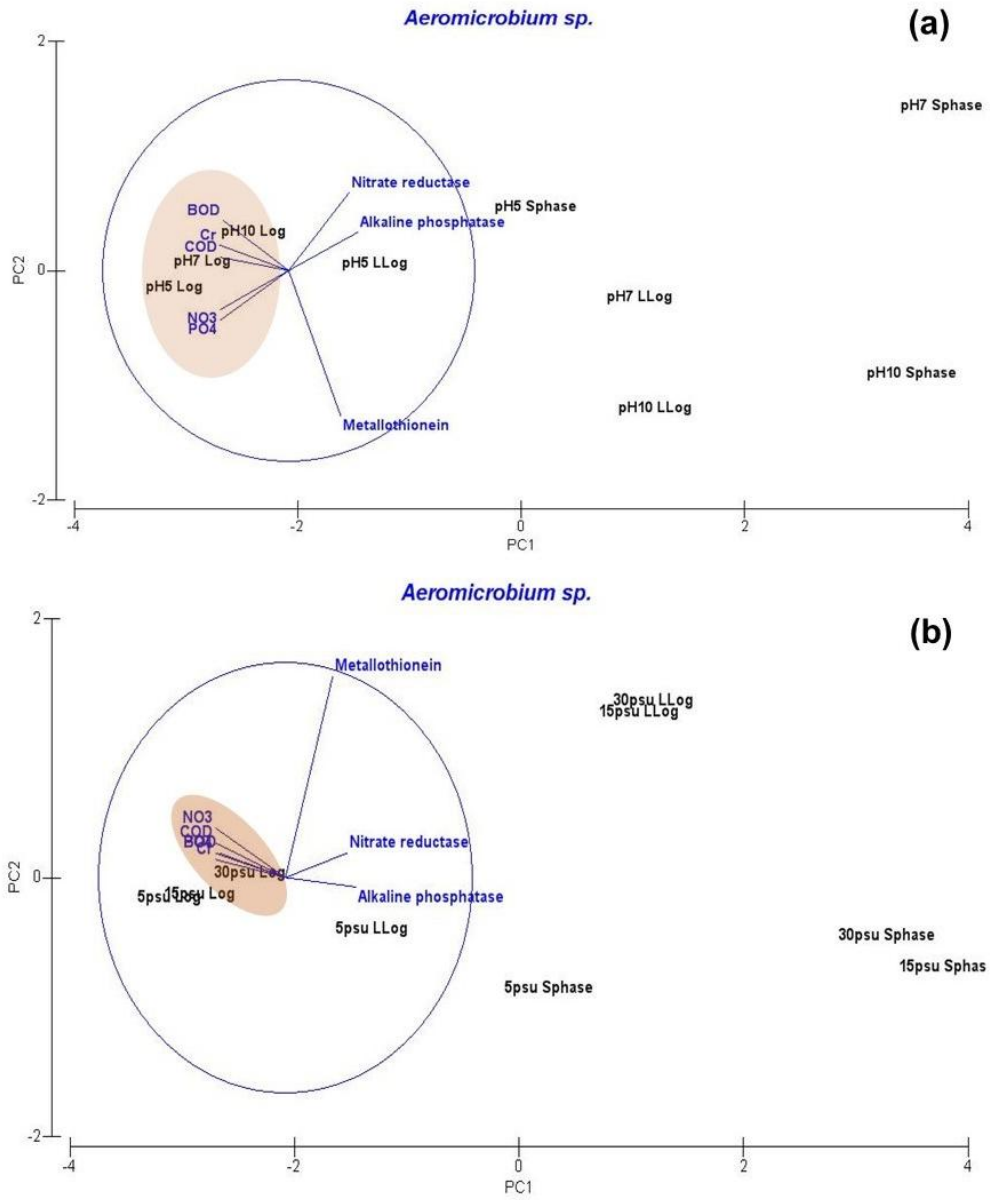


Fig. 6.2 Effect of (a) pH and (b) salinity on the pollutant reduction efficiencies and MT, NR and AP concentrations of *Aeromicrobium* sp.

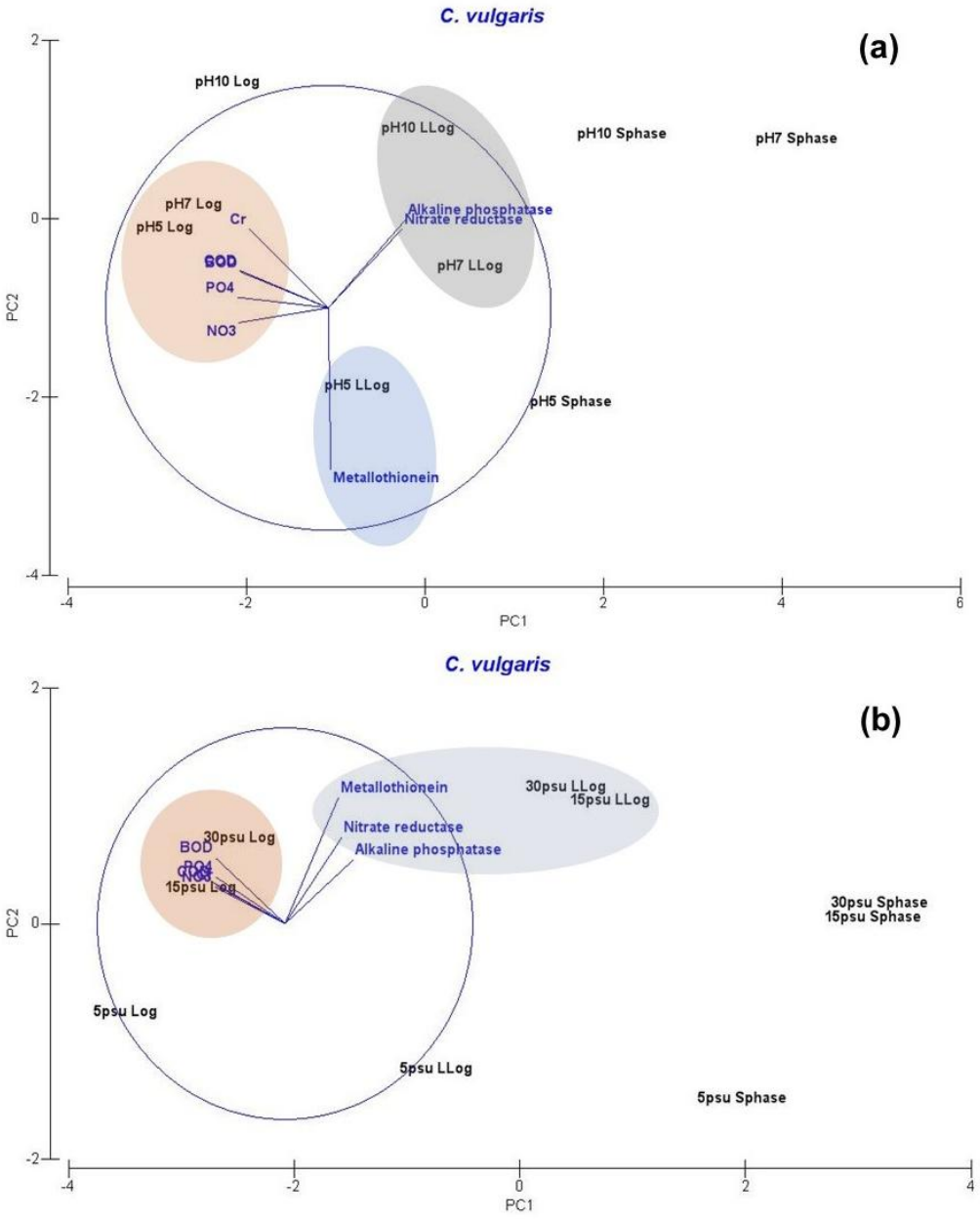


Fig. 6.3 Effect of (a) pH and (b) salinity on the pollutant reduction efficiencies and MT, NR and AP concentrations of *Chlorella vulgaris*

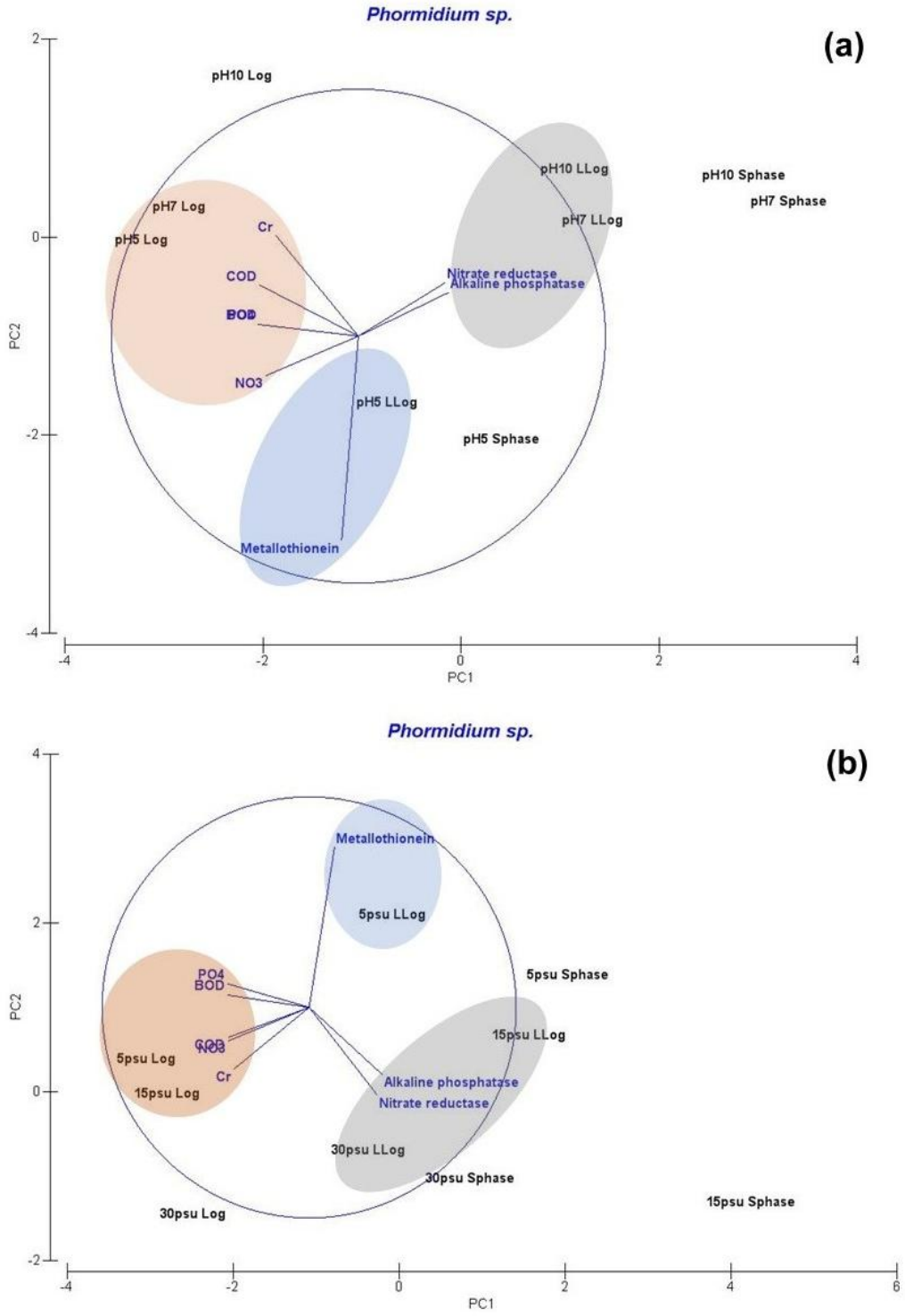


Fig. 6.4 Effect of (a) pH and (b) salinity on the pollutant reduction efficiencies and MT, NR and AP concentrations of *Phormidium* sp.

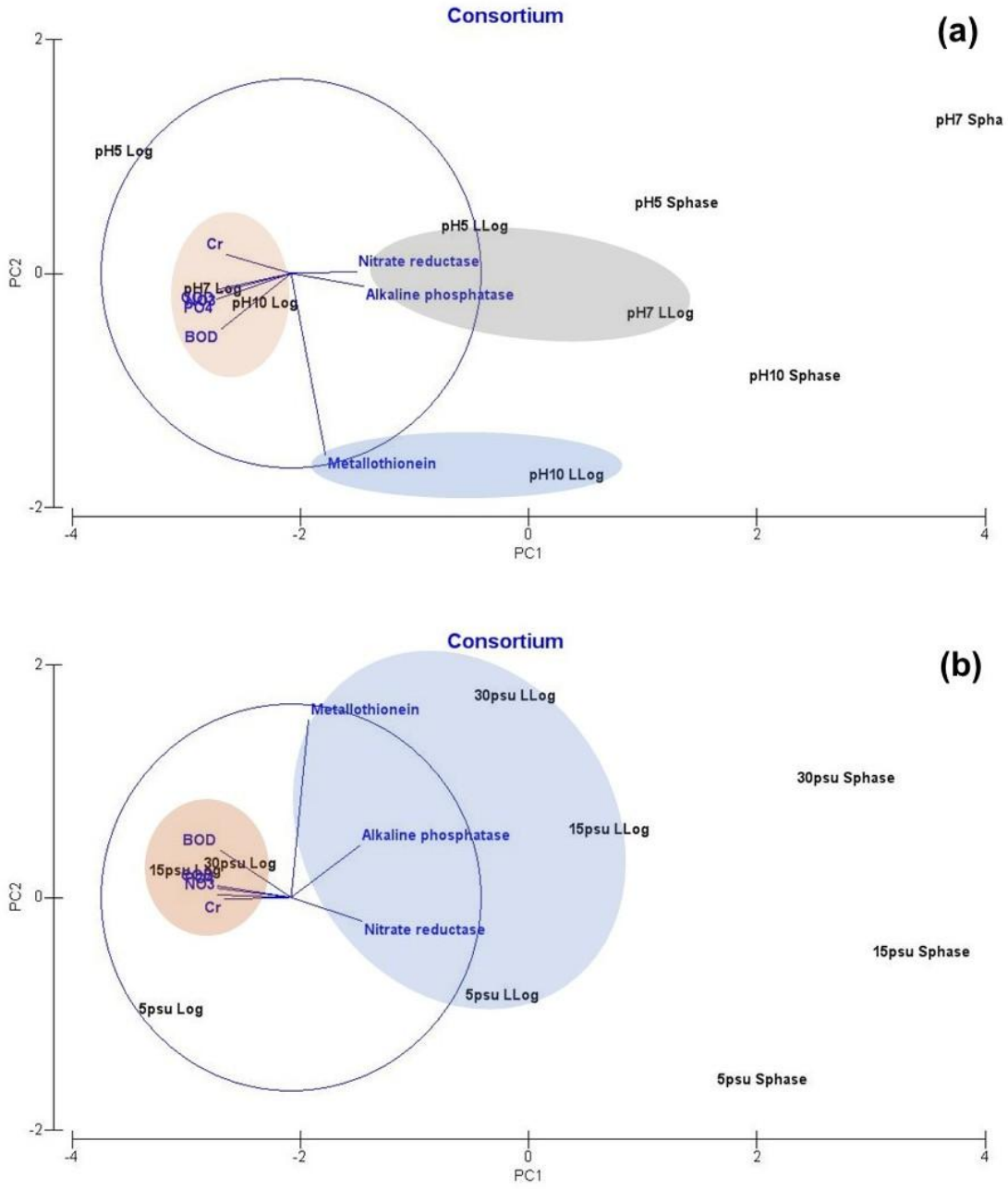


Fig. 6.5 Effect of (a) pH and (b) salinity on the pollutant reduction efficiencies and MT, NR and AP concentrations of bacterial-algal consortium comprising of *Bacillus cereus, Aeromicrobium* sp., *Chlorella vulgaris* and *Phormidium* sp.

6.4 Discussion

By employing metallothionein assays, the molecules produced by any organism that deal with pollutant detoxification can be estimated. In that, in the presence of a pollutant, the elaboration of MT is profuse and therefore, its assay can be adapted to easily illustrate the ability of any aquatic organism to deal with the hazardous pollution of a specific nature. A case in hand, in this study was the chromium reduction efficiencies of the microalgae and higher metallothionein concentrations at acidic pH. As reported in the literature, sorption of metal ions is mainly affected by pH of the media or the wastewater. Most of the algae species have optimum pH and various studies have been conducted to find the optimum pH value for maximal removal of heavy metals (Chalivendra, 2014). Cossich et. al (2002) reported that maximum Cr(III) sorption on *Sargassum* sp. can be achieved at pH 4. Sheng et al. (2004) found that the optimum pH for Cr(VI) and Cd sorption by *Padina* sp. and *Sargassum* sp was at pH 2. Zhou et al (1998) showed that the sorption of Cd on *Sargassum kjellmanianum* was optimum at pH between 4 and 5.

In general, pH range of 3 to 5 (acidic) is best optimum pH for the microalgal-sorption of metal ions. This is because most of the metal binding groups of algae are acidic (Chalivendra, 2014). At acidic pH, these groups cause electrostatic interactions between cationic species and generate a negative charge at the cell surface on which all metals ions are adsorbed. However, at very low pH (< 2), high concentration of H^+ ions are present and they will prevent metals from binding to ligands on the cell surface (Campbell and Stokes, 1985; Peterson et al., 1984; Gensemer, 1991; Parent and Campbell, 1994). Some studies (Crist et al., 1981; Mehta and Gaur, 2001; Ozer et al.,

1994) proved this phenomenon and reported that algae possess low metal sorption ability at extremely acidic conditions (pH < 2).

The bacterial cultures *B. cereus* and *Aeromicrobium* sp., showed the highest Cr(VI) reduction efficiencies at alkaline pH 10. Langley and Beveridge (1999) elucidated that the accumulation of metals on the surface of microbial cells is a consequence of the net negatively-charged surface and is influenced by the chemistry of the cell wall, physicochemical characteristics of the environment, such as pH, and the sequence of metal hydrolysis. The cell wall contains amines, amides, and carboxylic functional groups that are protonated or deprotonated, depending on the pH of the aqueous medium (Guibal et al., 1992). Increasing the pH increases the negative charge at the surface of the cells until all relevant functional groups are deprotonated, which favors electrochemical attraction and adsorption of cations. Furthermore, the increase in metal uptake with an increase in pH may be the result of more efficient competition of cations with H^+ for binding sites on bacteria. Anions could be expected to interact more strongly with cells as the concentration of positive charges increases, as the result of protonation of functional groups at low pH values (Bedell and Darnall, 1990). This bacterium, belonging to the genus *Halomonas*, was found by Watts et al. (2015) to exhibit growth concomitant to Cr(VI) reduction under alkaline conditions of pH 10.

Murthy et al. (2013) isolated a metallothionein protein capable of binding Pb from *B. cereus* and found that there was an increase in the metallothionein biosynthesis when cells were exposed to increased Pb concentrations up to 500 mg L^{-1}. Blindauer (2011)

explained that MTs in *Synechococcus* PCC 7942 are involved in homeostasis of zinc, as deletion of the *smtA* gene transcribing MT leads to hypersensitivity to Zn(II).

Ma Clean et al. (1972) first reported the presence of Cd-binding material in fresh blue-green alga *Anacystis nidulans*. Mallich and Rai (1998) found that the cyanobacterium *Anabaena dolilolum* synthesized low molecular weight Cd-binding protein (3.3 kDa) in response to Cd and they concluded that, this protein may play a role in metal tolerance. Torres et al. (1997) concluded that marine algae in response to Cd synthesized metallothioneins that sequester the metal in harmless form. Occurrence of these metal-binding proteins in organisms growing in a mining refuse area also supports the postulate that they are involved in detoxification (Grill et al., 1988; Kubota et al., 1988).

The bacteria, microalgae and their consortium tested in this study were observed to possess higher nutrient removal efficiencies at pH 7 and 15 psu. Koot (1980) reported that the optimal pH for nitrification is 7.5, with nitrification being inhibited at pH values lower than 7.0. Very acidic (pH<5) or alkaline conditions (pH>9) are not conducive for nutrient removal. Zhou et al. (2015) studied the optimal pH ranges for *C. vulgaris* on nutrient removal and found the optimal pH range for ammonia and nitrogen removal was 7–8. Different results were established by Liang et al. (2013). In a co-cultured system of *Bacillus licheniformis* and *C. vulgaris*, it was shown that the optimal pH for NH_4-N removal was 7; while for phosphorus removal pH did not have significant impact (Liang et al., 2013; Zhou et al., 2015).

The statistical analyses (PCA) done in this study are useful to recognize very high chromium removal efficiency in *B. cereus* at alkaline pH 10, also the high pollutant

reductions at pH 7 and 10. Similarly, *Aeromicrobium* sp. was tolerant to a wider range of pH than *B. cereus*. Significantly higher concentrations of NR and AP activity were observed during late log phase indicates the possibility of accumulation of the enzymes which essentially help clean up the hazardous molecules. The highest concentrations of MT and maximum removal of Cr were in the set grown in pH 10 corroborate with the previous reports of Liang et al. (2013) and Zhou et al. (2015).

Maximum concentrations of nitrate reductase and alkaline phosphatase were observed at pH 7 and 10 for all the isolates. Increased concentrations of nitrate reductase and alkaline phosphatase were observed at 15 and 30 psu. The APA of algae is reported to be affected by salinity (Hernández et al., 1995; Lee et al., 1999). Enzymatic activity has been shown to be very low under low salinities and to increase with increasing salinity up to 45–50 psu, where maximum APA was found (Hernández et al., 1995). Phosphatase activity of algae usually shows an optimum alkaline pH (Hernández et al., 1996) for activity, mostly between 8.7 and 9.0.

In higher plants, these values ranged between 7.0 and 7.5 (Sym, 1984; Maurino et al., 1986), for some microalgae, between 7.5 and 7.6 (Hochman et al., 1986), and for *Ulva rigida* the optimum was found at pH 8.0 (Corzo and Niell, 1991), close to the pH of seawater (8.2). Borowitzka and Borowitzka, (1988) reported pH 8.5–9.0 for NR production as optimum.

The NR activity in all isolates tested in this study was found to be the highest at pH 7 and 10 and at 30 psu. In the case of *Phormidium*, pollutant reduction efficiencies significantly varied at 5 and 15 psu while synthesis of NR and AP was maximum at 15

and 30 psu. Synthesis of MT was higher at 5 psu during the late log phase. These results are useful to suggest that while being able to perform well in the acidic pH ranges, the ability of both microalgae, in particular in the mixed consortium of bacteria to reduce the pollutant concentrations is an indication of its usefulness in dealing with TW with very high concentrations of hazardous wastes. In this regard, the PCA plots of the consortium sets are useful to note the increased concentrations of NR and AP, MT in all tested salinities and wide ranging pH of 5 to 10 during late log phase that certainly highlight the innate bioremediation potential of the cultures examined in this study.

Chapter 7

Summary

Bioremediation is now-a-days acknowledged as eco-friendly, less expensive, energy saving and little-to-null-carbon foot-print technology for hazardous waste treatment. Thus, search of ecologically efficient biota is a global effort. While many studies on the use of efficient bacterial and microalgal cultures for bioremediation of wastewaters are known, isolation, screening and characterization of environmental isolates with bioremediation potential of reckon are essential to develop pragmatic and cost-effective bioremediation strategies. Further, development of novel microbial consortia of greater relevance in bioremediation through new searches needs to continue so as to obtain sets of native bacteria and microalgae that can effectively remediate hazardous wastewaters.

From recent literature on tannery wastewater treatment, it is discernible that most studies have either focused on diluting the wastewater, or on examining Cr(VI) reduction only. Moreover, the use of *marine* bacterial and microalgal strains for examining their bioremediation potential has lacked attention. The use of marine and salt-tolerant microbes for the removal of toxicants from tannery wastewaters offers many advantages for eco-sustainable bioremediation of effluent-polluted marine environments.

The research work presented in this thesis focused on identifying potent marine and tannery wastewater (TW) bacteria and microalgae capable of detoxifying hazardous TW, either as single culture(s) or in consortium, for eventual discharge into the environment or reuse for other processes. With this principal focus in the fore, it was aimed to evaluate these cultures to improve the tannery wastewater for safe discharge into the open or for various other uses. Outcome of this research work can be summarized as a lab-scale demonstration of an uncomplicated and effective method for treating 100% raw tannery

wastewater. This synthesis is based on careful assessment of the advantages, in particular of bacterial and microalgal consortium *vis-a-vis* monoalgal and single bacterial culture for bioremediation.

The following are the major findings ensued from this study.

- A total of 27 marine and 19 salt-tolerant bacterial cultures, all tolerant to at least 50 mg L^{-1} Cr(VI), were isolated from seawater and tannery wastewater, respectively. On further screening based on Cr(VI) tolerance, three marine bacterial isolates - *Exiguobacterium mexicanum*, *Exiguobacterium aurantiacum* and *Aeromicrobium* sp., able to tolerate 100, 400 and 300 mg L^{-1} Cr(VI) emerged as the highly potent ones. Similarly, among the salt-tolerant bacteria isolated from TW, *Gordonia* sp. and *Bacillus cereus*, tolerant to 200 and 500 mg L^{-1} Cr(VI), respectively, were selected. Salt-tolerant green microalga *Chlorella vulgaris* isolated from tannery wastewater and marine cyanobacterium *Phormidium* sp. isolated from seawater grew well in the media amended with 100 mg L^{-1} Cr(VI).

- Marine bacterial isolates, both individually and in consortium, efficiently brought down the biochemical oxygen demand (BOD), chemical oxygen demand (COD), and Cr(VI) from tannery wastewater. Most notably, the consortium of the bacteria was found to be more effective in the reduction of BOD, COD and Cr(VI) among other parameters to permissible, safe discharge limits within 72 h of their growth in TW which had far higher concentrations of these hazardous parameters. In fact, these parameters were at least five times greater than the BIS

permissible limits for safe discharge.

- The marine microalgae *Chlorella* sp. and *Phormidium* sp., both individually and in consortium, efficiently reduced the concentrations of most parameters measured from tannery wastewater. The levels of BOD, COD, TN, TP, Cr(VI) and TDS were reduced to permissible, safe discharge limits by ~15 days. Markedly, the consortium of *Chlorella* and *Phormidium* sp. brought down the concentrations of a number of hazardous parameters faster than both of them individually. In addition, the duration required to achieve safe discharge limits was reduced substantially, in particular in case of BOD, COD, nitrates and phosphates.

- Toxicity testing of tannery wastewater in this study, ascertained that the untreated wastewater is deleterious to prolonged survival of the hatchlings of *Artemia salina*. The treatment of tannery wastewater with bacterial and microalgal consortium remarkably improved the wastewater quality and promoted the survival of the nauplii which can be directly ascribed to the reduction of toxic effects of effluent on *A. salina* after its bioremediation by the select set of bacteria and microalgae. This can be attributed to the substantial reduction in concentrations of the toxicants that were way greater in concentrations than BIS (1994) permitted limits.

- The isolates *Bacillus. cereus* and *Chlorella vulgaris* were found to tolerate wide range of salinities from 5 to 30 psu.

- Maximum concentrations of metallothioneins (MTs) and chromium reduction efficiencies in the bacterial isolates, *Bacillus cereus* and *Aeromicrobium* sp. were observed at alkaline pH 10. The highest concentrations of MTs and chromium reduction efficiencies in the microalgal isolates *Chlorella vulgaris* and *Phormidium* sp. were observed at acidic pH 5. However, in the consortium containing both bacteria and microalgae, the highest concentrations of MT were observed at alkaline pH 10.

- Reduction of BOD, COD, nitrates and phosphates in the TW were optimally highest at neutral pH 7 and salinity of 15 psu when treated with the bacteria, microalgae and their consortium.

- Maximum concentrations of nitrate reductase and alkaline phosphatase were observed at pH 7 and 10 for the bacterial and microalgal isolates. Increased production of nitrate reductase and alkaline phosphatase were induced at 15 and 30 psu.

- Consortium of the bacteria and microalgae was more efficient in reducing the TW pollutants by virtue of being more tolerant to wide range of pH and salinities and by elaborating all tested enzymes.

- Results of this study are useful to highlight that employing carefully developed microbial consortia is an advantageous strategy for treating effluents across more varying conditions

Future prospects

Marine bacteria, owing to their ability to grow in higher salt concentrations than their terrestrial counterparts, can certainly be ideal candidates for bioremediation as alternative, low cost and eco-friendly resources for treatment of tannery wastewaters to achieve safe and permissible discharge limits. Efforts to up-scale TW treatment using these and other marine bacterial cultures, individually and in consortium essentially would help developing pragmatic bioremediation strategies.

Further, the substantial biomass generated from microalgae can be useful variously, for biofuel production in particular. Notably, the accumulation of troublesome sludge in conventional treatment methods is certainly avoided by bioremediation. Moreover, by separating the algal biomass -that could be of use as a feeder material for a variety of other uses- the treated water can be recycled in the industry itself or for controlled irrigation of forests, lawns, and other greeneries.

Suggested future prospects of this work would be to conduct experiments with the combination of various toxic heavy metals in wastewater to investigate the synergistic/antagonistic effect on metal removal efficiency by bacteria and algae. Importantly, removal of nutrients, toxic substances and heavy metals from wastewater using combination of bacterial and algal strains, comparing its reduction efficiencies with single strains is imperative.

In order to evaluate the efficiency of the bacteria and microalgae for commercial scale, pilot scale studies with tannery wastewaters may be done in outdoor conditions. In

principle, chemical extraction techniques for microalgae and bacteria bound metals need to be evaluated to realize the percentage of metal recovery from microbial biomasses.

References

Aguilera S, Aguilar ME, Chavez MP, Lopez-Meza JE, Pedraza-Reyes M, Campos-Garcia J, Cervantes C. 2004. Essential residues in the chromate transporter ChrA of *Pseudomonas aeruginosa*. FEMS Microbiol Lett, 232: 107-112.

Ajayan KV, Selvaraju M, Unnikannan P, Sruthi P. 2015. Phycoremediation of tannery wastewater using microalgae *Scenedesmus* species. Int J Phytorem, 17:907–916.

Ajayan KV, Selvaraju M. 2012. Heavy metal induced antioxidant defense system of green microalgae and its effective role in Phycoremediation of tannery effluent. Pak J Biol Sci, 15:1056-1062.

Apaydin O, Kurt U, Gonullu MT. 2009. An investigation on the tannery wastewater by electrocoagulation. Global NEST J, 11: 546-555.

APHA. 2005. Standard methods for the examination of water and wastewater. 20th edn. APHA-AWWA-WPCF, Washington DC.

Aswathi M, Rai LC. 2005. Health effects of environment. Ecology and Environmental Safety, 29: 90-93.

Balaji S, Kalaivani T, Sushma B, Varneetha Pillai C, Shalini M, Rajasekaran C. .2015. Characterization of sorption sites and differential stress response of microalgae isolates against tannery effluents from Ranipet industrial area – An application towards phycoremediation. Int J Phytoremediation, DOI: 10.1080/15226514.2015.1115960

Baldwin BR, Peacock AD, Park M, Ogles DM, Istok JD, McKinley JP et al. 2008. Multilevel samplers as microcosms to assess microbial response to bio stimulation. Ground water, 46: 295-304.

Barsanti L, Gualtieri P. 2006. Algae: anatomy, biochemistry, and biotechnology. Boca Raton, CRC Press.

Bates SS, Tessier A, Campbell PGC, and Buffle J. 1982. Zinc adsorption and transport by *Chlamydomonas variabilis* and *Scenedesmus subspicatus* (Chlorophyceae) grown in semi continuous culture. J Phycol, 18: 521–529.

Batool R, Yrjälä K, Hasnain S. 2012. Hexavalent chromium reduction by bacteria from tannery effluent. J Microbiol Biotechnol 22(4): 547–554.

Bedell GW, Darnall DW. 1990. Immobilization of nonviable, biosorbent, algal biomass for the recovery of metal ions. In: Biosorption of Heavy Metals. Volesky B. (ed), pp 313–326, CRC Press, Boca Raton, FL.

Bhatnagar A, Bhatnagar M, Chinnasamy S, Das KC. 2010. *Chlorella minutissima*—a promising fuel alga for cultivation in municipal wastewaters. Appl Biochem Biotechnol, 161: 523–536.

Bhattacharya A, Gupta A, Kaur A, Malik D. 2015. Simultaneous bioremediation of phenol and Cr(VI) from tannery wastewater using bacterial consortium. Int J Appl Sci Biotechnol, 3(1): 50-55.

Bitton G. 2005. Wastewater Microbiology, 3 edn. New Jersey, John Wiley & sons, Inc.

Blindauer CA. 2011. *Bacterial metallothioneins: past, present, and questions for the future.* JBIC J Biol Inorganic Chem, 6(7): 1011-1024.

Borowitzka MA, Borowitzka LJ. 1988. *Dunaliella*. In: Borowitzka MA, Borowitzka LJ (eds), Microalgal Biotechnology. Cambridge University Press, 27–58.

Bureau of Indian Standards (BIS). 1994. Quality tolerance for water for Tanning industry IS: 4221, New Delhi.

Burmolle M, et al. 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Appl Environ Microbiol, 72: 3916–3923.

Cai S, Park YS, Li Y. 2013. Nutrient recovery from wastewater streams by microalgae: Status and prospects. Renewable and Sustainable Energy Reviews.19: 360-369.

Camargo FAO, Bento FM, Okeke BC, Frankenberger WT. 2003. Chromate Reduction by chromium-resistant bacteria isolated from soils contaminated with dichromate. J Environ Quality, 32(4): 1228-1233.

Campbell PGC, Stokes PM. 1985. Acidification and toxicity of metals to aquatic biota. Can J Fish Aquat Sci, 42: 2034-2049.

Campos J, Martinez-Pacheco M, Cervantes C. 1995. Hexavalent-chromium reduction by a chromate-resistant *Bacillus* sp. strain. Antonie Van Leeuwenhoek, 68: 203-208.

Campos VL, Moraga R, Ya´nez J, Zaror CA, Mondaca MA. 2005. Chromate reduction by *Serratia marcescens* isolated from tannery effluent. Bull Environ Contam Toxicol, 75: 400–406.

Carey RO, Migliaccio KW. 2009. Contribution of wastewater treatment plant effluents to nutrient dynamics in aquatic systems: A review. Environmental Management, 44: 205-217.

Carucci A, Chiavola A, Malone M, Rolle E. 1999. Treatment of tannery wastewater in a sequencing batch reactor. Water Sci Technol, 40: 253–259.

Chalivendra S. 2014 Bioremediation of wastewater using microalgae. PhD Thesis. University of Dayton, Dayton, Ohio.

Chandra R, Pandey PK, Srivastava A. 2004. Comparative toxicological evaluation of untreated and treated tannery effluent with *Nostoc muscorum* L. (Algal assay) and microtox bioassay Environ Monit Assess, 95: 287–294.

Chapman DJ, Harrison PJ. 1988. Nitrogen metabolism and measurement of nitrate reductase activity. In: Experimental Phycology. A laboratory manual. Lobban CS, Chapman DJ, Kremer B (eds). Cambridge University Press, Cambridge, p.196-202.

Chaturvedi MK. 2011. Studies on chromate removal by chromium-resistant *Bacillus* sp. isolated from tannery effluent. Journal of Environmental Protection, 2: 76-82.

Chellam CT, Sampathkumar P. 2012. Bio removal of nutrients in tannery effluent water using marine micro algae, *Chlorella Marina*. Proceedings of International Forestry and Environment Symposium, Sri Lanka. Published by Department of Forestry and Environmental Science, University of Sri Jayewardenepura, Vol. 17.

Chen L, Li T, Guan L, Zhou Y, Li P. 2011. Flocculating activities of polysaccharides released from the marine mat-forming cyanobacteria *Microcoleus* and *Lyngbya*. Aquat Biol, 11: 243–248.

Cheung KH, Gu JD. 2003. Reduction of chromate (Cr_2O_4) by an enrichment consortium and an isolate of marine sulfate-reducing bacteria. Chemosphere, 52: 1523-1529.

Cheung KH, Lai HY, Gu JD. 2006. Membrane-associated hexavalent chromium reductase of *Bacillus megaterium* TKW3 with induced expression. J Microbiol Biotechnol, 16: 855-862.

Chinnasamy S, Bhatnagar A, Hunt RW, Das KC. 2010. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. Bioresour Technol, 101: 3097–3105.

Chourey K, Wei W, Wan XF, Thompson DK. 2008. Transcriptome analysis reveals response regulator SO2426- mediated gene expression in *Shewanella oneidensis* MR-1under chromate challenge. BMC Genomics, 9: 395.

Christenson L, Sims R. 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnol Adv, 29:686–702.

Christopher JG, Kumar G, Tesema AF, Thi NBD, Kobayashi T, Xu K. 2016. Bioremediation for tanning industry: A future perspective for zero emission, http://dx.doi.org/10.5772/63809.

Chu W-L, See Y-C, Phang S-M. 2008. Use of immobilized *Chlorella vulgaris* for the removal of colour from textile dyes. J Appl Phycol, 21: 641-648.

Colak O, Kaya Z. 1988. A study on the possibilities of biological wastewater treatment using algae. Doga Biyolji Serisi, 12(1): 18–29.

Conway HL, Williams SC. 1979. Sorption of cadmium and its effect on growth and the utilization of inorganic carbon and phosphorus of two freshwater diatoms. J Fish Res Board Can, 36: 579-586

Corzo A, Niell FX. 1991. Determination of nitrate reductase activity in *Ulva rigida* C. Agardh by the *in situ* method. J Exp Mar Biol Ecol, 146: 181–191.

Cossich ES, Tavares CRG, Ravagnani TMK. 2002. Biosorption of chromium (III) by *Sargassum* sp. biomass. Electronic J Biotechnol, 5: 133–140.

Cotner JB, Wetzel RG. 1992. Uptake of dissolved inorganic and organic compounds by phytoplankton and bacterioplankton. Limnol Oceanogr, 37: 232-243.

Covington AD. 2009. Tanning chemistry: The science of leather. Royal Society of Chemistry, Cambridge, UK.

Craggs R, Sutherland D, Campbell H. 2012. Hectare-scale demonstration of high rate algal ponds for enhanced wastewater treatment and biofuel production. J Appl Phycol, 24: 329-337.

Crist RH, Oberholser K, Shank N, Nguyen M. 1981. Nature of binding between metallic ions and algal cell walls. Environ Sci Technol, 15: 1212–1217.

Das C, Naseera K, Ram A, Meena RM, Ramaiah N 2016. Bioremediation of tannery wastewater by a salt-tolerant strain of *Chlorella vulgaris*. J Appl Phycol, DOI:10.1007/s10811-016-0910-8.

Daulton TL, Little BJ, Jones-Meehan J, Blom DA, Allard LF. 2007. Microbial reduction of chromium from the hexavalent to divalent state. Geochim Cosmochim Acta, 71: 556-565.

Dayan AD, Paine AJ. 2001. Mechanisms of chromium toxicity, carcinogenicity and allergenicity: Review of the literature from 1985 to 2000. Hum Exp Toxicol, 20(9): 439- 51.

Desai C, Jain K, Madamwar D. 2008. Evaluation of in vitro Cr(VI) reduction potential in cytosolic extracts of three indigenous *Bacillus* sp. isolated from Cr(VI) polluted industrial landfill. Bioresour Technol, 99: 6059-6069.

Desikachary TV. 1959. Cyanophyta. Indian Council of Agriculture Research, New Delhi. Gandhi, H.P., pp. 1952-1998.

Devi BD, Thatheyus AJ, Ramya D. 2012. Bioremeoval of hexavalent chromium, using *Pseudomonas fluorescens*. J Microbiol Biotechnol Res, 2: 727-735.

Ding J, Zhao F, Cao Y, Xing L, Liu W, Mei S, Li S. 2014. Cultivation of microalgae in dairy farm wastewater without sterilization. Int J Phytorem, DOI:10.1080/15226514.2013.876970.

Dixit S, Singh DP. 2014. An evaluation of phycoremediation potential of cyanobacterium *Nostoc muscorum*: characterization of heavy metal removal efficiency. J Appl Phycol, 26: 1331-1342.

Dominic VJ, Murali S, Nisha MC. 2009. Phycoremediation efficiency of three micro algae *Chlorella Vulgaris*, *Synechocystis Salina* and *Gloeocapsa Gelatinosa*. SB Academic Review, Xvi(1): 138–146.

Donmez G, Aksu Z. 2002. Removal of chromium(VI) from saline wastewaters by *Dunaliella* species. Process Biochemistry, 38:751-762.

Dunn K, Maart B, Rose P. 2013. *Arthrospira* (Spirulina) in tannery wastewaters. Part 2: Evaluation of tannery wastewater as production media for the mass culture of *Arthrospira* biomass. AJOL DOI: http://dx.doi.org/10.4314/wsa.v39i2.12

Dunn K. 1998. The biotechnology of high rate algal ponding systems in the treatment of saline tannery wastewaters. PhD thesis. Rhodes University, Grahamstown, South Africa.

Dyhrman ST, Palenik BP. 1999. Phosphate stress in cuntures and field populations of the dinoflagellate *Prorocentrum minimum* detected using a single-cell alkaline phosphatase activity assay. Appl Environ Microbiol, 65: 3205–3212.

Fakruddin M, Mazumdar RM, Tania TK, Islam S, Nipa MN, Iqbal A, Bhuiyan HR. 2009. Isolation and characterization of chromate resistant and reducing bacteria from tannery effluent of Chittagong, Bangladesh. J bio-sci, 17: 71-76.

FAO. 1999. The State of the World's forest. ISBN 92-5-104193-8. FAO Documentation Group Cataloguing in Publishing Data. FAO, Rome, Italy.

Farag S, Zaki S. 2010. Identification of bacterial strains from tannery effluent and reduction of hexavalent chromium. J Environ Biol, 31(5): 877-882.

Fay P. 1992. Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol Rev, 56: 340–73.

Filip SD, Peters T, Adams VD, Middle brooks EJ. 1979. Residual heavy metal removal by an algae-intermittent sand filtration system. Water Res, 13: 305-313.

Flores A, Perez JM. 1999. Cytotoxicity, apoptosis and in vitro DNA damage induced by potassium chromate. Toxicol Appl Pharmacol, 161: 75-81.

Francisco R, Moreno A, Morais PV. 2010. Different physiological responses to chromate and dichromate in the chromium resistant and reducing strain *Ochrobactrum tritici* 5bvl1. Biometals, 23: 713-725.

Fredrickson JK, Kostandarithes HM, Li SW, Plymale AE, Daly MJ. 2000. Reduction of Fe(III), Cr(VI), U(VI) and Tc(VII) by *Deinococcus radiodurans* R1. Applied Environ Microbiol, 66: 2006-2011.

Gadd GM. 1990. Heavy metal accumulation by bacteria and other microorganisms. Experientia, 46: 834-840.

Gadd GM. 1992. Metals and microorganisms: A problem of definition. FEMS Microbiol Lett, 100: 197-203.

Gadd GM. 2000. Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. Curr Opi Biotech, 11: 271-279.

Gaikar RB, Uphade BK, Gadhave AG, Kuchekar SR. 2010. Effect of dairy effluent on seed germination and early seedling growth of soybeans. Res J Chem, 3: 137–139.

Gaikwad GL, Wate SR, Ramteke DS, Roychoudhury K. 2014. development of microbial consortia for the effective treatment of complex wastewater. J Bioremed Biodeg, 5: 4.

Ganesh R, Balaji G, Ramanujam RA. 2006. Biodegradation of tannery wastewater using sequencing batch reactor-respirometric assessment. Bioresour Technol, 97: 1815-1821.

Gensemer RW. 1991. The effects of pH and aluminum on the growth of the acidophilic diatom *Asterionella ralfsii* var. Americana. Limnol Oceanogr, 36: 123–131.

Gheewala SH, Annachatre AP. 1997. Bio-degradation of aniline. Water Sci Technol, 36-53.

Ghigliazza R, Lodi A, and Rovatti M. 1998. Study on biological phosphorus removal process by *Acinetobacter lwoffi*: possibility to by-pass the anaerobic phase. Bioprocess Biosyst Eng, 18(3): 207-211.

Gin KY, Tang YZ, Aziz MA. 2002. Derivation and application of a new model for heavy metal biosorption by algae. Water Res, 36: 1313–1323.

Gopalan R, Veeramani H. 1994. Studies on microbial chromate reduction by *Pseudomonas* sp. in aerobic continuous suspended growth cultures. Biotechnol Bioeng, 43: 471-476.

Grill E, Winnacker EL, Zenk MH. 1988. Occurrence of heavy metal binding phytochelatins in plants growing in a mining refuse area. Experienta, 44: 539–540.

Grima EM, Belarbi EH, Acien Fernandez FG, Robles Medina A, Chisti Y. 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol Adv, 20: 491–515

Guibal E, Roulph C, Le Cloirec P. 1992. Uranium biosorption by a filamentous fungus *Mucor miehei*; pH effect on mechanisms and performances of uptake. Water Res, 26: 1139–1145.

Gupta S., Gupta R., Tamra R. 2007. Challenges faced by leather industry in Kanpur. Report.

Gupta VK, Shrivastava AK, Jain N. 2001. biosorption of chromium(VI) from aqueous solutions by green algae *Spirogyra* species. Wat Res, 35(17): 4079–4085.

Hafez M, Fouad A, El-Desouky W. 2002. Accumulation of some metal ions on *Bacillus licheniformis*. J Radioanal Nucl Chem, 251: 249–252.

Han X, Wong YS, Wong MH, Tama NFY. 2007. Biosorption and bioreduction of Cr(VI) by a microalgal isolate, *Chlorella miniate*. J Hazard Mater, 146: 65–72.

Hart BA. 1975. Bioconcentration and toxicity of cadmium in *Chlorella pyrenoidosa*. In: The effect of cadmium on freshwater phytoplankton. Office of Water Research and Technology, Washington, DC, PB 257-547, pp. 1-31.

Hasegawa MC, de Souza Daniel JF, Takashima K, Batista GA, da Silva SMCP. 2014. COD removal and toxicity decrease from tannery wastewater by zinc oxide-assisted photocatalysis: a case study. Environ Technol, 35: 1589–1595.

Hassan BA, Venkateshwaran AA, Fredrickson JK, Daly MJ. 2003. Engineering *Deinococcus geothermalis* for bioremediation of high temperature radioactive waste environments. Appl Environ Microbiol, 69: 4575- 4582.

He ZG, Li SZ, Wang LS, et al. 2014. Characterization of five chromium-removing bacteria isolated from chromium-contaminated soil. Water Air Soil Poll, 225: 1-10.

Hecky RE, Kilham P. 1988. Nutrient limitation of phytoplankton in freshwater and marine environments: A review of recent evidence on the effects of enrichment. Limnol Oceanogr, 33: 796-822.

Heitzer A, Sayler GS. 1993. Monitoring efficacy of bioremediation. Trends Bio technol, 11: 334-343.

Henze M, Harremoës P, La Cour Jansen J, Arvin E. 2002. Wastewater treatment. biological and chemical processes, 3 edn. Ed. Förstner U, Murphy RJ, Rulkens WH. New York, Springer-Verlag Berlin Heidelberg.

Hernández I, Fernández JA, Niell FX. 1995. A comparative study of alkaline phosphatase activity of two species of *Gelidium* (Gelidiales, Rhodophyta). Eur J Phycol, 30: 69–77.

Hernández I, Hwang SJ, Heath RT. 1996. Measurement of phosphomonoesterase activity with a radiolabelled glucose-6-phosphate. Role in the phosphorus requirement of phytoplankton and bacterioplankton in a temperate mesotrophic lake. Arch Hydrobiol, 137: 265–280.

Hernandez I, Niell FX, Fernandez JA. I992. Alkaline phosphatase activity in *Porphyra umbilicalis* (L.) Kutzing. Exp Mar Biol Ecol, 159: 1-13.

Hernández-Zamora M, Cristiani-Urbina E, Martínez-Jerónimo F, Perales-Vela HV, Ponce-Noyola T, Montes-Horcasitas MdC, Cañizares-Villanueva RO. 2015. Bioremoval of the azo dye Congo Red by the microalga *Chlorella vulgaris*. Environ Sci Pollut Res, 22: 10811–10823.

Hochman A, Nissany A, Wynne D, Kaplan B, Berman T. 1986. Nitrate reductase: an improved assay method for phytoplankton. J Plankton Res, 8: 385–392.

Hoffmann JP. 1998. Wastewater treatment with suspended and non-suspended algae. J Phycol, 34: 757-763.

Hollibaugh JT, Seibert DLR, Thomas WH. 1980. A comparison of the acute toxicities of ten heavy metals to phytoplankton from Saanich Inlet, B.C., Canada. Estuarine Coastal Marine Sci, 10: 93-105.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. 2000. Bergey's manual of determinative bacteriology. Ninth edition, Lipincott Williams and Wilkins,USA.

Hori K, Si I, Ikeda G, Ji O, Tanji Y, Weeraphasphong C. 2002. Behavior of filamentous cyanobacterium *Anabaena* spp. in water column and its cellular characteristics. Biochem Eng J, 10: 217–25.

Hudson JJ, Taylor WD, Schindler DW. 2000. Phosphate concentrations in lakes. Nature, 406: 54-56.

Isart NR, Vasak M. 2002. Advances in the structure and chemistry of metallothioneins, J. Inorg. Biochem. 88: 388–396.

Islam LN, Mahmud ARZ, Nurun Nabi AHM, Hossain M, Mohasin M. 2014. Assessment of physicochemical and biochemical qualities of tannery effluents of Hazaribagh, Dhaka, and comparison with non-tannery water samples. Int J Environ, 4: 68-81.

Jaysudha S, Sampathkumar P. 2014. Nutrient removal from tannery effluent by free and immobilized cells of marine microalgae *Chlorella salina.* Int J Environ Biol, 4(1): 21-26.

Ji L, Xie S, Feng J, Li Y, Chen L. 2011. Heavy metal uptake capacities by the common fresh water green alga *Cladophora fracta*. J Appl Phycol, 24: 979-983.

Ji, Min-Kyu, Abou-Shanab, Reda AI, Hwang, Jae-Hoon. 1999. Removal of nitrogen and phosphorus from piggery wastewater effluent using the green microalga *Scenedesmus obliquus*. J Environ Eng, 139 (9): 1198-1205.

Kabdasli I, Tünay O, Orhon D. 1999. Wastewater control and management in a leather tanning district. Water Sci Technol, 40: 261–267.

Kabdasli NI, Tünay O, Orhon D. 1993. The treatability of chromium tannery wastes. Water Sci Technol, 28(2): 97-105.

Kabir ES *et al.* 2002. Assessment of effluent quality of Dhaka export processing zone with special emphasis to the textile and dying industries. Jahangir Nagar University Journal of Science, 25: 137-138.

Kader J, Sannasi P, Othman O, Ismail BS, Salmijah S. 2007. Removal of Cr(VI) from aqueous solutions by growing and non-growing populations of environmental bacterial consortia. Global J Environ Res, 1: 12–17.

Kadirvelu K, Kavipriya M, Karthika C, Radhika M, Vennilamani N, Pattabhi S. 2003. Utilization of various agricultural wastes for activated carbon preparation and application for the removal of dyes and metal ions from aqueous solutions. Bioresour Technol, 87: 129-132.

Kägi JHR, Schäffer A. 1988. Biochemistry of metallothionein. Biochemistry, 27: 8509–8515.

Kamala-Kannan S, Krishnamoorthy R, Lee KJ, Purusothaman A, Shanthi K, Rao NR. 2007. Aerobic chromate reducing *Bacillus cereus* isolated from the heavy metal contaminated Ennore Creek sediment, North of Chennai, Tamil Nadu, South East India. Res J Microbiol, 2: 133–140.

Kashefi K, Lovley DR. 2000. Reduction of Fe(III), Mn(IV) and toxic metals at 100°C by *Pyrobaculum islandicum*. Appl Environ Microbiol, 66: 1050-1056.

Khan SR, Kawaja MA, Khan AM, Ghani H, Kazmi S. 1999. Environmental impacts and mitigation costs associated with cloth and leather exports from Pakistan. A Report on trade and sustainable development submitted by sustainable development policy institute and IUCNP to IISD Canada for the IISD/IUCN/IDRC Project on Building Capacity for Trade and Sustainable Development in Developing Countries, Islamabad.

Khanafari A, Eshghdoost S, Mashinchian A. 2008. Removal of lead and chromium from aqueous solution by *Bacillus circulans* biofilm. J Environ Health Sci Eng, 5: 195-200.

Kim SB, Lee SJ, Kim CK, Kwon GS, Yoon BD and Oh HM. 1998. Selection of microalgae for advanced treatment of swine wastewater and optimization of treatment condition. Korean J Appl Microbiol Biotechnol, 26: 76-82.

Kiviranta J, Sivonen K, Niemela SI, Huovinen K. 1991. Detection of toxicity of cyanobacteria by *Artemia salina* bioassay. Environ Toxicol Water Qual, 6: 423-436.

Kongjao S, Damronglerd S, Hunsom M. 2008. Simultaneous removal of organic and inorganic pollutants in tannery wastewater using electrocoagulation technique. Korean J Chem Eng, 25: 703-709.

Koot ACJ. 1980. Treatment of wastewater, Waltman Publishers, Delft (NL).

Koteswari YN, Ramanibai R. 2003. The effect of tannery effluent on the colonization rate of plankets: A microcosm study. Turk J Bioi, 27: 163-170.

Kubota K, Nishizono H, Suzuki S, Ishii F. 1988. A copper binding protein in root cytoplasm of *Polygonum cuspidatum* growing in a metalliferous habitat. Plant Cell Physiol, 29: 1029–1033.

Kumar A, Kumar R, Tiku DK, Sharma P, Chaturvedi R. 2007. Biological process for reducing chemical and biochemical oxygen demand of pulp and paper industrial effluent. United States Patent 7267772-B2.

Langley S, Beveridge TJ. 1999. Effect of O-side-chain-lipopolysaccharide chemistry on metal binding. Appl Environ Microbiol, 65: 489–498.

Langton J, Chesman BS, Burt GR, Pope ND, McEvoy J. 2002. Metallothionein in liver of eels. *Anguilla anguilla* from Thames Estuary: an indicator of environmental quality? Mar Environ Res, 53: 263–293.

Larsdotter K. 2006. Microalgae for phosphorus removal from wastewater in a Nordic climate. PhD dissertation, Royal Institute of Technology, Sweden.)

Lea PJ, Leegood RC. 1995. Plant biochemistry and molecular biology. John Wiley & Sons, New York.

Lee TM, Tsai CC, Shih MC. 1999. Induction of phosphorus deficiency and phosphatase activity by salinity (NaCl) stress in *Gracilaria tenuistipitata* (Gigartinales, Rhodophyta). Phycologia, 38: 428–433.

Lefebvre O, Vasudevan N, Torrijos M, Thanasekaran K, Maletta R. 2005. Halophilic biological treatment of tannery soaks liquor in a sequencing batch reactor. Water Res, 39: 1471-1480.

Leta S, Assefa F, Gumaelius L, Dalhammar G. 2004. Biological nitrogen and organic matter removal from tannery wastewater in pilot plant operations in Ethiopia. Appl Microbial Biotechnol, 66: 333-339.

Liang Z, Liu Y, Ge F, Xu Y, Tao N, Peng F, Wong M. 2013. Efficiency assessment and pH effect in removing nitrogen and phosphorus by algae-bacteria combined system of *Chlorella vulgaris* and *Bacillus licheniformis*. Chemosphere, 92: 1383–1389.

Linde AR, Garcia-Vazquez E. 2006. A simple assay to quantify metallothionein helps to learn about bioindicators and environmental health. Biochem Molecular Biol Edu, 34(5): 360–363.

Linde AR, Sanchez-Galan S, Valles-Mota P, Garcia-Vazquez E. 2001. Metallothionein as bioindicator of freshwater metal pollution: European eel and brown trout. Ecotoxicol Environ Saf, 49: 60–63.

Liu Y-G, Pan C, Xia W-B, Zeng G-M, Zhou M, Liu Y-Y, Ke J, and Huang C. 2008. Simultaneous removal of Cr(VI) and phenol in consortium culture of *Bacillus* sp. and *Pseudomonas putida* Migula (CCTCC AB92019). Trans. Nonferrous Met. Soc. China, 18: 1014-1020. DOI: 10.1016/S1003-6326(08)60174-0

LLovera S, Bonet R, Simon-Pujol MD, Congregado F. 1993. Effect of culture medium ions on chromate reduction by resting cells of *Agrobacterium radiobacter*. Appl Microbiol Biotechnol, 39: 424-426.

Lobban CS, Harrison PJ. 1994. Seaweed ecology and physiology. Cambridge University Press, Cambridge.

Lovley DR. 1993. Dissimilatory metal reduction. Annu Rev Microbiol, 47: 263-290.

Ma X, Novak PJ, Ferguson J, Sadowsky M, Lapara TM, Semmens MJ et al. 2007. The impact of H_2 addition on dechlorinating microbial communities. Bioremediation Journal, 11: 45-55.

MaClean FI, Lucis OJ, Shaiki ZA, Janson ER. 1972. The uptake and subcellular distribution of Cd and Zn in microorganisms. Proc Fed Am Soc Exp Biol, 31: 1-699.

Mahdavi H, Liu Y, Ulrich YC. 2013. Partitioning and bioaccumulation of metals from oil sands process affected water in indigenous *Parachlorella kessleri*. Chemosphere, 90: 1893-1899.

Mallich N, Rai LC. 1998. Characterization of Cd-induced low molecular weight protein in a N_2-fixing cyanobacterium *Anabena doliolum* to Co-:multiple tolerance. Biometals, 11: 55–61.

Mandal T, Dasgupta D, Mandal S, Datta S. 2010. Treatment of leather industry wastewater by aerobic biological and Fenton oxidation process. J Hazard Materials, 180, 204-211.

Manu B, Chandhani S. 2002. Anaerobic decolorization of simulated textile wastewater containing azo dyes. Bioresour Technol, 82: 225-231.

Manu KJ, Mohana VS, Ganeshaiah KN. 2011. Effluent generation by the dairy units: characterization and amelioration for irrigation. Int J Res Chem Environ, 1: 173–182.

Mara DD, Pearson HW, Silva SA, Rose PD, Maart BA, Dunn KM, Rowswell RA, Britz P. 1996. Waste stabilization ponds: Technology and applications high rate algal oxidation ponding for the treatment of tannery effluents. Water Sci Technol, 33: 219-227.

Mata TM, Melo AC, Simoes M, Caetano NS. 2012. Parametric study of a brewery effluent treatment by microalgae *Scenedesmus obliquus*. Bioresour Technol, 107: 151–158.

Mateju V, Cizinská S, Krejcí J, and Janoch T. 1992. Biological water denitrification-A review. Enzyme Microb Technol, 14(3): 170-183.

Maurino SG, Echevarria C, Mejias JA, Vargas MA, Maldonado JM. 1986. Properties of the *in vivo* nitrate reductase activity assay in maize, soybean and spinach leaves. J Plant Physiol, 24: 123–130.

Mbwele LA. 2006. Microbial phosphorus removal in waste stabilisation pond wastewater treatment systems. Licentiate Thesis from the School of Biotechnology, Royal Institute of Technology.

McLean JS, Beveridge TJ, Phipps D. 2000. Isolation and characterization of a chromium-reducing bacterium from a chromated copper arsenate-contaminated site. Environ Microbiol, 2: 611-619.

Megharaj M, Avudainayagam S, Naidu R. 2003. Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. Curr Microbiol, 47: 51-54.

Mehta S K, Gaur J P. 2001. Removal of Ni and Cu from single and binary metal solutions by free and immobilized *Chlorella vulgaris*. European J Protistology,37: 261-271.

Mehta SK and Gaur JP. 2005. Use of algae for removing heavy metal ions from wastewater: Progress and prospects. Crit Rev Biotechnol, 25: 113-152.

Mergeay M, Monchy SB, Vallaeys T, Auquier V, Benotmane A, et al. 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: Towards a catalogue of metal-responsive genes. FEMS Microbiol Rev, 27: 385-410.

Middleton SS, Latmani RB, Mackey MR, Ellisman MH, Tebo BM, Criddle CS. 2003. Cometabolism of Cr(VI) by *Shewanella oneidensis* MR-1 produces cell-associated reduced chromium and inhibits growth. Biotechnol Bioeng, 83: 627-637.

Misha A, Yadav A, Agaiwal M, Bajipai M. 2004. Fenugreek mucilage for solid removal from tannery effluent. Reactive and Functional Polymers, 59*:* 99–104.

Mohanty MS. 1997. Raw hides and skin problem of procurement, curing preservation and marketing, presucted at CLRI, Madras.

Mongkolthanaruk W, Dharmsthiti S. 2002. Biodegradation of lipid-rich wastewater by a mixed bacterial consortium. Int Biodeterior Biodegradation, 50: 101-105.

Munawar M, Munawar IF. 1982. Phycological studies in Lakes Ontario, Erie, Huron and Superior. Can J Botany, 60: 1837-1858.

Murthy S, Bali G, Sarangi SK. 2013. Effect of lead metallothionein concentration in lead resistant bacteria *Bacillus cereus* isolated from industrial effluent. African Journal of Biotechnology, 10(71): 15966-15972.

Mustafa E-M, Phang S-M, Chu W-L. 2012. Use of an algal consortium of five algae in the treatment of landfill leachate using the high-rate algal pond system. J Appl Phycol, 24: 953-963.

Nanda S, Sarangi PK, Abraham J. 2010. Cyanobacterial remediation of industrial effluents I. Tannery effluents. New York Science Journal, 3: 32-36.

Nandy T, Kaul SN, Shastry S, Manivel W, Deshpande CV. 1999. Wastewater management in cluster of tanneries in Tamil Nadu through implementation of common treatment plants. J Sci Ind Res, 58: 475-516.

Nordberg M. 1998. Metallothioneins: Historical review and state of knowledge. Talanta, 46: 243–254.

Nourbakhsh M, Sag Y, Ozer D, Aksu Z, Kustal T, Caglar A. 1994. A comparative study of various biosorbents for removal of chromium(VI) ions from industrial waste waters. Process Biochem, 29: 1-5.

Nourbakhsh MN, Kilicarslan S, Ilhan S, Ozdag H. 2002. Biosorption of Cr^{6+}, Pb^{2+} and Cu^{2+} ions in industrial waste water on *Bacillus* sp. Chem Eng J, 85: 351–355.

Oehmen A, et al. 2007. Advances in enhanced biological phosphorus removal: From micro to macro scale. Wat Res, 41(11): 2271-2300.

Oh SJ, Yamamoto T, Kataoka Y, Matsuda O, Matsuyama Y, Kotani Y. 2002. Utilization of dissolved organic phosphorus by the two toxic dinoflagellates, *Alexandrium tamarense* and *Gymnodinium catenatum* (Dinophyceae). Fisheries Science, 68: 416-424.

Oliveira EC, Plastino EM. 1994. Gracilariaceae. In: Biology of economic algae Akatsuka I, (ed). SBP Academic Publishing, The Hague, Netherlands, p. 185-226.

Orandi S, Lewis DM. 2013. Biosorption of heavy metals in a photorotating biological contactor – a batch process study. Appl Microbiol Biotechnol, 97: 5113-5123.

Orhon D, Ates E, Sozen S. 2000. Experimental evaluation of the nitrification kinetics for tannery wastewaters. Water SA, 26: 43-52.

Oswald WJ. 1988. Micro-algae and waste-water treatment. In: Borowitzka MA, Borowitzka LJ (eds) Microalgal Biotechnology. Cambridge University Press, Cambridge, pp 305-328.

Ozer D, Aksu Z, Kutsal T, Caglar A. 1994. Adsorption isotherms of lead (II) and chromium (VI) on *Cladophora crispata*. Environ Technol, 15: 439–448.

Pandian K, Thatheyus AJ, Ramya D. 2014. Bioremoval of chromium, nickel and zinc in electroplating effluent by *Pseudomonas aeruginosa*. Open J Water Pollut Treat, 1: 75-82.

Parent L, Campbell PGC. 1994. Aluminum bioavailability to the green alga *Chlorella pyrenoidosa* in acidified synthetic soft water. Environ Toxicol Chem, 3: 587–598.

Park CH, Gonzalez D, Ackerley D, Keyhan M, Martin A. 2002. Molecular engineering of soluble bacterial proteins with chromate reductase activity. In: Hinchee RE, Porta A, Pellei M (eds). Remediation and beneficial reuse of contaminated sediments. Batelle Press, Columbus, USA., ISBN-13: 9781574771299, pp: 103-112.

Parsons et al. 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Press. Potts Point, N.S.W. Australia.

Pattanapipitpaisal P, Brown NL, Macaskie LE. 2001a. Chromate reduction and 16SrRNA identification of bacteria isolated from a Cr(VI) contaminated site. Appl Microbiol Biotechnol, 57: 257-261.

Pattanapipitpaisal P, Brown NL, Macaskie LE. 2001b. Chromate reduction by *Microbacterium liquefaciens* immobilised in polyvinyl alcohol. Biotechnol Lett, 23: 61-65.

Perpetuo EA, Souza CB, Nascimento CAO. 2011. Engineering bacteria for bioremediation. In: Carpi A, ed. Progress in molecular and environmental bioengineering - From analysis and modeling to technology applications. http://www.intechopen.com/books/progress-in-molecular-and-environmental-bioengineeringfrom- analysis-and-modeling-to-technology-applications/engineering-bacteria-forbioremediation: InTech, p. DOI: 10.5772/19546.

Peterson HG, Healey FP, Wagemann R. 1984. Metal toxicity to algae: a highly pH dependent phenomenon. Can J Fish Aquat Sci, 41: 974–979.

Petria V. 1978. Effect of chromium salts from water sediments on physiological processes in the alga *Chlorella vulgaris.* Rev Roum Biol Ser Biol Veg, 23: 55-57.

Philip L, Iyengar L, Venkobachar C. 1998. Cr(VI) reduction by *Bacillus coagulans* isolated from contaminated soils. J Environ Eng, 124: 1165-1170.

Pittman JK, Dean AP, Osundeko O. 2011. The potential of sustainable algal biofuel production using wastewater resources. Bioresour Technol, 102: 17–25.

Poopal AC, Laxman RS. 2008. Hexavalent chromate reduction by immobilized *Streptomyces griseus*. Biotechnol Lett, 30: 1005-1010.

Posadas E, Bochon S, Coca M, García-González MC, García-Encina PA, Muñoz R. 2014. Microalgae-based agro-industrial wastewater treatment: a preliminary screening of biodegradability. J Appl Phycol, 26: 2335-2345.

Prakasam TBS, Dondero NS. 1978. Aerobic hetrotrophic bacterial population of tannery effluent and activated sludge. Appl Microbiol, 115: 1122–1127.

Pun R, Raut P, Pant BR. 2013. Removal of chromium(VI) from leachate using bacterial biomass. Sci World, 11: 63-65.

Rahman MS et al. 2012. Water quality characterization of the Sundarbans Reserve Forest (SRF), Khulna for biodiversity consideration, Bangladesh. In: Silver jubilee conference, Bangladesh Chemical Society, 20: 67-68.

Rai UN, Dwivedi S, Tripathi RD, Shukla OP, Singh NK. 2005. Algal biomass: an economical method for removal of chromium from tannery effluent. Bull Environ Contam Toxicol, 75: 297–303.

Ram B, Bajpai PK, Parwana HK. 1999. Kinetics of chrome-tannery effluent treatment by the activated-sludge system. Process Biochem, 35: 255-265.

Ramakrishnan B. 2012. Microbial community tracking in bioremediation. J Bioremed Biodeg, 3: 10.

Rao HP, Ranjith Kumar R, Raghavan B, Subramanian V, Sivasubramanian V. 2011. Application of phycoremediation technology in the treatment of wastewater from a leather-processing chemical manufacturing facility. Water SA, 37: 07-14.

Rawat I, Kumar RR, Mutanda T, Bux F. 2011. Dual role of microalgae: phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Appl Energy, 88: 3411–3424.

Rehman A, Farah RS, Shakoori AR. 2007. Heavy metal resistant *Distigmaproteus* (Euglenophyta) isolated from industrial effluents and its possible role in bioremediation of contaminated wastewaters. World J Microbiol Biotechnol, 23: 753–758.

Rehman A, Zahoor A, Muneer B, Hasnain S. 2008. Chromium tolerance and reduction potential of *Bacillus* sp. ev3 isolated from metal contaminated wastewaster. Bull Environ Contam Toxicol, 81: 25–29.

Rehman A. 2011. Heavy metals uptake by *Euglena proxima* isolated from tannery effluents and its potential use in wastewater treatment. Russian J Ecology, 42: 44–49.

Reichardt W, Overbeck J, Steubing L. 1967. Free dissolved enzymes in lake waters. Nature, 216: 1345-1347.

Renuka N, Sood A, Ratha SK, Prasanna R, Ahluwalia AS. 2013. Nutrient sequestration, biomass production by microalgae and phytoremediation of sewage water. Int J Phytoremed, 15(8): 789–800.

Riano B, Molinuevo B, Garcia-Gonzalez MC. 2011. Treatment of fish processing wastewater with microalgae-containing microbiota. Bioresour Technol, 102: 10829–10833.

Roh Y, Liu SV, Li G, Huang H, Phelps TJ, Zhou J. 2002. Isolation and characterization of metal-reducing *Thermoanaerobacter* strains from deep subsurface environments of the Piceance Basin, Colorado. Applied Environ Microbiol, 68: 6013-6020.

Romanenko VI, Korenkov VN. 1977. A pure culture of bacterial cells assimilating chromates and bichromates as hydrogen acceptors when grown under anaerobic conditions. Mikrobiologiya, 46: 414-417.

Safi C, Zebib B, Merah O, Pontalier P, Vaca-Garcia C. 2014. Morphology, composition, production, processing and applications of Chlorella vulgaris: a review. Renewable and Sustainable Energy Reviews; 35: 265–278.

Sannasi P, Kader J, Ismail BS, Salmijah S. 2006. Sorption of Cr(VI), Cu(II) and Pb(II) by growing and non-growing cells of a bacterial consortium. Bioresour Technol, 97: 740–747.

Schindler DW.1977. Evolution of phosphorus limitation in lakes. Science, 195: 260-262.

Shan Y, McKelvie ID, Hart BT. 1994. Determination of alkaline phosphatase-hydrolyzable phosphorus in natural water systems by enzymatic flow injection. Limnol Oceanogr, 39: 1993–2000.

Shen H, Wang YT. 1994. Biological reduction of chromium by *E. coli*. J Environ Eng, 120: 560-572.

Shen H, Wang YT. 1995. Simultaneous chromium reduction and phenol degradation in a coculture of *Escherichia coli* ATCC 33456 and *Pseudomonas putida* DMP-1. Applied Environ Microbiol, 61: 2754-2758.

Sheng PX, Tan LH, Chen JP, Ting YP. 2004. Biosorption performance of two brown marine algae for removal of chromium and cadmium. J Dispersion Sci Technol, 25: 679–686.

Shi J, Podola B, Melkonian M. 2007. Removal of nitrogen and phosphorus from wastewater using microalgae immobilized on twin layers: an experimental study. J Appl Phycol, 19: 417-423.

Silva-Benavides AM, Torzillo G. 2011. Nitrogen and phosphorus removal through laboratory batch cultures of microalgae *Chlorella vulgaris* and cyanobacterium *Planktothrix isothrix* grown as monoalgal and as co-cultures. J Appl Phycol, 24: 267–276.

Singh HN, Vaishampayan A, Singh RK. 1978. Evidence for the involvement of a genetic determinant controlling functional specificity of Group VI B elements in the metabolism of nitrogen and nitrate in the blue-green alga *Nostoc muscorium*. Biochem Biophys Res Commun, 81: 67-74.

Singh M, Reynolds DL, Das KC. 2011. Microalgal system for treatment of effluent from poultry litter anaerobic digestion. Bioresour Technol, 102: 10841–10848.

Sinha SN, Biswas M, Paul D, Rahaman S. 2011. Biodegradation potential of bacterial isolates from tannery effluent with special reference to hexavalent chromium. Biotechnology, Bioinformatics and Bioengineering, 1: 381-386.

Smith WL, Gadd GM. 2000. Reduction and precipitation of chromate by mixed culture sulphate-reducing bacterial biofilms. J Applied Microbiol, 88: 983-991.

Srinath T, Verma T, Ramteke PW, Garg SK. 2002. Chromium(VI) biosorption and

bioaccumulation by chromate resistant bacteria. Chemosphere, 48: 427-435.

Srivastava S, Srivastava AK. 2005. Studies on phosphate uptake by *Acinetobacter calcoaceticus* under aerobic conditions. Enzyme Microb Technol, 36(2-3): 362-368.

Sultan S, Hasnain S. 2007. Reduction of toxic hexavalent chromium by *Ochrobactrum intermedium* strain SDCr-5 stimulated by heavy metals. Bioresour Technol 98: 340-344.

Suzuki T, Miyata N, Horitsu H, Kawai K, Takamizawa K, Tai Y, Okazaki M. 1992. NAD(P)H-dependent chromium(VI) reductase of *Pseudomonas ambigua* G-1: A Cr(V) intermediate is formed during the reduction of Cr(VI) to Cr(III). J Bacteriol, 174: 5340-5345.

Sym GJ. 1984. Optimisation of the *in vivo* assay conditions for nitrate reductase in barley (*Hordeum vulgare* L. cv. Igri). J Sci Food Agric, 35: 725–730.

Szpyrkowicz L, Kaul SN, Neti RN. 2005. Tannery wastewater treatment by electro-oxidation coupled with a biological process. J Applied Electrochem, 35: 381-390.

Tadesse I, Isoaho S, Green F, Puhakka J. 2003. Removal of organics and nutrients from tannery effluent by advanced integrated wastewater pond systems technology. Water Sci Technol, 48: 307-314.

Tang CY, Criddle QS, Fu CS, Leckie JO. 2007. Effect of flux (transmembrane pressure) and membranes properties on fouling and rejection of reverse osmosis and nanofiltration membranes treating perfluoro octane sulfonate containing waste water. J Environ Sci Technol, 41.

Tchobanoglous G, Burton FL and Stensel HD. 2003. Wastewater Engineering: Treatment, Disposal, and Reuse, New York: McGraw-Hill.

Tekerlekopoulou AG, Tsiamis G, Dermou E, Siozios S, Bourtzis K, Vayenas DV. 2010. The effect of carbon source on microbial community structure and Cr(VI) reduction rate. Biotechnol Bioeng, 107: 478-487.

Thacker U, Parikh R, Shouche Y, Madamwar D. 2006. Hexavalent chromium reduction by *Providencia* sp. Process Biochem, 41: 1332-1337.

Thacker U, Parikh R, Shouche Y, Madamwar D. 2007. Reduction of chromate by cell-free extract of *Brucella* sp. isolated from Cr(VI) contaminated sites. Bioresour Technol, 98: 1541-1547.

Thanigavel M. 2004. Biodegradation of tannery effluent in fluidized bed bioreactor with low density biomass support. M.Tech. Thesis, Annamalai University, Tamilnadu, India.

Thompson IP, Gast CJvd, Ciric L, Singer AC. 2005. Bioaugmentation for bioremediation: The challenge of strain selection. Environmental Microbiology, 7: 909-915.

Tiwari DP et al. 1989. Removal of toxic metals from electroplating industries. Indian J Environ Health, 31: 122-126.

Torres E, Cid A, Fidalgo P, Herrero C, Abalde J. 1997. Long-chain class III: metallothioneins as a mechanism of cadmium tolerance in the marine diatom *Phaeodactylum tricornutum* Bohlin. Aquatic Toxicol, 39: 231–246.

Travieso L, Benítez F, Sánchez E, Borja R, León M, Raposo F, Rincón B. 2008. Assessment of a microalgae pond for post-treatment of the effuent from an anaerobic fixed bed reactor treating distillery wastewater. Environ Technol, 29: 985-992.

Uberoi NK. 2003. Environmental Management. Excel Books Publisher, New Delhi, p. 269.

UNIDO. 1991. In: Proceedings of the Conference on Ecological Sustainable Industrial Development, Copenhagen, vol. 8. p.44.

UNIDO. 2000. The scope for decreasing pollution load in leather processing. Regional Programme for Pollution Control in the Tanning Industry in South-East Asia. www.elaw.org/system

USEPA. 1998. How wastewater treatment works. The Basics. Cincinnati, OH: U.S. Environmental Protection Agency.

Vaneechoutte M, Kampfer P, De Baere T, Falsen E, Verschraegen G. 2004. Wautersia gen. nov., a novel genus accommodating the phylogenetic lineage including *Ralstonia eutropha* and related species and proposal of *Ralstonia* [*Pseudomonas*] *syzygii* (Roberts et al. 1990) comb. nov. Int J Syst Evol Microbiol, 54: 317-327.

Vanhaecke P, Persoone G, Claus C, Sorgeloos P. 1981. Proposal for a short-term toxicity test with *Artemia nauplii*. Ecotox Environ Safe, 5: 382–387.

Verheijen LAHM, Weirsema D, Hwshoffpol LW, Dewit J. 1996. Live stock and the environment: Finding a balance management of waste from animal product processing. International Agriculture Centre, Wageningen, The Netherlands.

Vishakha SS, Kulkarni SW, Minal W. 2013. Physicochemical characterization of dairy effluents. Int J Life Sc Bt Pharm Res, 2: 2250–3137.

Vitic C, Giovannetti L. 2001. The impact of chromium contamination on soil heterotrophic and photosynthetic microorganisms. Ann Microbiol, 51: 201-213.

Wagner M, et al. 2002. Microbial community composition and function in wastewater treatment plants. Antonie Van Leeuwenhoek. 81(1-4): 665-680.

Wagner M, Loy A. 2002. Bacterial community composition and function in sewage treatment systems. Curr Opin Biotechnol, 13(3): 218-227.

Wang Y, Xiao C. 1995. Factors affecting hexavalent chromium reduction in pure cultures of bacteria. Water Res, 29: 2467-2474.

Wang YT, Shen H. 1995. Bacterial reduction of hexavalent chromium. J Ind Microbiol, 14: 159-163.

Watts MP, Khijniak TV, Boothman C, Lloyd JR. 2015. Treatment of alkaline Cr(VI)-contaminated leachate with an alkaliphilic metal-reducing bacterium. Appl Environ Microbiol, 81(16): 5511–5518.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol, 173: 697–703.

Whitton BA, Grainger SLJ, Hawley GRW, Simon JW. 1991. Cell bound and extracellular phosphatase activities of cyanobacterial isolates. Microb Ecol, 21: 85–98.

Wood LM, Wang HK. 1983. Microbial resistance to heavy metals. Environ Sci Technol, 17: 582A-590A.

Xue HB, Stumm W, and Sigg L. 1988. The binding of heavy metals to algal surfaces. Water Resour, 22: 917–926.

Yelda S, Mitra A, Bandyopadhyay M. 2002. Purification of pulp and paper mill effluent using *Eichornia crassipes*. Environ Technol, 23: 453-465.

Yu RQ, Wang WX. 2004. Bio kinetics of cadmium, selenium, and zinc in freshwater alga *Scenedesmus obliquus* under different phosphorus and nitrogen conditions and metal transfer to *Daphnia magna*. Environmental Pollution, 129: 443–456.

Zahoor A, Rehman A. 2009. Isolation of Cr (VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing wastewater. J Environ Sci, 21: 814-820.

Zengin G, Ölmez T, Dogruel S, Kabdasli I, Tünay O. 2002. Assessment of sourced-based nitrogen removal alternative in leather tanning industry, Water Sci Technol, 45: 205–215.

Zhou JL, Huang PL, Lin RG. 1998. Sorption and desorption of Cu and Cd by macroalgae and microalgae. Environ Pollut, 101:67–75.

Zhou L, Wu F, Zhao Z, Wang B. 2015. Effects of environmental factors on nitrogen and phosphorus removal by *Chlorella vulgaris* in wastewater. Current Biotechnology, 5: 60–65.

Zhu W, Chai L, Ma Z, Wang Y, Xiao H, Zhao K. 2008. Anaerobic reduction of hexavalent chromium by bacterial cells of *Achromobacter* sp. strain Ch1. Res Microbiol, 163: 616-623.

Publications

Das C., Naseera K., Ram A., Meena R. M., Ramaiah N. 2017. Bioremediation of tannery wastewater by a salt-tolerant strain of *Chlorella vulgaris*. Journal of Applied Phycology 29(1): 235 – 243.

Das C., Ramaiah N., Pereira E., Naseera K. 2018. Efficient bioremediation of tannery wastewater by monostrains and consortium of marine *Chlorella* sp. and *Phormidium* sp. International Journal of Phytoremediation 20(3): 284 – 292.

Conference proceeding and presentations

Poster presented at "ASEAN-India Marine Biotechnology workshop" held during 19th to 22nd March, 2013, organized by National Institute of Oceanography (CSIR), Goa, India.

Title: Biodetoxification of tannery effluent

Authors: Das C., Sabu E., Naseera K. and Savant S.

Poster presented at "International Conference on New Horizons in Biotechnology" held at National Institute for Interdisciplinary Science and Technology (CSIR), Trivandrum, from 22nd to 25th November, 2015

Title: Tannery effluent detoxification potential of a marine strain of *Chlorella vulgaris*.

Authors: Das C., Naseera K., Aswathi A., Ram A., Meena R.M. and Ramaiah N.

J Appl Phycol
DOI 10.1007/s10811-016-0910-8

Bioremediation of tannery wastewater by a salt-tolerant strain of *Chlorella vulgaris*

Cindrella Das [1] · K. Naseera [1] · Anirudh Ram [2] · Ram Murti Meena [1] · Nagappa Ramaiah [1]

Received: 28 April 2016 / Revised and accepted: 7 July 2016

Abstract Phycoremediation is the use of algae for removal or reduction of inorganic nutrients and xenobiotics from wastewaters. It is a reliable process for biotransformation and detoxification of a variety of pollutants. This study focused on the potential of a strain of the green microalga, *Chlorella vulgaris*, to reduce various pollutants in tannery wastewater (TW). The microalga was grown in TW for a culture period of 21 days and the resultant removal/reduction/biotransformation of biochemical oxygen demand (BOD), chemical oxygen demand (COD), nitrates (NO_3–N), phosphates (PO_4–P), sulphates (SO_4–S), dissolved solids and chromium (Cr) was monitored. The isolate was efficient in the removal of excess nutrients in wastewater. Most notably, complete removal (100 % reduction) of NO_3–N and Cr was observed by the 6th and 12th day of culture period, respectively. Removal of phosphates was as high as 91.73 % by day 6 and over 99 % by day 21. This strain also reduced sulphate concentrations to 67.4 % by day 21. Levels of chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in TW were reduced by 94.74 and 95.93 %, respectively, after 21 days. Our results are useful to suggest that this isolate of *C. vulgaris* is promising for bioremediating and detoxifying tannery wastewater to improve its quality to meet up recommended effluent discharge limits.

Keywords *Chlorella* · Microalgae · Tannery · Wastewater · BOD · Bioremediation

Introduction

Globally, an estimated total of more than 52 billion domesticated animals are slaughtered every year for human consumption (FAO 2004) and the hides of larger animals are processed for varied uses. Common commercial hides include leather from cattle and other livestock animals which are used for manufacturing shoes, clothes, upholstery, interior decoration among many others.

The tanning industry is a significant contributor to economy and provides large scale employment opportunities in particular to unskilled and/or semiskilled people. Unfortunately, it is one of the worst anthropogenic polluters (Khwaja et al. 2001). Tanneries process the foul-smelling, damp, hairy, and assorted-sized hides employing cheap labor. In spite of the impending deleterious ecological consequences, the developing world still practices traditional, archaic processing technologies (Kennedy 1999). To circumvent the obnoxious impacts of tannery wastewater, several treatment methods have emerged during the early 1990s (Durai and Rajasimman 2011; Saranraj and Sujitha 2013).

Notably, most of the world's tanneries are located in the Indian subcontinent. India alone has about 3,000 tanneries with an annual processing capacity of 700,000 t of hides and skins. These tanneries produce and discharge an estimated 30 billion liters of effluent annually (Srivastav 2012). Conventional leather tanning technology is highly polluting and produces large amounts of organic and chemical pollutants (Kabdasli et al. 2002). These pollutants, in the discharged effluent, pose serious threats to the environment.

✉ Nagappa Ramaiah
ramaiah@nio.org

[1] CSIR—National Institute of Oceanography, Dona Paula, Goa 403004, India

[2] CSIR—National Institute of Oceanography, Regional Centre, Mumbai 400053, India

Published online: 02 September 2016

Tannery wastewaters are characterized mainly by high levels of biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), total dissolved solids (TDS), chromium, and sulphides (Leta et al. 2004). Conventional wastewater treatment schemes typically include primary and secondary treatment. Primary treatment consists of sulphide oxidation, solids separation, and chromium precipitation. Secondary treatment is usually accomplished by activated sludge systems (Dotro 2003). The large amounts of sludge generated by wastewater treatment processes must be sent off-site for disposal. Handling and disposal of this sludge is typically the largest single cost component in the operation of a wastewater treatment plant. Activated sludge systems require very high capital as well as operation and maintenance costs. The cost factor coupled with the recent but increasingly stringent environmental regulations and standards (Bosnic et al. 2000; Kabdasli et al. 2002), has led the industry to search for new effective treatment technologies.

With these concerns, there has been increased interest in using biological methods for remediation of different industrial wastewaters. Most studies have concentrated on the use of fungi and bacteria to treat wastewater (McMullan et al. 2001; Tastan et al. 2010). However, additional carbon sources are required for such systems. Hence, the use of microalgae in bioremediation of industrial wastewaters is of practical interest due to their central role in autotrophic carbon dioxide fixation. The process of wastewater treatment using algae has low energy requirement, reduced sludge formation as well as GHG emission along with production of variously useful algal biomass. It has been shown to be a more cost effective method for removal of BOD, certain microbial pathogens, phosphorus, and nitrogen than activated sludge process and other secondary treatment processes (Sheehan et al. 1998).

Various techniques are in place for exploiting faster growth rates and nutrient removal efficiencies of certain microalgae for the treatment/detoxification of a variety of wastewaters. During the last four decades, phycoremediation has been evaluated by numerous investigators (e.g., Oswald 1988; Mara et al. 1996; Tadesse et al. 2003; Shi et al. 2007; Chu et al. 2008; Craggs et al. 2012; Mustafa et al. 2012; Dixit and Singh 2014; Posadas et al. 2014). Dunn (1998) and Dunn et al. (2013) examined the detoxification of tannery wastewater and nutrient removal using high-rate algal pond systems. Different microalgae and cyanobacteria such as species of *Oscillatoria*, *Phormidium*, *Ulothrix*, *Chlamydomonas, Scenedesmus* have been evaluated for their ability to grow in tannery effluent and accumulate chromium (Rai et al. 2005; Balaji et al. 2015; Ajayan et al. 2015). Notable reports on the use of *Chlorella* for bioremediation of tannery wastewater are those by Chellam and Sampathkumar (2012) and by Jaysudha and Sampathkumar (2014). Both free as well as immobilized cells of *Chlorella salina* or *Chlorella marina* have been examined mainly for removal of nutrients in tannery wastewater. *Chlorella vulgaris* was used by Rao et al. (2011) for removal of nutrients from tannery wastewater and by Hernández-Zamora et al. (2015) for removal of Congo Red, an azo-dye used for dyeing cotton, jute, leather, paper, silk, and wool.

In view of such advantages offered by microalgae, this study aimed at evaluating the microalga, *C. vulgaris* for its potential in reducing BOD, COD, sulphates, inorganic nutrients, dissolved solids, and chromium in the effluent. Our results demonstrate a very highly efficient, minimal energy requiring improvement of tannery wastewater by this salt-tolerant microalgal strain.

Materials and methods

Salt tolerant microalga *Chlorella vulgaris* NIOCCV was used in this study. It was grown in algal culture medium (ACM, HiMedia, Mumbai, India) at a constant temperature of 28 ± 0.5 °C under fluorescent lights at 150–300 μmol photons m^{-2} s^{-1} (with 10:14-h light/dark photoperiod).

Characteristics of tannery wastewater

The tannery wastewater (TW) was collected from outside the discharge point of a tannery industry located in Kanpur, in Northern India on the banks of River Ganges. Various physical and chemical parameters of the wastewater collected were analyzed using standard methods in APHA (American Public Health Association 2005).

Experimental design

Initially, the growth of NIOCCV was tested in different strengths of TW viz., 100 % (no dilution), 70 % (7:3), 50 % (1:1), 30 % (3:7), and 10 % (1:9). The original TW was diluted to these strengths using tap water. Even after several trials of altering inoculum size, light periods, and aeration, very poor growth was observed in the 100 and 70 % TW unlike the vigorous growth (vis-a-vis that in standard algal culture medium) in 50, 30, or 10 % TW. Hence, we tested the bioremediation potential of this strain in 1:1 diluted TW.

Tannery wastewater was diluted to 50 % (1:1) with tap water and 100 mL aliquots of this dilution were dispensed into several 250-mL Erlenmeyer flasks. Ten milliliter of exponential algal culture, standardized to an optical density (OD 620 nm) of 0.2, were inoculated into eight triplicate sets of 250-mL flasks containing 100 mL of 1:1 TW. The culture was grown for 21 days at a constant temperature of 28 ± 0.5 °C under fluorescent lights at 150–300 μmol photons m^{-2} s^{-1} (10:14-h light/dark photoperiod). Cells from a set of three flasks each were harvested on day 0, 3, 6, 9, 12, 15, 18, and 21. Two controls (one of TW without any algae while another of standard algae culture broth (HiMedia) with algal cells)

were included to confirm and check the effect of TW on the growth of algae during the experiment. On all sampling days, analysis of different parameters was carried out both from un-inoculated and algae-inoculated flasks with 1:1 TW.

For chlorophyll *a* measurement, 10 mL filtered sample (Whatman GF/C), extracted with acetone overnight and measured spectrophotometrically (Strickland and Parsons 1968). Cells were also counted microscopically at ×400 on all sampling days.

Analytical procedures

The physico-chemical parameters BOD, COD, nitrates (NO_3–N), phosphates (PO_4–P), sulphates (SO_4–S), total dissolved solids (TDS), total chromium (Cr), and hexavalent chromium (Cr^{6+}) were measured from the samples harvested during the experiment. Measurements of BOD and COD were made following standard methods described in APHA (2005). The BOD was determined using the 5-day BOD test and the COD, using open reflux oxidation method. TDS was determined by gravimetric method as per APHA (2005). TDS was calculated by measuring the residual weight after drying known sample volumes filtered through 0.7-μm glass microfiber filters at 180 °C. The algal cells were separated from wastewater through filtration using 0.45-μm glass microfiber filters, and the filtrate was used for measurement of dissolved nitrates, phosphates, and sulphates. Nitrate concentrations were colorimetrically measured following the Cd column reduction method (APHA 2005). Phosphates were measured using the ascorbic acid method (APHA 2005). Sulphates were measured following the barium chloride precipitation method (APHA 2005). For total chromium concentrations, the samples were digested with concentrated nitric acid followed by filtration through 0.45-μm filter paper, while for hexavalent chromium, 0.45-μm filtered samples without any pre-digestion were analyzed. The chromium contents were measured using the colorimetric diphenylcarbazide (DPC) method (APHA 2005).

Toxicity bioassay

Brine shrimp (*Artemia salina*) bioassay (Kiviranta et al. 1991) was used to assess the reduction in toxicity in the remediated tannery effluent. Briefly, 1-day-old *Artemia* hatchlings were placed in multi-well plates (10 well^{-1}). Survival was monitored from 1 mL treated and untreated wastewater samples and seawater (positive control). The assay was conducted in five replicates. The number of dead nauplii after 24, 48, and 72 h were counted and percent survival calculated.

Statistical analysis

Statistical analyses were carried out using XLSTAT 7. One-way analysis of variance (ANOVA) was used to check the effect of *C. vulgaris* on its ability to reduce various toxic parameters from the wastewater. Subsequent pair-wise comparisons were performed using post hoc Tukey's HSD (Honestly Significant Difference) tests.

Results

Characteristics of tannery wastewater

The physico-chemical characteristics of the raw tannery wastewater are presented in Table 1. The wastewater, collected from the outlets of a tannery, had dark brown color, salinity of 15 PSU, high BOD, COD, nitrates, phosphates, sulphates, TDS, and chromium concentrations. Both BOD and COD were quite high with average concentrations of 1,350 (±42.5) mg L^{-1} and 4,000 (±51.2) mg L^{-1}, respectively. Untreated TW had very high concentrations of nitrate (0.93 ± 0.01 mg L^{-1}), phosphate (6.01 ± 0.05 mg L^{-1}), and sulphate (178.69 ± 0.98 mg L^{-1}). Total dissolved solids (TDS) were higher compared to total suspended solids (TSS), indicating high contents of dissolved salts in the wastewater. Total chromium (3.22 mg L^{-1}) concentrations in the wastewater were found to be higher than the permissible limits (<2.0 mg L^{-1}) for discharge of effluents. Concentrations of chlorides, Ca, and Mg in the wastewater were also higher than those permitted by Bureau of Indian Standards (BIS 1994).

Growth of *C. vulgaris* in tannery wastewater

Cell counts of *C. vulgaris*, enumerated once every 3 days during the culture period of 21 days, increased by over seven times the initial counts of 5×10^3 cells mL^{-1}. Also the chlorophyll *a*, 0.25 mg mL^{-1} was higher on day 21 (Fig. 1). In comparison to standard algal culture medium, the growth of cells was lower in the 1:1 diluted tannery effluent.

Reduction of different physico-chemical parameters

The concentrations of physico-chemical parameters analyzed from the 1:1 TW before treatment (day 0) and after treatment with *C. vulgaris* (day 21) are presented in Table 1. The concentrations of different parameters in tannery wastewater at 3-day intervals after treatment with *C. vulgaris* are shown in Table 2. After 21 days of treatment, the BOD level was reduced from 672 to 27 mg L^{-1}, and the COD from 1,680 to 88 mg L^{-1} (Table 2). In case of BOD, the reduction was 95.92 %, whereas for reduction of COD, the efficiency rate was 94.74 % by day 21 (Fig. 2). Similarly, significant reduction of nitrates, phosphates and sulphates occurred within 21 days (Table 2). Indeed, 100 % reduction of nitrates was seen by day 6. Removal of phosphates by *C. vulgaris* was as high as 91.74 % by day 6 and over 99 % by day 21. The sulphate

Table 1 Physicochemical characteristics of raw, diluted (1:1) untreated and treated tannery wastewater (TW)

Parameter	Raw TW	Untreated 1:1 TW (Day 0)	Treated 1:1 TW (Day 21)	Maximum permissible limits (BIS 1994)
pH	7.45 ± 0.00	7.00 ± 0.00	7.20 ± 0.00	5.5–9.0
Salinity (PSU)	15.00 ± 0.30	12 ± 0.38	10 ± 0.25	–
Total solids (mg L^{-1})[a]	5,000.00 ± 0.50	–	–	2,200
Total suspended solids (mg L^{-1})[a]	500.00 ± 30.40	–	–	100
Total dissolved solids (mg L^{-1})	4,333.33 ± 288.70	3,333.33 ± 0.00	1,766.70 ± 288.68	2,100
BOD (mg L^{-1})	1,350.00 ± 42.50	658.40 ± 26.42	27.33 ± 4.04	30
COD (mg $L^{-1}L$)	4,000.00 ± 51.20	1,747.24 ± 62.09	88.33 ± 3.82	250
H_2S (S^{2-}) (mg L^{-1})[b]	11.75 ± 11.28	–	–	2
Sulphates (SO_4^{2-}) (mg L^{-1})	178.69 ± 0.98	75.34 ± 4.84	25.16 ± 2.37	1,000
Nitrates (mg L^{-1})	0.93 ± 0.01	0.39 ± 0.02	0.00	–[d]
NH_3 (mg L^{-1})[b]	2,734.16 ± 1.12	–	–	50
Phosphates (mg L^{-1})	6.01 ± 0.05	3.85 ± 0.25	0.002 ± 0.00	–
Phenols (mg L^{-1})	3.80 ± 0.58	–	–	5-50
Cr (mg L^{-1})	3.22	0.88 ± 0.03	0.00	2
Mn (mg L^{-1})[c]	0.10	–	–	–
Ni (mg L^{-1})[c]	0.05	–	–	3
Cu (mg L^{-1})[c]	0.02	–	–	3
Zn (mg L^{-1})[c]	0.08	–	–	15
Ca (mg L^{-1})[c]	265.10	–	–	–
Mg (mg L^{-1})[c]	33.40	–	–	–

Standard deviation, $n = 3$

[a] This parameter was not analyzed in the treated TW because the algal biomass adds to the total/suspended solids

[b] Untraceable in both untreated and treated TW

[c] Since the concentrations were within safe limits in raw TW, they were not measured

[d] No set limit

concentrations in TW were reduced by 67.4 % by day 21. The very high concentration of TDS of >3,000 mg mL^{-1} in untreated wastewater was reduced to 2,066 mg mL^{-1} by *C. vulgaris* after 12 days (Fig. 2) and to 1,766 mg mL^{-1} by day 21 (Table 2) with an overall TDS removal of 41 % (Fig. 2). Concentrations of total chromium in the tannery effluent, following treatment with *C. vulgaris* are presented against control TW (Fig. 3). Hexavalent chromium was completely transformed from the TW by day 12.

One-way ANOVA with post hoc Tukey's HSD test showed that the concentrations of all the measured parameters decreased significantly after the treatment process of 21 days. For TDS, the reduction was significant ($p < 0.001$), and highly significant ($p < 0.0001$) for BOD, COD, NO_3–N, PO_4–P, SO_4–S, total and hexavalent chromium (Table 3).

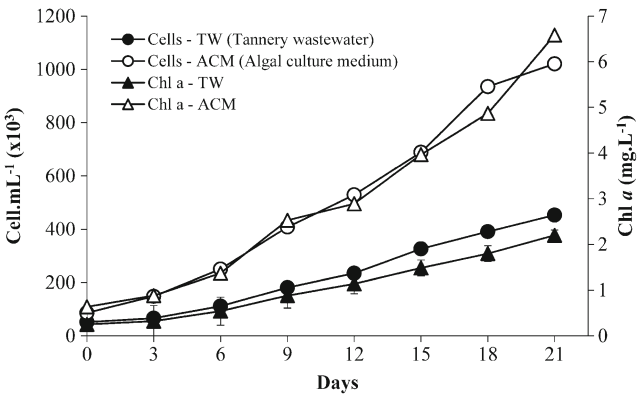


Fig. 1 Growth of *C. vulgaris* in tannery wastewater

Toxicity assay

Percent survival of brine shrimp (*A. salina*) nauplii/hatchlings subjected to treated and untreated effluent for 24, 48 and 72 h differed rather significantly (Table 4). For instance, in the *C. vulgaris* treated effluent the survival of *Artemia* nauplii was 83.9 % on Day 1 and increased to 100 % by day 9. A positive trend was apparent in the survival of *Artemia* hatchlings in the treated effluent when exposed for 24, 48, and 72 h. Survival was significantly less in the untreated effluent. It was at least 28–32 % lower compared to that in treated effluent.

Table 2 Effect of *C. vulgaris* in reducing toxic pollutant concentrations from TW

Days		BOD	COD	NO_3–N	PO_4–P	SO_4–S	Cr	Cr^{6+}	TDS
0	Treatment	672.02 ± 36.79	1,680.05 ± 19.93	0.371 ± 0.03	3.712 ± 0.34	77.41 ± 4.56a	0.821 ± 0.06	0.561 ± 0.01	3,000.00 ± 577.35a
	Control	658.40 ± 26.42	1,747.24 ± 62.09	0.385 ± 0.02	3.852 ± 0.25a	75.34 ± 4.84a	0.877 ± 0.03a	0.577 ± 0.03	3,333.33 ± 0.00a
3	Treatment	480.67 ± 31.00	1,401.67 ± 60.37	0.146 ± 0.03	1.455 ± 0.09	72.06 ± 1.91ab	0.524 ± 0.09a	0.224 ± 0.06a	2,666.70 ± 115.47ab
	Control	516.67 ± 35.12	1,414.17 ± 82.53	0.271 ± 0.01a	3.707 ± 0.09ab	72.52 ± 1.78ab	0.825 ± 0.04ab	0.325 ± 0.04a	3,066.67 ± 254.70a
6	Treatment	271.67 ± 28.43	779.17 ± 30.03	0.000	0.307 ± 0.04a	63.99 ± 2.34bc	0.453 ± 0.10a	0.053 ± 0.01a	2,333.30 ± 0.00abc
	Control	423.33 ± 25.17a	894.50 ± 59.14ab	0.229 ± 0.00a	3.293 ± 0.05bc	64.41 ± 0.89bc	0.820 ± 0.05ab	0.242 ± 0.06ab	3,000.00 ± 577.35a
9	Treatment	185.33 ± 25.50	253.33 ± 55.14a	0.000	0.273 ± 0.04a	56.91 ± 4.40c	0.029 ± 0.01b	0.004 ± 0.00b	2,233.30 ± 100.00bc
	Control	373.33 ± 20.82ab	777.40 ± 48.92ab	0.122 ± 0.01b	3.217 ± 0.06cd	62.92 ± 1.12c	0.819 ± 0.04ab	0.199 ± 0.04b	2,900.00 ± 251.66a
12	Treatment	86.67 ± 7.64a	206.67 ± 15.51ab	0.000	0.215 ± 0.03a	44.84 ± 4.80d	0.004 ± 0.00b	0.000	2,066.70 ± 115.47bc
	Control	366.67 ± 11.55ab	720.67 ± 27.14b	0.175 ± 0.02	2.754 ± 0.23e	59.88 ± 4.51c	0.785 ± 0.05ab	0.088 ± 0.01c	2,866.67 ± 115.47a
15	Treatment	46.67 ± 7.64a	125.00 ± 18.03bc	0.000	0.156 ± 0.02a	36.62 ± 4.97d	0.000	0.000	1,933.30 ± 152.75bc
	Control	346.67 ± 36.86bc	626.67 ± 86.61c	0.106 ± 0.00bc	3.057 ± 0.03cde	59.80 ± 0.68c	0.737 ± 0.07b	0.023 ± 0.01c	2,833.33 ± 57.74a
18	Treatment	28.33 ± 7.64a	98.80 ± 3.54c	0.000	0.070 ± 0.01a	35.48 ± 3.54de	0.000	0.000	1,900.00 ± 251.66c
	Control	333.33 ± 11.55bc	548.33 ± 27.14c	0.083 ± 0.02bc	2.830 ± 0.17de	58.35 ± 3.40c	0.712 ± 0.02b	0.021 ± 0.01c	2,766.67 ± 0.00a
21	Treatment	27.33 ± 4.04a	88.33 ± 3.82c	0.000	0.002 ± 0.00a	25.16 ± 2.37e	0.000	0.000	1,766.70 ± 288.68c
	Control	278.33 ± 17.56c	518.90 ± 41.26c	0.065 ± 0.01c	2.250 ± 0.15	49.01 ± 2.86	0.685 ± 0.07b	0.019 ± 0.01c	2,666.67 ± 152.75a

Means ± SD in a column that have no lowercase letters (a, b, c, d, e) in common are significantly different from each other (ANOVA with post hoc Tukey's HSD, $p < 0.05$)

Standard deviation, $n = 3$

Discussion

Industrial effluent generation is on the rise in fast growing economies as India. While stringent regulatory norms are available statutorily, their implementation at fundamental levels is an obvious missing reality, in particular for tannery effluent discharges.

Concentrations of different physico-chemical parameters of the tannery wastewater were comparable to those reviewed by Durai and Rajasimman (2011). The observations of Munawar (1970) and Kannan (2006) suggest that algae grow luxuriantly with great variety and abundance in waters rich in calcium. The present data of the effluent characteristics also showed that calcium is possibly one of the favorable factors for growth of *C. vulgaris* in Kanpur tannery effluent. Besides calcium, high amounts of nitrates and phosphates and other oxidizable organic matter in the effluent also could be contributing to the growth of this microalgal strain as suggested by Murugesan and Sivasubramanian (2005) and Burch et al. (2001).

The biochemical oxygen demand (BOD) and chemical oxygen demand (COD) are important parameters to determine the water quality. Both of these parameters are greatly affected by the pollution load resulting from tannery industries (Islam and Suravi 2010). BOD in TW was reduced significantly ($p < 0.001$) by 95.93 % and that of COD by 94.74 % after treatment. Our strain, *C. vulgaris,* was efficient in reducing the levels to within the standards set by BIS (1994). These reductions were much higher within 21 days than those reported by Nanda et al. (2010) for *Nostoc* sp.-treated TW. Their strain of *Nostoc* sp. could bring down BOD by only 57.5 % and COD by only 37.8 % in 28 days using 1:5 TW diluted with BG11 medium. Therefore, our approach of diluting the TW by 50 % merely with tap water seems a more pragmatic and inexpensive approach. In order to achieve adequate improvement of the TW for safe discharge, the wastewater could be diluted and growing *C. vulgaris* in it for a fortnight or so would prove ecologically advantageous.

Results from this study demonstrate higher reduction of nutrients (nitrates, phosphates and sulphates) by *C. vulgaris* from TW when compared with those reported by Ajayan et al. (2015), for *Scenedesmus* sp. and Adam et al. (2015), for *Tetraselmis* sp. Remarkably, the nutrient levels were reduced to well below the maximum permissible limits of BIS.

Total dissolved solids (TDS) is an important chemical parameter of water, which mainly indicates the presence of various minerals including nitrate, nitrite, phosphate, sulphates, metallic ions, alkalis, and acids in both colloidal and dissolved forms (Rahman et al. 2012; Kabir et al. 2002). The *C. vulgaris* strain we tested has only a moderate potential for reducing the TDS contents in the tannery wastewater when compared to the BIS (1994) permissible limit.

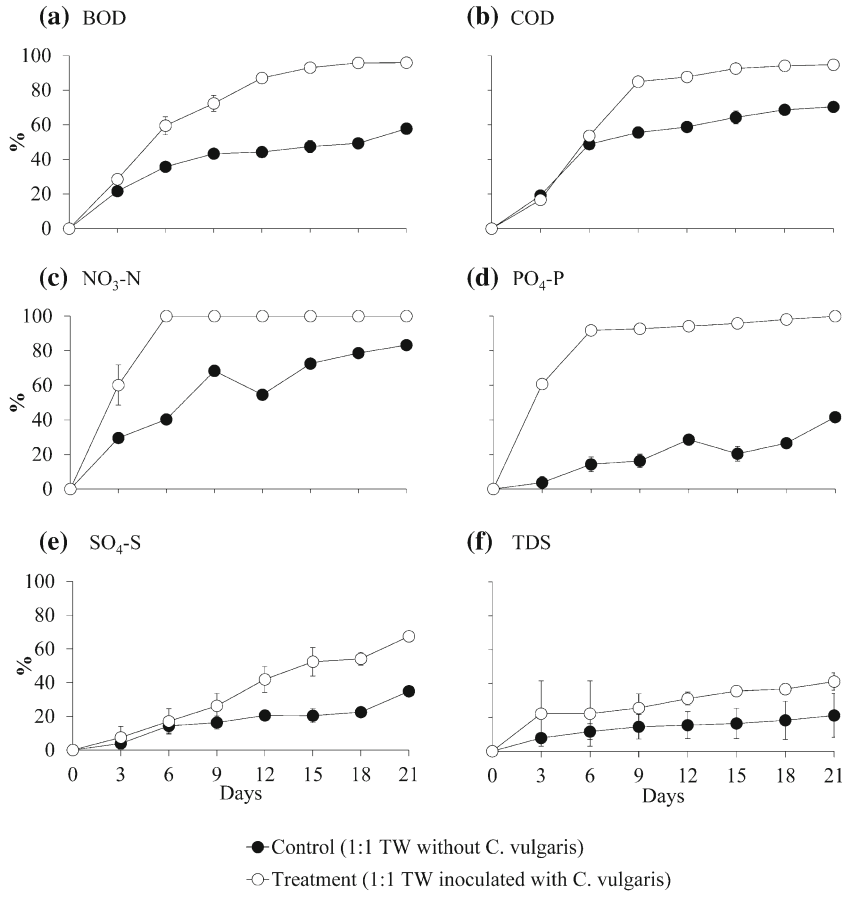


Fig. 2 Reduction efficiencies for pollutants of tannery effluent after treatment with *C. vulgaris*

Most pertinently, the strain of *C. vulgaris* we studied could efficiently biotransform Cr^{6+} from Kanpur tannery wastewater. This culture could achieve 100 % biotransformation of Cr^{6+} from the TW in about 12 days. As Cervantes et al. (2001) also noted in the case of *Oscillatoria*, *Phormidium*, *Scenedesmus*, and *Pandorina* spp., the present isolate seems to be useful for bio-sorption studies for the removal and/or biotransformation of Cr from contaminated sources. These kinds of algae biotransform/detoxify heavy metal ions usually through the process of biosorption, adsorption, and bioaccumulation

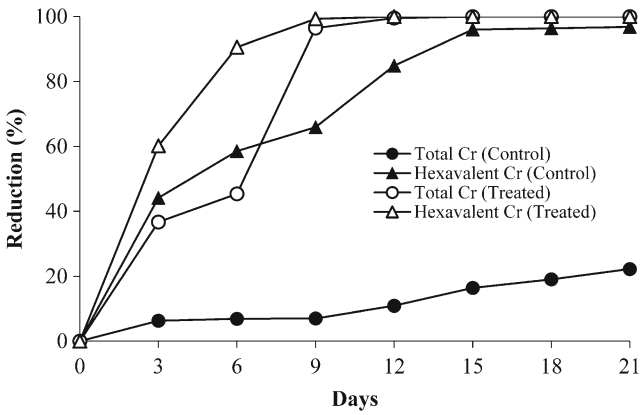


Fig. 3 Reduction efficiencies for total and hexavalent chromium concentrations of TW after treatment with *C. vulgaris*

(Rehman and Shakoori 2001; Gin et al. 2002; Rehman et al. 2007). Rehman (2011) reported 68 % reduction in Cr concentrations from TW in 20 days using *Euglena proxima*. In comparison to the previous results, the transformation potential of *C. vulgaris* is far superior.

Toxicity tests are indispensable tools to evaluate the quality and pollutant charge of the effluent. The brine shrimp, *Artemia salina* is a microcrustacean used frequently in toxicity studies, due to the ease of culture, short generation time, low commercial cost as dormant eggs (cysts), and cosmopolitan distribution (Vanhaecke et al. 1981). Hasegawa et al. (2014) determined the toxicity of tannery wastewater, before and after zinc oxide-assisted photocatalytic treatment, using dry shrimp eggs. Islam et al. (2014) studied the toxic effects of varying dilutions of tannery wastewater on brine shrimp nauplii.

Toxicity testing of tannery wastewater in this study, ascertained that the untreated wastewater is deleterious to the hatchlings of *A. salina*. The treatment of tannery wastewater with *C. vulgaris* unambiguously promoted the survival of the nauplii which can be directly ascribed to the reduction of toxic effects of treated effluent on *A. salina*. The results are useful to ascertain the decrease in toxicity in *C. vulgaris*-treated effluent to *Artemia* larvae. This can be attributed to the substantial reduction in concentrations of the toxicants that were in far higher concentrations than BIS (1994) permitted limits.

Table 3 Significant differences in the removal efficiencies after treatment for each parameter (one-way ANOVA, $p < 0.05$)

	Source of variance	*DF*	Sum of squares	Mean of squares	*F* value	*P* value
BOD	Between days	7	1,192,896.328	170,413.761	343.881	3.16E-16
$n = 24$	Within days	16	7,928.965	495.560	—	—
	Total	23	1,200,825.293	—	—	—
COD	Between days	7	8,553,938.972	1,221,991.282	1,139.862	2.27453E-20
$n = 24$	Within days	16	17,152.825	1,072.052	—	—
	Total	23	8,571,091.798	—	—	—
NO_3–N	Between days	7	0.377	0.054	253.230	3.56942E-15
$n = 24$	Within days	16	0.003	0.0002	—	—
	Total	23	0.380	—	—	—
PO_4–P	Between days	7	34.053	4.865	306.068	7.96224E-16
$n = 24$	Within days	16	0.254	0.016	—	—
	Total	23	34.307	—	—	—
SO_4–S	Between days	7	7,487.319	1,069.617	74.234	5.26261E-11
$n = 24$	Within days	16	230.539	14.409	—	—
	Total	23	7,717.859	—	—	—
Total Cr	Between days	7	2.205	0.315	117.286	1.51693E-12
$n = 24$	Within days	16	0.043	0.0027	—	—
	Total	23	2.248	—	—	—
Cr^{6+}–Cr	Between days	7	0.837	0.120	254.923	3.38616E-15
$n = 24$	Within days	16	0.008	0.00047	—	—
	Total	23	0.845	—	—	—
TDS	Between days	7	3,696,250	528,035.714	7.823	0.000347
$n = 24$	Within days	16	1,080,000	67,500	—	—
	Total	23	4,776,250	—	—	—

Table 4 Toxicity testing in *A. salina* exposed to untreated water and in water treated with *C. vulgaris*

		Artemia survival (%)		
Days		24 h	48 h	72 h
0	Treatment	83.9 ± 1.7a	81.4 ± 5.4ab	38.1 ± 23.7b
	Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3	Treatment	95.8 ± 6.4a	95.8 ± 6.4a	72.5 ± 8.8
	Control	91.4 ± 1.5	75.9 ± 1.4	17.6 ± 3.5
6	Treatment	91.5 ± 20.0a	71.3 ± 20.6ab	21.7 ± 9.8b
	Control	78.5 ± 2.3	69.2 ± 6.1	4.9 ± 3.1
9	Treatment	100.0 ± 0.0a	78.6 ± 18.9a	5.6 ± 3.2
	Control	76.9 ± 4.6	59.7 ± 5.1	6.7 ± 10.2
12	Treatment	100.0 ± 0.0a	87.2 ± 8.9a	14.1 ± 12.8
	Control	77.4 ± 1.5a	77.9 ± 11.2a	11.6 ± 5.6
15	Treatment	100.0 ± 0.0a	88.0 ± 3.0a	27.1 ± 9.8
	Control	78.0 ± 0.0a	78.3 ± 8.6a	8.1 ± 4.6
18	Treatment	100.0 ± 0.0a	90.0 ± 3.5a	18.9 ± 9.7
	Control	76.9 ± 4.6	64.3 ± 12.7	3.1 ± 4.6
21	Treatment	100.0 ± 0.0a	93.0 ± 7.1a	20.6 ± 14.7
	Control	72.0 ± 0.0	61.9 ± 3.7	2.6 ± 3.9
Seawater		100.0 ± 0.0a	100.0 ± 0.0a	74.8 ± 17.8

Means ± SD in a row that have no lowercase letter (a, b) in common are significantly different from each other (ANOVA with post hoc Tukey's HSD, $p < 0.05$)

Standard deviation, $n = 5$

Control—tannery wastewater without inoculated algae, *Treatment*—tannery wastewater inoculated with *C. vulgaris*

Further, the biotransformation of toxic metals, in general, hexavalent chromium from the wastewater by *C. vulgaris* ought to be facilitating the prolonged survival. Therefore, the toxicity results of this work showed that survival of *A. salina* was an important tool to evaluate the efficacy of TW bioremediation by *C. vulgaris*.

Our salt-tolerant *Chlorella* strain grew the least in 100 % and quite poorly in 70 % TW, as explained in Methods section. This was the case even after increasing inoculum size, lengthening of light periods and elevated aeration. While the growth of this strain was far superior in the 3:7 or 1:9 TW, to ensure that the salt-tolerant strain would not experience undue hypo-osmotic stress with more diluted TW, we resorted to test its bioremediation efficiency in 1:1 TW. Since tannery effluent is a mixture of innumerable alkalis/chorides/salts, our approach of using 1:1 dilution is very likely to be effective in the reduction of COD, BOD, nutrients, and chromium. Further, as can be deciphered from the growth curve, the profuse growth of *Chlorella* NIOCCV in 1:1 TW generates substantial biomass that can be useful variously, as has been proposed by Huang et al (2010), for biofuel production in particular. Notably, the accumulation of troublesome sludge in conventional treatment methods is certainly avoided by phycoremediation. Additionally, unlike the use of costly chemical medium, as done for instance by Nanda et al (2010), our approach of using fresh water for dilution ought to be beneficial for the industry in some production processes. In that, by separating the algal biomass—that could be of use as a feeder material in a variety of other uses—the treated water can be recycled in the industry itself or for controlled irrigation of forests, lawns, and other

greeneries. The latter intent of the use of microalga-treated TW for irrigating the non-food crops is an eco-friendly approach of carbon sequestration. Further, as Orandi and Lewis (2013) also proposed, the rigorous recovery of accumulated metals from the algal biomass can be achieved through washes with dilute acid or via adsorption/desorption cycles of alga itself.

In conclusion, this study is useful to ascertain that treatment by *C. vulgaris* has significance in reducing pollution load from tannery wastewater with high efficiency. Therefore, this algal species can be used as an alternate, potentially low cost method for treating tannery effluent before releasing into natural systems. Such methods seem to offer economic treatment and may be effective in minimizing the environmental impact. These very promising results call for elaborated studies on the use of algal species for promulgating functional phycoremediation strategies in the future.

Acknowledgments We thank the Director, CSIR—National Institute of Oceanography for facilities and encouragement. The work was supported under the CSIR project BSC0111 "Integrated NextGen Approaches in Health, Disease and Environmental Toxicity (INDEPTH)". This is CSIR-NIO contribution number 5913.

References

Adam S, Suresh Kumar P, Santhanam P, Dinesh Kumar S, Prabhavathi P (2015) Bioremediation of tannery wastewater using immobilized marine microalga *Tetraselmis* sp.: experimental studies and pseudo-second order kinetics. J Mar Biol Oceanogr 4:1

Ajayan KV, Selvaraju M, Unnikannan P, Sruthi P (2015) Phycoremediation of tannery wastewater using microalgae *Scenedesmus* species. Int J Phytoremed 17:907–916

APHA (2005) Standard methods for the examination of water and wastewater, 20th edn. APHA-AWWA-WPCF, Washington DC

Balaji S, Kalaivani T, Sushma B, Varneetha Pillai C, Shalini M, Rajasekaran C (2015) Characterization of sorption sites and differential stress response of microalgae isolates against tannery effluents from Ranipet industrial area—an application towards phycoremediation. Int J Phytoremediation. doi:10.1080/15226514.2015.1115960

Bosnic M, Buljan J, Daniels RP (2000) Pollutants in tannery effluents. US/RAS/92/120, United Nations Industrial Development Organization, Regional Programme for Pollution Control in the Tanning Industry in South-East Asia

Burch MD, Chow CWK, Hobson P (2001) Algaecides for control of toxic cyanobacteria. Proceedings of the 2001 Water Quality Technology Conference. American Water Works Association, Nashville

Bureau of Indian Standards (BIS) (1994) Quality tolerance for water for Tanning industry. IS: 4221, New Delhi

Cervantes C, Campos-Garcia J, Devars S, Gutierrez-Corona F, Loza-Tavera H, Torres-Guzman JC, Moreno-Sanchez R (2001) Interactions of chromium with microorganisms and plants. FEMS Microbiol Rev 25:335–347

Chellam CT, Sampathkumar P (2012) Bio removal of nutrients in tannery effluent water using marine micro algae, *Chlorella marina*. Proceedings of International Forestry and Environment Symposium, Sri Lanka. Published by Department of Forestry and Environmental Science, University of Sri Jayewardenepura, Vol. 17

Chu W-L, See Y-C, Phang S-M (2008) Use of immobilised *Chlorella vulgaris* for the removal of colour from textile dyes. J Appl Phycol 21:641–648

Craggs R, Sutherland D, Campbell H (2012) Hectare-scale demonstration of high rate algal ponds for enhanced wastewater treatment and biofuel production. J Appl Phycol 24:329–337

Dixit S, Singh DP (2014) An evaluation of phycoremediation potential of cyanobacterium *Nostoc muscorum*: characterization of heavy metal removal efficiency. J Appl Phycol 26:1331–1342

Dotro GC (2003) Assessment and design framework for the use of constructed wetlands to treat tannery effluents. Master Thesis, University of Calgary

Dunn K (1998) the biotechnology of high rate algal ponding systems in the treatment of saline tannery wastewaters. PhD thesis. Rhodes University, South Africa.

Dunn K, Maart B, Rose P (2013) *Arthrospira* (Spirulina) in tannery wastewaters. Part 2: evaluation of tannery wastewater as production media for the mass culture of *Arthrospira* biomass. Water SA 39:279–284

Durai G, Rajasimman M (2011) Biological treatment of tannery wastewater—a review. J Envi Sci Technol 4:1–17

Food and Agriculture Organization (FAO) (2004) The State of Food and Agriculture. FAO Agriculture Series No. 35

Gin KY, Tangm YZ, Aziz MA (2002) Derivation and application of a new model for heavy metal biosorption by algae. Water Res 36:1313–1323

Hasegawa MC, de Souza Daniel JF, Takashima K, Batista GA, da Silva SMCP (2014) COD removal and toxicity decrease from tannery wastewater by zinc oxide-assisted photocatalysis: a case study. Environ Technol 35:1589–1595

Hernández-Zamora M, Cristiani-Urbina E, Martínez-Jerónimo F, Perales-Vela HV, Ponce-Noyola T, Montes-Horcasitas MC, Cañizares-Villanueva RO (2015) Bioremoval of the azo dye Congo Red by the microalga *Chlorella vulgaris*. Environ Sci Pollut Res 22:10811–10823

Huang G, Chen F, Wei D, Zhang X, Chen G (2010) Biodiesel production by microalgal biotechnology. Appl Energ 87:38–46

Islam MS, Suravi MNT (2010) Investigation on water quality in the Ashuliabeel, Dhaka. Bangladesh J Fish Res 14:55–64

Islam LN, Mahmud ARZ, Nurun Nabi AHM, Hossain M, Mohasin M (2014) Assessment of physicochemical and biochemical qualities of tannery effluents of Hazaribagh, Dhaka, and comparison with non-tannery water samples. Int J Environ 4:68–81

Jaysudha S, Sampathkumar P (2014) Nutrient removal from tannery effluent by free and immobilized cells of marine microalgae *Chlorella salina*. Int J Environ Biol 4:21–26

Kabdasli I, Tunay O, Cetin MS, Olmez T (2002) Assessment of magnesium ammonium phosphate precipitation for the treatment of leather tanning industry wastewaters. Water Sci Technol 46:231–239

Kabir ES, Kabir M, Islam SM, Mia C, Begum N, Chowdhury DA, Sultana SM, Rahman SM (2002) Assessment of effluent quality of Dhaka export processing zone with special emphasis to the textile and dying industries. Jahangirnagar Univ J Sci 25:137–138

Kannan S (2006) Biodiversity of cyanobacteria in freshwater ponds of Poondi, Thanjavur. M.Phil. dissertation, Bharathidasan University, Tiruchirapalli

Kennedy L (1999) Cooperating for survival: tannery pollution and joint action in the Palar Valley (India). World Dev 27:1673–1691

Khwaja AR, Singh R, Tandon SN (2001) Monitoring of Ganga water and sediments vis-a-vis tannery pollution at Kanpur (India): a case study. Environ Monit Assess 68:19–35

Kiviranta J, Sivonen K, Niemela SI, Huovinen K (1991) Detection of toxicity of cyanobacteria by *Artemia salina* bioassay. Environ Toxicol Water Qual 6:423–436

Leta S, Assefa F, Gumaelius L, Dalhammar G (2004) Biological nitrogen and organic matter removal from tannery wastewater in pilot plant operations in Ethiopia. Appl Microbiol Biotechnol 66:333–339

Mara DD, Pearson HW, Silva SA, Rose PD, Maart BA, Dunn KM, Rowswell RA, Britz P (1996) Waste Stabilization Ponds:

Technology and Applications. High rate algal oxidation ponding for the treatment of tannery effluents. Water Sci Technol 33:219–227

McMullan G, Meehan C, Conneely A, Kirby N, Robinson T, Nigam P, Banat IM, Marchant R, Smyth WF (2001) Microbial decolourization and degradation of textile dyes. Appl Microbiol Biotechnol 56:81–87

Munawar M (1970) Limnological studies on freshwater ponds of Hyderabad, India. II The biocoenose-distribution of unicellular and colonial phytoplankton in polluted and unpolluted environments. Hydrobiologia 36:105–128

Murugesan S, Sivasubramanian V (2005) Cyanobacteria of Porur Lake, Chennai, Tamilnadu. Indian Hydrobiol 8:49–54

Mustafa E-M, Phang S-M, Chu W-L (2012) Use of an algal consortium of five algae in the treatment of landfill leachate using the high-rate algal pond system. J Appl Phycol 24:953–963

Nanda S, Sarangi PK, Abraham J (2010) Cyanobacterial remediation of industrial effluents, I. Tannery effluents. N Y Sci J 3:32–36

Orandi S, Lewis DM (2013) Biosorption of heavy metals in a photorotating biological contactor—a batch process study. Appl Microbiol Biotechnol 97:5113–5123

Oswald WJ (1988) Micro-algae and waste-water treatment. In: Borowitzka MA, Borowitzka LJ (eds) Micro-algal biotechnology. Cambridge University Press, Cambridge, pp 305–328

Posadas E, Bochon S, Coca M, García-González MC, García-Encina PA, Muñoz R (2014) Microalgae-based agro-industrial wastewater treatment: a preliminary screening of biodegradability. J Appl Phycol 26: 2335–2345

Rahman MS et al (2012) Water quality characterization of the Sundarbans Reserve Forest (SRF), Khulna for biodiversity consideration, Bangladesh. In: Silver jubilee conference. Bangladesh Chemical Society 20:67–68

Rai UN, Dwivedi S, Tripathi RD, Shukla OP, Singh NK (2005) Algal biomass: an economical method for removal of chromium from tannery effluent. Bull Environ Contam Toxicol 75:297–303

Rao HP, Ranjith Kumar R, Raghavan B, Subramanian V, Sivasubramanian V (2011) Application of phycoremediation technology in the treatment of wastewater from a leather-processing chemical manufacturing facility. Water SA 37:7–14

Rehman A (2011) Heavy metals uptake by *Euglena proxima* isolated from tannery effluents and its potential use in wastewater treatment. Russ J Ecol 42:44–49

Rehman A, Shakoori AR (2001) Heavy metal resistant *Chlorella* spp. isolated from tannery effluents, and their role in remediation of hexavalent chromium in industrial wastewater. Bull Environ Contam Toxicol 66:542–547

Rehman A, Farah RS, Shakoori AR (2007) Heavy metal resistant *Distigma proteus* (Euglenophyta) isolated from industrial effluents and its possible role in bioremediation of contaminated wastewaters. World J Microbiol Biotech 23:753–758

Saranraj P, Sujitha D (2013) Microbial bioremediation of chromium in tannery effluent: a review. Int J Microbiol Res 4:305–320

Sheehan J, Dunahay T, Benemann J, Roessler P (1998) A look back at the U.S. Department of Energy's Aquatic Species Program—biodiesel from algae, Golden, CO, National Renewable Energy Institute, Report No. NREL/TP-580-24190, p. 328

Shi J, Podola B, Melkonian M (2007) Removal of nitrogen and phosphorus from wastewater using microalgae immobilized on twin layers: an experimental study. J Appl Phycol 19:417–423

Srivastav AK (2012) Chromium removal from tannery industry waste water by fungus. Thesis, Thapar University, Punjab

Strickland JDH, Parsons TR (1968) A practical handbook of seawater analysis. Bull Fish Res Board Canada 167:49–62

Tadesse I, Isoaho S, Green F, Puhakka J (2003) Removal of organics and nutrients from tannery effluent by advanced integrated wastewater pond systems technology. Water Sci Technol 48:307–314

Tastan BE, Ertugrul S, Donmez G (2010) Effective bioremoval of reactive dye and heavy metals by *Aspergillus versicolor*. Bioresour Technol 101:870–876

Vanhaecke P, Persoone G, Claus C, Sorgeloos P (1981) Proposal for a short-term toxicity test with *Artemia nauplii*. Ecotox Environ Saf 5: 382–387

International Journal of Phytoremediation

ISSN: 1522-6514 (Print) 1549-7879 (Online) Journal homepage: http://www.tandfonline.com/loi/bijp20

Efficient bioremediation of tannery wastewater by monostrains and consortium of marine Chlorella sp. and Phormidium sp.

Cindrella Das, Nagappa Ramaiah, Elroy Pereira & K. Naseera

To cite this article: Cindrella Das, Nagappa Ramaiah, Elroy Pereira & K. Naseera (2018) Efficient bioremediation of tannery wastewater by monostrains and consortium of marine Chlorella sp. and Phormidium sp., International Journal of Phytoremediation, 20:3, 284-292, DOI: 10.1080/15226514.2017.1374338

To link to this article: https://doi.org/10.1080/15226514.2017.1374338

Accepted author version posted online: 20 Oct 2017.
Published online: 20 Oct 2017.

INTERNATIONAL JOURNAL OF PHYTOREMEDIATION
2018, VOL. 20, NO. 3, 284–292
https://doi.org/10.1080/15226514.2017.1374338

Efficient bioremediation of tannery wastewater by monostrains and consortium of marine *Chlorella* sp. and *Phormidium* sp.

Cindrella Das, Nagappa Ramaiah, Elroy Pereira, and K. Naseera

Biological Oceanography Division, CSIR - National Institute of Oceanography, Dona Paula, Goa, India

ABSTRACT

This study evaluated the bioremediation potential of two marine microalgae *Chlorella* sp. and *Phormidium* sp., both individually and in consortium, to reduce various pollutants in tannery wastewater (TW). The microalgae were grown in hazardous 100% TW for 20 days, and the reductions in biochemical oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (TN), total phosphorous (TP), chromium (Cr) and total dissolved solids (TDS) of the wastewater monitored periodically. Both marine isolates reduced the BOD and COD by ≥90% in the consortium and by over 80% individually. Concentrations of TN and TP were reduced by 91.16% and 88%, respectively, by the consortium. Removal/biosorption efficiencies for chromium ranged from 90.17–94.45%. Notably, the TDS, the most difficult to deal with, were reduced by >50% within 20 days by the consortium. The novel consortium developed in this study reduced most of the ecologically harmful components in the TW to within the permissible limits of discharge in about 5 to 15 days of treatment. Thus, both the tested marine strains of *Chlorella* and *Phormidium* sp. are promising for bioremediating/detoxifying TW and adequately improve the water quality for safe discharge into open water bodies, in particular when used as a consortium.

KEYWORDS

bioremediation; tannery wastewater; microalgae; consortium

Introduction

Growing economies, affluent lifestyles, and technology advancements of the recent decades have ushered in a plethora of complex and environmentally unsustainable situations. To meet up with the consumer demands, the tanning industry that caters to leather products and goods, is also growinhg rapidly. In spite of fierce competition from synthetic analogues, demand for genuine leather products is continually increasing the world over. The highly labour intensive tanning industry employs a large number of low-skilled artisans who are mostly poverty-stricken and socially disadvantaged (Varadarajan and Krishnamoorthy 1993). It is known for causing deleterious impacts on the environment through the discharge of voluminous, highly toxic, chemically laden wastewaters into natural water bodies. Literature is burgeoning with reports on how tanneries process animal hides into leather through many successive complex stages which consume large quantities of water and chemicals, for instance, lime, sodium sulfide, ammonium sulphate, sodium chloride and chromium salts (Thanikaivelan et al. 2005; Covington and Covington 2009; Lufrano et al. 2013). The resulting wastewater is highly saline, high in organic loads and specific pollutants such as sulfides and chromium (Tunay et al. 1999; Song et al. 2000).

The toxic effects of chromium are valence dependent. Hexavalent chromium — Cr (VI) is highly soluble, mutagenic and carcinogenic whereas the trivalent form — Cr (III) is less soluble, thus less mobile and less bioavailable (Ackerley et al. 2006; Xu et al. 2009). Reviews of Biradar et al. (2012) and Prabhahar et al. (2012) highlight that hexavalent chromium is the most dangerous environmental pollutant from tanneries due to its corrosive effect. It affects the skin and respiratory tract, irritates mucus membranes, and is a causative agent of tubular necrosis of kidneys, chronic bronchitis, mutations, and lung carcinoma in humans. A lot of new chemical and physical techniques have been tried and are being developed to strip and recover Cr (VI) from the effluent. However, these require high inputs of energy and expensive chemicals (Xu et al. 2009). Moreover, the large amounts of sludge generated by these conventional wastewater treatment processes must be sent off-site for treatment and disposal, further increasing operational costs. Hence, the high-cost factor, coupled with recent increasingly stringent environmental regulations and safety standards (Bosnic et al. 2000; Kabdasli et al. 2002), has led to the search for new and effective wastewater treatment technologies.

In comparison with physicochemical treatment technologies for wastewaters, microalgae based treatments are proving to be far more efficient and safe, besides removing toxicants including heavy metals at less cost (Christenson et al. 2011; Ding et al. 2014). Microalgae are among the fastest growing, photosynthesizing organisms and can complete an entire growth cycle every half-a-day if adequate amounts of sunlight, water, carbon dioxide, and nutrients are available. The eukaryotic microalgae and cyanobacteria are ideal for economic and ecofriendly removal of nutrients from wastewaters because of their high N and P requirement for growth (Mata et al. 2012). For most microalgae, wastewater serves as a suitable medium as it supplies most of the necessary nutrients for their growth. This attribute can be used to significantly reduce the cost associated

CONTACT Dr. Nagappa Ramaiah ramaiah@nio.org Biological Oceanography Division, CSIR - National Institute of Oceanography, Dona Paula, Goa 403004, India.

with wastewater treatment, as well as to mitigate greenhouse gas emissions (Pittman et al. 2011). As also suggested by Rawat et al. (2011), microalgal biomass generated from wastewater treatment plants, as opposed to the accumulation of hazardous sludge in conventional treatment methods, can be utilized for production of bioenergy, animal feed, pharmaceuticals, and fertilizers.

Various reports are available on bioremediation which employ microalgae for the treatment/detoxification of a variety of wastewaters (Oswald 1988; Mara et al. 1996; Tadesse et al. 2003; Shi et al. 2007; Chu et al. 2008; Craggs et al. 2012; Mustafa et al. 2012; Dixit and Singh 2014; Posadas et al. 2014). Studies of Silva-Benavides and Torzillo (2011), Singh et al. (2011), Renuka et al. (2013), Chinnasamy et al. (2010), Riano et al. (2011) emphasize the advantages of wastewater treatment and biomass production by consortia of microalgae. The importance of consortia, as against single organisms, has been illustrated by the studies of Bhatnagar et al. (2010) and Silva-Benavides and Torzillo (2011), regarding survival, biomass production, as well as nutrient removal. Also, the algal consortia can be of practical value as some of them may grow far better together in wastewaters, or the loss of one alga from the consortium may be compensated by the continued/luxuriant growth of other alga(e) (Chinnasamy et al. 2010). Hence, there is urgent need to screen promising native microalgae from wastewaters and other water sources that not only have the potential to sequester excessive nutrients but also can form consortia, with extensive biomass applications after harvesting from the contaminated sites.

While many studies on the use of microalgal cultures for bioremediation of wastewaters are known, isolation, screening, and characterization of environmental isolates with better bioremediation potential are essential to developing efficient bioremediation strategies. Further, development of microalgal consortia through new searches needs to continue to obtain sets of microalgae that can effectively remediate hazardous wastewaters. From the recent literature on TW treatment (Ajayan et al. 2015; Ajayan and Selvaraju 2012; Rehman 2011; Chandra et al. 2004), it is discernible that the studies have either focused on diluting the wastewater, or on examining Cr (VI) reduction only. Moreover, the use of *marine* microalgal strains for examining their potential in this regard needs focus. This is to not only reduce/biotransform Cr (VI) but also to find out whether other toxic components convert to nonhazardous state. Hence, in this study, we aimed at examining the bioremediation potential of marine strains of *Chlorella* sp. and *Phormidium* sp., individually and in the consortium, on hazardous tannery wastewater. Through this approach, it was aimed to improve the tannery wastewater for safe discharge into the open or for various other uses listed in Das et al. (2017). Our study proposes an uncomplicated and effective method for treating 100% raw tannery wastewater and assesses the advantages of consortium versus monoalgal culture for bioremediation.

Materials and methods

Characteristics of tannery wastewater

Tannery wastewater (TW) sample was collected from a tannery CETP located at Pallavaram, Chennai, Tamil Nadu, India. Various physical and chemical parameters of the wastewater were analyzed using standard methods listed in APHA (2005). Measurements of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were made following standard methods described in APHA (2005). The BOD was determined using the 5-day BOD test and the COD, using open reflux oxidation method. The algal cells were separated from wastewater by filtration using 0.45μm glass microfiber filters, and the filtrate was used for measurement of total nitrogen (TN) following the Kjeldahl method and total phosphorous (TP) using the ascorbic acid method (APHA 2005). For measuring total chromium concentrations, the samples were digested with concentrated nitric acid followed by filtration through 0.45μm filter paper. The filtrate was completely oxidized to hexavalent chromium using potassium permanganate and analyzed by colorimetric Diphenylcarbazide (DPC) method (APHA 2005). Total solids (TS), total suspended solids (TSS) and total dissolved solids (TDS) were determined by the gravimetric method as per APHA (2005). TS was calculated by measuring the weight of residual solids from TW after drying known sample volumes in pre-weighed beakers at 180°C. For measurement of TSS and TDS known sample volumes were filtered through pre-weighed 0.7μm glass microfiber filter papers. The residual solids on the filter paper and in the filtrate were dried at 180°C and calculated as TSS and TDS, respectively. Bacterial counts in the tannery wastewater were determined using standard dilution plate method. For this, 0.1, 0.2 and 0.3 mL aliquots of 1000X diluted tannery wastewater were plated on nutrient agar (HiMedia, Mumbai), incubated at 28±0.5°C for 48 h and final counts of colony forming units were noted.

Isolation of microalgae

Marine green alga *Chlorella* sp. and marine cyanobacterium *Phormidium* sp. used in this study were isolated from coastal waters off Goa and cultured in algal culture medium (ACM, HiMedia, Mumbai) grown at a constant temperature of 28± 0.5°C, under fluorescent illumination of 150–300 μmol photons m^{-2} s^{-1} (with 12:12 h light:dark photoperiod). The acclimatized algae were used in the following experiments.

Experimental design

The bioremediation experiments were carried out in triplicates in 15L polypropylene tanks. These tanks were washed with diluted HNO_3 and then rinsed with deionized water. Ten litre aliquots of 100% strength TW were dispensed into the tanks. The effluent was not subjected to any pretreatment or additional nutrient supplementation. Three sets of treatments were set up; the first two sets were inoculated with single alga, *Chlorella* sp. or *Phormidium* sp., and one set with these two microalgae together, as a consortium. To each tank, 8.0 g of the wet algal cells were added on day 0. In the consortium tank, 4.0 g of each alga were added together. The experiment was carried out for 20 days at a constant temperature of 28±0.5°C under fluorescent illumination of sufficiently adequate light intensity 225 μmol photons m^{-2} s^{-1} (12:12 h light:dark photoperiod). This light intensity was chosen to ensure optimum illumination throughout the 10 L medium (fluid depth

15 cm). Two controls (one of TW without any algal inoculum and another of standard algae culture medium with algal cells) were included to compare the effect of TW on the growth and bioremediation potential of algae. Sub-samples of 100 mL aliquots from all experimental tanks were drawn on day 0, 5, 10, 15, 20 and analyzed for algal growth and different physicochemical parameters.

Analytical procedures

Measurements of BOD, COD, TN, TP, total Cr and TDS from the experimental setup were done as described above for TW characteristics. The TS and TSS were not analyzed because the algal biomass adds to the total/suspended solids, interfering with accurate measurements. Similarly, the increase in algal biomass was also not measured due to interference from suspended solid contents in the wastewater. Microscopic enumeration of *Chlorella* sp. was done by drawing triplicate aliquots of 0.1 mL culture from ACM on day 0 and day 20 and counting the individual cells at 400X. To avoid underestimation of *Phormidium* sp. cells, measuring Chl *a* was sought as a reliable option since the filaments of *Phormidium* cells clumped both in the ACM and TW. Hence, growth analyses of both *Chlorella* sp. and *Phormidium* sp. were done using Chl *a* measurements. On all sampling days, samples were stirred to maintain homogeneity, 10 mL aliquots drawn from all treatments, filtered through GF/C (0.7 μm, Whatman, USA) and the filter paper transferred into a vial with 10 mL 90% (v/v) methanol and held overnight for extraction. Chl *a* in this extract was measured spectrophotometrically following Parsons et al. (1984).

Reduction efficiency

The reduction/removal efficiencies (%) of the various parameters were calculated by following Ji et al. (2011).

$$R = \frac{(Ci - Ce)}{Ci} \times 100$$

Where,

R = the removal/reduction percentage at each measurement time,
Ci = the initial concentration of a given parameter, and
Ce = the remainder concentration on a given sampling day

Statistical analyses

The growth of algae individually or in the consortium, measured as an increase in Chl *a*, was correlated with reduction of various pollutant concentrations in the wastewater during the experimental period. Results are expressed as Pearson correlation coefficients of which values of $\geq$0.4, $\geq$0.6 and $\geq$0.8 are statistically significant with $p < 0.05$, < 0.01 and < 0.001, respectively. Two-way analysis of variance (ANOVA) was done to check the efficiency of microalgae, individually and in the consortium. This was to check if the reduction of measured toxicants were statistically significant. Subsequently, pair-wise comparisons were performed using post hoc Tukey's HSD (Honestly Significant Difference) tests to evaluate at which period of the experiment, the reduction of measured toxicants was statistically significant at $p < 0.05$.

Table 1. Physico-chemical characteristics of raw untreated tannery wastewater (TW).

Parameter	Raw TW	Max. permissible limits (BIS, 1994)
pH	7.76 ± 0.00	5.5–9.0
Salinity (g.L^{-1})	15.00 ± 0.30	—
TS (mg.L^{-1})	6761.00 ± 50.20	2200
TSS (mg.L^{-1})	1595.00 ± 30.40	100
TDS (mg.L^{-1})	5166.67 ± 288.70	2100
BOD (mg.L^{-1})	1520.00 ± 42.50	100
COD (mg.L^{-1})	3070.00 ± 51.20	250
TN (mg.L-1)	822.00 ± 51.20	100
TP (mg.L^{-1})	1.89 ± 0.05	1.0
Cr (mg.L^{-1})	9.57 ± 0.28	2.0
Bacterial count (cfu.mL^{-1})	3.82×10^4	—

(Standard deviation, n=3).

Results

Characteristics of tannery wastewater

The physicochemical characteristics of the tannery wastewater are presented in Table 1. The wastewater collected from the tannery CETP before its treatment process, had dark brown colour, salinity of 15 g.L^{-1}, high BOD, COD, TN, TP, Cr and TDS concentrations. The BOD and COD concentrations were as high as 1520 (±42.5) mg.L^{-1} and 3070 (±51.2) mg.L^{-1}, respectively. This untreated TW had high concentrations of TN (822±51.2 mg.L^{-1}), TP (1.89±0.05 mg.L^{-1}) and total chromium (9.57±0.28 mg.L^{-1}). Notably, the TDS (5166.67± 288.7 mg.L^{-1}) were higher than TSS (1595±30.4 mg.L^{-1}), indicating high contents of dissolved salts in the wastewater. Counts of bacterial colony forming units in the tannery effluent sample were 3.82×10^4 cfu.mL^{-1}.

Growth of algae in tannery wastewater

The growth of the microalgae, individually and as a consortium, in TW was about 1.5 times lower than that in standard algal culture media. Chlorophyll *a* concentrations in the TW were observed to be the highest in the consortium (12.5± 0.49 mg.L^{-1}) by day 20, followed individually by *Chlorella* sp. (11.8±0.26 mg.L^{-1}) and *Phormidium* sp. (9.21±0.84 mg.L^{-1}) (Figure 1). In the consortium, the microalgal growth in TW was 1.34 times the growth of *Chlorella* and 1.5 times the growth of *Phormidium* strains in unialgal culture setups. Cell counts of

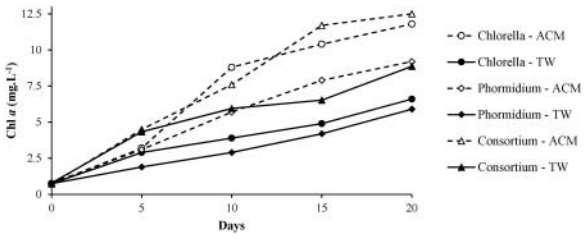


Figure 1. Growth of *Chlorella* sp., *Phormidium* sp. and their consortium in tannery wastewater. ACM=Algal culture medium; TW=Tannery wastewater.

Chlorella sp. were measured only at the beginning and end of the experiment. The initial cell count of *Chlorella* sp. on day 0 was 1.02×10^5 cells.mL^{-1} which increased to 9.0×10^5 cells. mL^{-1} by day 20. It is likely that the cell counts increased steadily (as is evident from the increasing trend in the concentration of Chl *a* (see Figure 1).

Reduction of different physicochemical parameters following algal growth

There was a significant reduction in all measured parameters in TW after treatment with algae (Table 2). Percent reduction of all examined parameters was significantly higher ($p<0.001$) in the monoalgal and consortium sets than in the control set. Maximum reduction of BOD from 1520±261 mg.L^{-1} to 100.25±8.5 mg.L^{-1} was observed in the consortium (Figure 2). As much as 93.4% of BOD was reduced by the algal consortium which was statistically highly significant ($p<0.001$). The level of BOD was brought down to near the BIS (1994) permissible limit of 100 mg.L^{-1} by day 20 in the consortium. Individually, *Chlorella* sp. and *Phormidium* sp. also reduced the BOD by 89.87% and 86.97%, respectively. Similarly, the COD reduction was the highest in the consortium (92.60±11.11%), followed by *Chlorella* sp. (86.43±13.82%) and *Phormidium* sp. (79.13± 3.95%). Algal consortium effectively brought down COD levels in TW from 3048±407 mg.L^{-1} to 225.46±12.9 mg.L^{-1} by day 20, which is within permissible limit of 250 mg.L^{-1} for safe discharge. Initial concentrations of TN (822±182 mg.L^{-1}) and TP (1.89±0.27 mg.L^{-1}) were reduced to 72.66±19.76 mg.L^{-1} and 0.21 mg.L^{-1}, respectively by the algal consortium, thereby meeting the BIS safe discharge standards by day 15. Removal of total chromium concentrations was as high as 94.45% with algal consortium followed individually by *Phormidium* sp. (93.18±4.65%) and *Chlorella* sp. (90.17±14.42%) (Table 2). In the consortium set up, the initial Cr content (9.57±1.6 mg. L^{-1}) was quite efficiently reduced, leaving behind about 0.52—0.98 mg.L^{-1}, which is far below the safe permissible discharge limit of 2 mg.L^{-1}. The high concentrations of TDS (>5000 mg.mL^{-1}) in untreated wastewater were reduced to 2155—2546 mg.mL^{-1} by day 20 (Figure 2) with an overall TDS removal of 53.46%. Highest TDS reduction of 58.28% was observed in the consortium. Notably, only the consortium brought down TDS levels close to the safe discharge limit of 2100 mg.mL^{-1}.

Statistically significant reduction ($p<0.001$) of all measured parameters was observed for the three treatments compared to the control set (Table 3), though these reduction efficiencies were observed to be significant on different days of treatments (Table 4). For instance, statistically significant reduction (post hoc Tukey's HSD, $p<0.01$) of BOD occurred up to day 5 of treatment in the *Chlorella* and consortium sets; and up to day 10 in the *Phormidium* set. However, reduction of BOD to permissible limits by the consortium was achieved by day 20. Similarly, the algal consortium aided significant ($p<0.01$) reduction of COD up to day 15, while the BIS permissible discharge limit was attained by day 20. Reduction of TN levels by the consortium was statistically significant ($p<0.01$) up to day 10 and safe discharge limit were attained by day 20. Significant reductions of TP and Cr were achieved within 5–10 days by *Chlorella* sp., *Phormidium* sp. and their consortium and permissible discharge limits by day 15. The permissible discharge limit for TDS was reached by day 20 in the consortium set with significant reductions occurring up to day 10.

Treatment of the tannery effluent with the consortium of *Chlorella* sp. and *Phormidium* sp. brought down the levels of BOD, COD and total chromium to within the permissible limits for safe discharge of effluents (Figure 2). The reduction of BOD, COD, TN, TP, Cr, and TDS in the TW correlated positively with the growth (measured as the increase in Chl *a*) of *Chlorella* sp. and *Phormidium* sp., individually and in the consortium. The positive trend in the correlation of Chl *a* with different parameters during the experimental period (Figure 3) can be ascribed to the growth-related reduction of these toxicants.

Discussion

A general apprehension about employing certain unicellular microalgae in wastewater treatment is their microscopic

Table 2. Percent reductions of different parameters in tannery wastewater after treatment with *Chlorella* sp., *Phormidium sp.* and their consortium.

	Day	BOD	COD	TN	TP	Cr	TDS
Control	5	10.52 ± 2.10	1.09 ± 0.21	20.33 ± 4.07	17.46 ± 3.49	0.51 ± 0.10	6.44 ± 1.29
	10	18.42 ± 3.68	10.47 ± 2.09	27.26 ± 5.45	31.22 ± 6.24	1.73 ± 0.35	7.73 ± 1.55
	15	35.52 ± 7.10	25.11 ± 5.02	52.89 ± 10.58	46.03 ± 9.20	18.90 ± 3.78	39.67 ± 7.93
	20	43.81 ± 8.76	39.35 ± 7.87	65.75 ± 13.15	55.55 ± 11.11	26.05 ± 5.20	42.35 ± 8.47
Chlorella sp.	5	51.25 ± 8.20	55.46 ± 8.87	14.02 ± 2.24	39.01 ± 6.24	32.84 ± 5.25	4.50 ± 0.72
	10	63.52 ± 10.16	65.81 ± 10.53	69.49 ± 11.12	45.97 ± 7.36	70.05 ± 11.21	43.86 ± 7.02
	15	81.26 ± 13.00	79.84 ± 12.78	86.85 ± 13.89	72.94 ± 11.67	81.38 ± 13.02	46.44 ± 7.43
	20	89.86 ± 14.37	86.43 ± 13.82	89.75 ± 14.36	83.07 ± 13.29	90.17 ± 14.42	51.37 ± 8.21
Phormidium sp.	5	49.58 ± 2.47	35.56 ± 1.78	26.79 ± 1.34	38.73 ± 1.94	45.76 ± 2.29	21.92 ± 1.10
	10	72.41 ± 3.62	58.27 ± 2.91	63.65 ± 3.18	42.33 ± 2.12	76.20 ± 3.81	43.22 ± 2.16
	15	79.53 ± 3.97	70.61 ± 3.53	81.74 ± 4.09	56.82 ± 2.84	90.45 ± 4.52	43.86 ± 2.19
	20	86.97 ± 4.34	79.13 ± 3.95	87.55 ± 4.38	76.19 ± 3.80	93.18 ± 4.66	50.71 ± 2.54
Consortium	5	62.29 ± 7.47	40.98 ± 4.92	32.49 ± 3.89	12.17 ± 1.46	58.75 ± 7.05	35.48 ± 4.26
	10	75.29 ± 9.03	64.25 ± 7.71	74.69 ± 8.96	50.79 ± 6.09	69.86 ± 8.38	56.77 ± 6.81
	15	84.94 ± 10.19	83.40 ± 10.00	88.90 ± 10.67	83.07 ± 9.97	92.51 ± 11.10	54.19 ± 6.50
	20	93.40 ± 11.21	92.60 ± 11.11	91.16 ± 10.93	88.88 ± 10.66	94.45±11.33	58.28 ± 6.99

(Standard deviation, n=3).

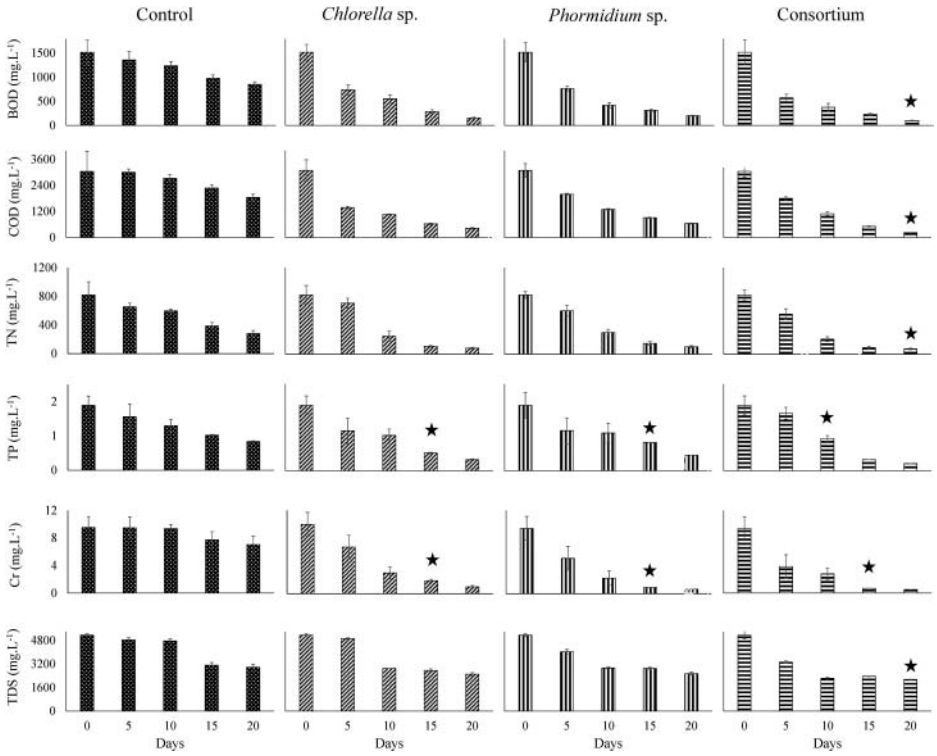


Figure 2. Reduction of pollutant concentrations from TW by *Chlorella* sp., *Phormidium* sp. and their consortium. (⋆) indicates concentrations that fall below the BIS permissible limit for safe discharge.

dimensions, which –as indicated by Grima et al. (2003)– makes biomass harvesting cumbersome and time-consuming. In this context, filamentous microalgae, with their larger dimensions (aiding with filtration/flocculation) and aggregate/mat forming properties, help to significantly reduce the harvesting cost as has been suggested by Chen et al. (2011) and Hori et al. (2002). Moreover, as demonstrated in this study, using the fastgrowing unicellular

Table 3. Significant differences in the removal efficiencies between treatments and days for each parameter (Two-way ANOVA).

	Source of variance	Sum of squares	Degree of freedom	Mean of squares	F value	P value
BOD	Intercept	34756804	1	34756804	2474.533	0.000000
n = 60	Treatment	3769403	3	1256468	89.455	0.000000
	Day	10610668	4	2652667	188.858	0.000000
	Treatment*Day	1010264	12	84189	5.994	0.000008
	Error	561832	40	14046	—	—
COD	Intercept	174205734	1	174205734	2321.208	0.000000
n = 60	Treatment	16044669	3	5348223	71.263	0.000000
	Day	38953965	4	9738491	129.761	0.000000
	Treatment*Day	4754580	12	396215	5.279	0.000031
	Error	3001983	40	75050	—	—
TN	Intercept	10685616	1	10685616	2391.952	0.000000
n = 60	Treatment	341605	3	113868	25.489	0.000000
	Day	4187391	4	1046848	234.335	0.000000
	Treatment*Day	235655	12	19638	4.396	0.000189
	Error	178693	40	4467	—	—
TP	Intercept	72.06757	1	72.06757	1503.244	0.000000
n = 60	Treatment	1.10735	3	0.36912	7.699	0.000355
	Day	15.69348	4	3.92337	81.837	0.000000
	Treatment*Day	1.30472	12	0.10873	2.268	0.026266
	Error	1.91766	40	0.04794	—	—
Cr	Intercept	1537.396	1	1537.396	1082.822	0.000000
n = 60	Treatment	265.290	3	88.430	62.283	0.000000
	Day	421.180	4	105.295	74.162	0.000000
	Treatment*Day	80.993	12	6.749	4.754	0.000089
	Error	56.792	40	1.420	—	—
TDS	Intercept	777045699	1	777045699	22939.98	0.000000
n = 60	Treatment	9586023	3	3195341	94.33	0.000000
	Day	58106037	4	14526509	428.85	0.000000
	Treatment*Day	8050715	12	670893	19.81	0.000000
	Error	1354920	40	33873	—	—

Table 4. Statistically significant differences in the reduction of different parameter (post-hoc Tukey's HSD, $p < 0.05$). Numbers in parenthesis indicate the days in which permissible limits for safe discharge were reached.

		No. of days*	Tukey HSD p-value	Tukey HSD inference
BOD	*Chlorella* sp.	5	0.0010053	** p<0.01
	Phormidium sp.	10	0.0010053	** p<0.01
	Consortium	5 (20)	0.0010053	** p<0.01
COD	*Chlorella* sp.	5	0.0010053	** p<0.01
	Phormidium sp.	10	0.0010053	** p<0.01
	Consortium	15 (20)	0.0014486	** p<0.01
TN	*Chlorella* sp.	10	0.0010053	** p<0.01
	Phormidium sp.	15	0.019637	* p<0.05
	Consortium	10 (20)	0.0010053	** p<0.01
TP	*Chlorella* sp.	5 (15)	0.0150289	* p<0.05
	Phormidium sp.	5 (15)	0.0451089	* p<0.05
	Consortium	15 (10)	0.0036347	** p<0.01
Cr	*Chlorella* sp.	10 (15)	0.0184453	* p<0.05
	Phormidium sp.	5 (15)	0.0083061	** p<0.01
	Consortium	5 (15)	0.0011342	** p<0.01
TDS	*Chlorella* sp.	20	0.0315698	* p<0.05
	Phormidium sp.	20	0.0192649	* p<0.05
	Consortium	10 (20)	0.0078187	** p<0.01

*Statistically significant reduction ($p < 0.05$) in pollutant concentration.

microalgae along with filamentous cyanobacteria in consortium appears to be a more advantageous approach for TW treatment.

Biochemical oxygen demand (BOD) and chemical oxygen demand (COD) are important parameters for assessing the water quality. The effectiveness of any treatment process is judged chiefly by the reduction of BOD and COD from the wastewater. These are measures of the amount of oxygen needed by microorganisms and chemical oxidants to oxidize the organics in the wastewater. High BOD and COD lead to

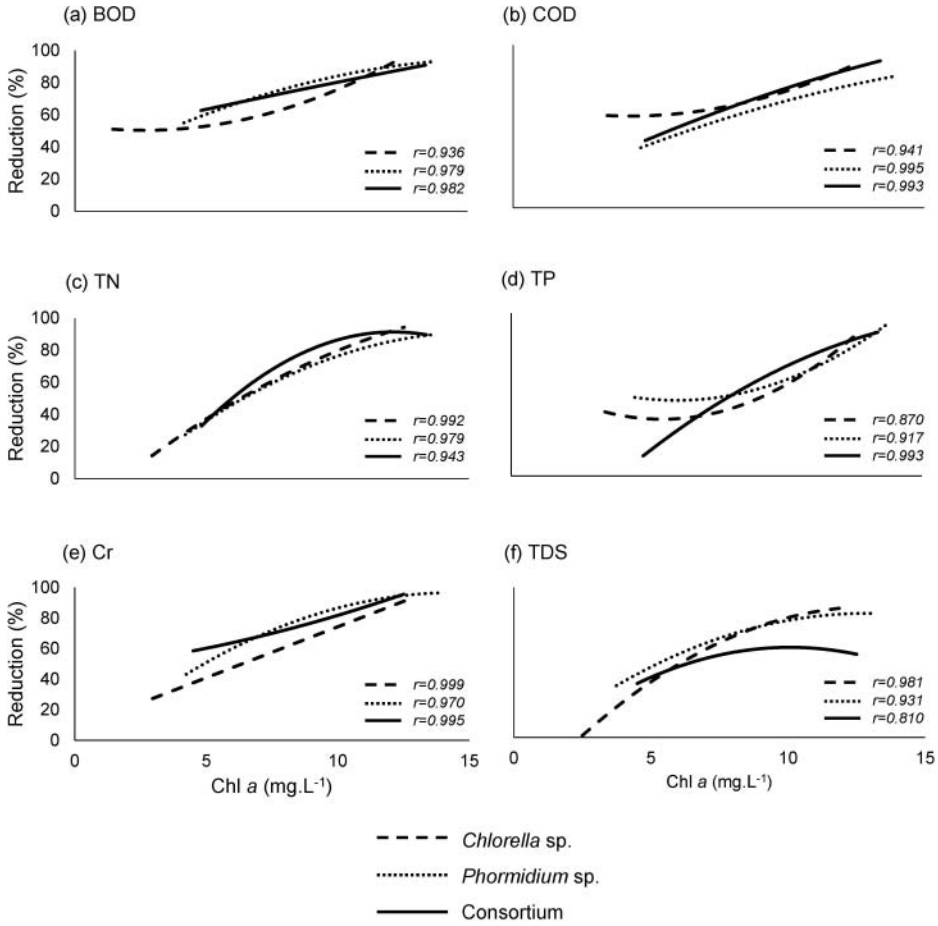


Figure 3. Correlation plots of the various pollutant removal efficiencies with the Chl *a* concentrations of *Chlorella* sp., *Phormidium* sp. and their consortium.

depletion of oxygen from water causing deleterious effects on *in situ* flora and fauna. The consortium set examined in this study brought down the BOD levels of TW remarkably to safe/ acceptable discharge limits. The highest reductions of BOD and COD were observed for the consortium treatment as compared to treatment with single alga. The concentration of BOD and COD in all three treatments were significantly ($p<0.05$) reduced by day 20.

The growth of both algae we examined in consortium strongly correlated with the reduction of BOD ($r=0.982$) and COD ($r=0.993$). These substantial reduction efficiencies of BOD (93.40%) and COD (92.60%) by day 20 are way higher than those reported by Nanda et al. (2010) for *Nostoc* sp., where BOD and COD in dilute TW (~15% strength) were reduced by 57.5% and 37.8%, respectively, in a longer treatment period of 28 days.

A major problem in wastewater treatment is the removal of nitrogenous and phosphorus compounds. Total nitrogen (TN) and total phosphorous (TP) are essential nutrients for growth of plants. Excess amounts of these in waters may lead to low levels of dissolved oxygen and negatively alter various plant life and organisms. Our approach of co-culturing *Chlorella* sp. and *Phormidium* sp. seems useful, in that, these algae in consortium reduced the TN concentration of TW to below the permissible limit of 100 $mg.L^{-1}$ by day 20. Similarly, TP concentrations were also efficiently reduced by day 15. These results are superior to those reported earlier by Silva-Benavides and Torzillo (2011) for co-culture of *Chlorella* sp. and *Plantothrix* sp. in municipal wastewater which is far more benign than the noxious full-strength TW. They demonstrated reduction of TN by 20% and that of PO_4-P by 25% in 4 days. Renuka et al. (2013) carried out bioremediation of sewage wastewater using a consortium of filamentous strains of microalgae and reduced the initial concentrations of NO_3-N and PO_4-P by 90% and 97% after 10^{th} and 6^{th} day of treatment. Although we did not quantify the protein content in the biomass produced in TW, it is likely that the TN taken up from the TW is assimilated to build the protein content and for other related metabolic functions.

The tolerance limit for total Cr for discharge into inland surface waters is 2 $mg.L^{-1}$ (BIS 1994). To comply with this limit, it is essential that industries treat their effluents to reduce its chromium concentration. A number of methods for the removal of metal ions from contaminated solutions have been reported including reduction, ion exchange, chemical and electrochemical precipitation, evaporation, solvent extraction, reverse osmosis and adsorption (Tiwari et al. 1989). Most of these methods suffer from drawbacks such as high capital and operational costs or the disposal of the residual and recalcitrant metal sludge. Algae, on the other hand, efficiently biotransform/detoxify heavy metal ions usually through the process of biosorption, adsorption and bioaccumulation (Gin et al. 2002; Rehman et al. 2007). As Orandi and Lewis (2013) proposed, the recovery of accumulated metals from the algal biomass can be achieved through washes with dilute acid or via adsorption/ desorption cycles of alga itself. Our marine strains of *Chlorella* sp. and *Phormidium* sp. substantially reduced chromium concentrations in TW by 81.38% and 90.45%, respectively. Furthermore, algal consortium reduced 92.51% of Cr by day 15. Rehman (2011) reported 68% reduction in Cr concentrations from TW in 20 days using *Euglena proxima*. Apparently, the chromium transformation potential of *Chlorella* and *Phormidium* sp. consortium is far greater.

The TDS content of wastewater is indicative of the presence of various inorganic solutes, minerals, chlorides, metallic ions, alkalis and acids in both colloidal and dissolved forms (Kabir et al. 2002; Rahman et al. 2012). These inorganic dissolved solids are difficult to remove/ameliorate in the natural receiving waters, and their high concentration can be detrimental to living organisms. Therefore, it is important that the TDS of industrial effluent be necessarily reduced to its safe discharge levels before the effluent is let into open water bodies (Misha et al. 2004). The only feasible and economical way to remove TDS from wastewaters is by using microorganisms, which either adsorb or metabolically utilize the dissolved solids so that they become part of the microbial cells, i.e. particulate matter. Bioremediation is a promising alternative method for TDS removal in the treatment of industrial wastewaters, as it is well-known to be a sustainable and cost-effective. However, the efficiency of TDS removal by algae mainly depends on the tolerance to the wastewater and their TDS uptake capability. Treatment of the TW with the consortium of *Chlorella* and *Phormidium* sp. effectively enhanced the removal of TDS to 58.28% compared to that of the individual culture of *Chlorella* sp. (51.37%) and *Phormidium* sp. (50.71%). TDS reduction strongly correlated ($r=0.810$) with the growth of consortium in the TW. Notably, only the consortium of the two algae could achieve TDS reductions close to the permissible limit set by BIS (1994). Furthermore, results of this study are useful to highlight that the use of marine and salt-tolerant microalgae offer the advantage of treating TW replete with high amount of salts.

Notably, some reduction in concentrations of most examined parameters was observed in the control set without added microalga. The reduction, in this case, might be due to the activity of indigenous microorganisms of the wastewater. However, the indigenous bacterial count in the effluent was quite low compared to those reported by Sinha et al. (2011) and Das et al. (2010) for tannery effluents. Hence, no attempt was made to characterize the indigenous bacterial population or quantify the reduction of pollutants due to it. Zahoor and Rehman (2009) observed about 52–62% reduction of Cr (VI) in control set due to native microflora of wastewater. However, treating the wastewaters with added microalgae is more practical and would enhance/ hasten the remediation process (Perpetuo et al. 2011). The microalgae used in this study were able to thrive and compete well in the presence of indigenous microflora with no apparent decrease in pollutant removal/detoxifying capability. The concentrations of hazardous parameters, in particular, reduced rather more quickly in the sets with added microalgae, individually or as consortium.

Post-hoc Tukey's HSD test is useful to elucidate the number of days required to achieve a significant reduction of the hazardous parameters. Greater percent reduction of most pollutants occurred within 5–10 days. It is apparent that maximum utilization/transformation of toxicants occurs during the logarithmic growth phase. Although significant reductions of the parameters are achieved within 5–10 day period, our analysis shows that reduction of all hazardous parameters to safe limits occurs by day 20. From our first attempts of employing marine microalgal strains for TW bioremediation, it is clear that there

is a greater ecological advantage. In that, the hazard levels of TW are brought down drastically within 5–10 days. Additional trials of employing more marine microalgae and/or suitable modifications in the treatment process might prove not only far eco-friendlier but also economical and in the long run prove very sustainable.

Experimental results describing the use of two or more microalgal species to treat TW are scarce. In this regard, results from this study strongly suggest that a pragmatic approach would be to adopt the consortium based bioremediation strategies to detoxify and bring down the pollutant loads from such hazardous effluents as TW. The growth response of different types of microalgae in wastewater also varies, because they differ in their inherent abilities like nutrient uptake, tolerance to harsh/extreme environmental conditions, and competition with indigenous organisms. Further, the difficulties that arise with the use of monostrains of microalgae, such as growth in diverse environments and harvesting problems, highlight that the consortial approach may be a more effective alternative for wastewater treatment. Such consortia show synergistic interactions and have wider potential to treat different types of wastewaters, than monostrains. Algae represent the base of food pyramids and primary consumer in food chains. Hence, their deployment needs to be an integral part of wastewater remediation in the global scenario, as an environment-friendly strategy.

Conclusion

The marine microalgae *Chlorella* sp. and *Phormidium* sp., both individually and in consortium, efficiently reduced the concentrations of most parameters measured from tannery wastewater. The levels of BOD, COD, TN, TP, Cr and TDS were reduced to permissible, safe discharge limits by ∼15 days. The consortium of *Chlorella* and *Phormidium* sp. brought down the concentrations of a number of hazardous parameters faster than both of them individually. Results from this study are useful to highlight that marine microalgae, owing to their ability to grow in higher salt concentrations than their terrestrial counterparts, can be employed in bioremediation as alternative, low cost and eco-friendly methods for treatment of tannery wastewaters to achieve safe and permissible discharge limits. Efforts to up-scale TW treatment using these and other marine microalgae would be essential for developing pragmatic bioremediation strategies.

Acknowledgments

We thank the Director, CSIR - National Institute of Oceanography for facilities and encouragement. The work was supported under the CSIR project BSC0111 'Integrated NextGen Approaches in Health, Disease and Environmental Toxicity (INDEPTH)'. The authors also wish to thank the concerned authorities at Pallavaram Tanners Industrial Effluent Treatment Co Ltd., Chennai, for generous supply of the tannery wastewater used in this study. This is CSIR-NIO contribution no. ____.

References

Ackerley DF, Barak Y, Lynch SV, Curtin J, Matin A. 2006. Effect of chromate stress on *Escherichia coli* K-12. J Bacteriol. 188:3371–81.

Ajayan KV, Selvaraju M, Unnikannan P, Sruthi P. 2015. Phycoremediation of tannery wastewater using microalgae *Scenedesmus* species. Int J Phytorem. 17:907–16.

Ajayan KV, Selvaraju M. 2012. Heavy metal induced antioxidant defense system of green microalgae and its effective role in Phycoremediation of tannery effluent. Pak J Biol Sci. 15:1056–62.

APHA. 2005. Standard *methods for the examination of water and wastewater*. 20th ed. Washington DC: APHA-AWWA-WPCF.

Bhatnagar A, Bhatnagar M, Chinnasamy S, Das KC. 2010. *Chlorella minutissima*—a promising fuel alga for cultivation in municipal wastewaters. Appl Biochem Biotechnol. 161:523–36.

Biradar NV, Sindagi AS, Reddy J, Ingalhalli SS. 2012. Bioremediation of chromium in pulp and paper processing industrial effluents by indigenous microbes. Bioremediation & Biodegradation. 3(12):1–7.

Bosnic M, Buljan J, Daniels RP. 2000. Pollutants in tannery effluents. Report for United Nations Industrial Development Organization (UNIDO) US/RAS/92/120, Regional Programme for Pollution Control in the Tanning Industry in South-East Asia.

Bureau of Indian Standards (BIS). 1994. Quality tolerance for water for Tanning industry IS: 4221. New Delhi.

Chandra R, Pandey PK, Srivastava A. 2004. Comparative toxicological evaluation of untreated and treated tannery effluent with *Nostoc muscorum* L. (Algal assay) and microtox bioassay Environ Monit Assess. 95:287–94.

Chen L, Li T, Guan L, Zhou Y, Li P. 2011. Flocculating activities of polysaccharides released from the marine mat-forming cyanobacteria *Microcoleus* and *Lyngbya*. Aquat Biol. 11:243–8.

Chinnasamy S, Bhatnagar A, Hunt RW, Das KC. 2010. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. Bioresour Technol. 101:3097–105.

Christenson L, Sims R. 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnol Adv. 29:686–702.

Chu W-L, See Y-C, Phang S-M. 2008. Use of immobilized *Chlorella vulgaris* for the removal of colour from textile dyes. J Appl Phycol. 21:641–8.

Covington AD, Covington T. 2009. Tanning Chemistry: The Science of Leather. Illustrated edition. Royal Society of Chemistry.

Craggs R, Sutherland D, Campbell H. 2012. Hectare-scale demonstration of high rate algal ponds for enhanced wastewater treatment and biofuel production. J Appl Phycol. 24:329–37.

Das C, Naseera K, Ram A, Meena RM, Ramaiah N. 2017. Bioremediation of tannery wastewater by a salt-tolerant strain of *Chlorella vulgaris*. J Appl Phycol. 29(1):235–243. doi:10.1007/s10811-016-0910-8.

Das M, Ahmed MD. K, Begum F, Parveen S, Islam MD. M, Begum M. 2010. Microbial load in tannery and textile effluents and their receiving rivers of Dhaka. Dhaka Univ J Bio Sci. 19(1):73–81.

Ding J, Zhao F, Cao Y, Xing L, Liu W, Mei S, Li S. 2014. Cultivation of microalgae in dairy farm wastewater without sterilization. Int J Phytorem. 17(3):222–227. doi:10.1080/15226514.2013.876970.

Dixit S, Singh DP. 2014. An evaluation of phycoremediation potential of cyanobacterium *Nostoc muscorum*: characterization of heavy metal removal efficiency. J Appl Phycol. 26:1331–42.

Gin KY, Tang YZ, Aziz MA. 2002. Derivation and application of a new model for heavy metal biosorption by algae. Water Res. 36:1313–23.

Grima EM, Belarbi EH, Acien Fernandez FG, Robles Medina A, Chisti Y. 2003. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol Adv. 20:491–515.

Hori K, Si I, Ikeda G, Ji O, Tanji Y, Weeraphasphong C. 2002. Behavior of filamentous cyanobacterium *Anabaena* spp. in water column and its cellular characteristics. Biochem Eng J. 10:217–25.

Ji L, Xie S, Feng J, Li Y, Chen L. 2011. Heavy metal uptake capacities by the common fresh water green alga *Cladophora fracta*. J Appl Phycol. 24:979–83.

Kabdasli I, Tunay O, Cetin MS, Olmez T. 2002. Assessment of magnesium ammonium phosphate precipitation for the treatment of leather tanning industry wastewaters. Water Sci Technol. 46:231–9.

Kabir ES, et al. 2002. Assessment of effluent quality of Dhaka export processing zone with special emphasis to the textile and dying industries. Jahangir Nagar University Journal of Science. 25:137–8.

Lufrano G, Meriç S, Zengin G E, Orhon D. 2013. Chemical and biological treatment technologies for leather tannery chemicals and wastewaters: A review. Science of The Total Environment. 461–462:265–81.

Mara DD, Pearson HW, Silva SA, Rose PD, Maart BA, Dunn KM, Rowswell RA, Britz P. 1996. Waste stabilization ponds: Technology and applications high rate algal oxidation ponding for the treatment of tannery effluents. Water Sci Technol. 33:219–27.

Mata TM, Melo AC, Simoes M, Caetano NS. 2012. Parametric study of a brewery effluent treatment by microalgae *Scenedesmus obliquus*. Bioresour Technol. 107:151–8.

Misha A, Yadav A, Agaiwal M, Bajipai M. 2004. Fenugreek mucilage for solid removal from tannery effluent. *Reactive and Functional Polymers*. *59*:99–104.

Mustafa E-M, Phang S-M, Chu W-L. 2012. Use of an algal consortium of five algae in the treatment of landfill leachate using the high-rate algal pond system. J Appl Phycol. 24:953–63.

Nanda S, Sarangi PK, Abraham J. 2010. Cyanobacterial remediation of industrial effluents I. Tannery effluents. New York Science Journal. 3:32–6.

Orandi S, Lewis DM. 2013. Biosorption of heavy metals in a photorotating biological contactor – a batch process study. Appl Microbiol Biotechnol. 97:5113–23.

Oswald WJ. 1988. Micro-algae and waste-water treatment. In: Borowitzka MA, Borowitzka LJ, editors. Micro-algal Biotechnology. Cambridge: Cambridge University Press. p. 305–28.

Parsons et al. 1984. A manual of chemical and biological methods for seawater analysis. Potts Point (N.S.W., Australia): Pergamon Press.

Perpetuo EA, Souza CB, Nascimento CA. 2011. Engineering Bacteria for Bioremediation. In: A. Carpi, editor. Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications (p. DOI: 10.5772/19546). http://www.intechopen.com/books/progress-in-molecular-and-environmental-bioengineering-from-analysis-and-modeling-to-technology-applications/engineering-bacteria-for-bioremediation:InTech.

Pittman JK, Dean AP, Osundeko O. 2011. The potential of sustainable algal biofuel production using wastewater resources. Bioresour Technol. 102:17–25.

Posadas E, Bochon S, Coca M, García-González MC, García-Encina PA, Muñoz R. 2014. Microalgae-based agro-industrial wastewater treatment: a preliminary screening of biodegradability. J Appl Phycol. 26:2335–45.

Prabhahar C, Saleshrani K, Tharmaraj K. 2012. Seasonal variation of heavy metals distribution and sediments in Palar River in and around Vaniyambadi segment, Vellore District, Tamil Nadu, India. International Journal of Pharmaceutical & Biological Archives. 3 (1):112–6.

Rahman MS, et al. 2012. Water quality characterization of the Sundarbans Reserve Forest (SRF), Khulna for biodiversity consideration, Bangladesh. In: Silver jubilee conference, Bangladesh Chemical Society. Vol. 20. p. 67–8.

Rawat I, Kumar RR, Mutanda T, Bux F. 2011. Dual role of microalgae: phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Appl Energy. 88:3411–24.

Rehman A, Farah RS, Shakoori AR. 2007. Heavy metal resistant *Distigmaproteus* (Euglenophyta) isolated from industrial effluents and its possible role in bioremediation of contaminated wastewaters. World J Microbiol Biotechnol. 23:753–8.

Rehman A. 2011. Heavy metals uptake by *Euglena proxima* isolated from tannery effluents and its potential use in wastewater treatment. Russian J Ecology. 42:44–9.

Renuka N, Sood A, Ratha SK, Prasanna R, Ahluwalia AS. 2013. Nutrient sequestration, biomass production by microalgae and phytoremediation of sewage water. Int J Phytoremed. 15:789–800.

Riano B, Molinuevo B, Garcia-Gonzalez MC. 2011. Treatment of fish processing wastewater with microalgae-containing microbiota. Bioresour Technol. 102:10829–33.

Shi J, Podola B, Melkonian M. 2007. Removal of nitrogen and phosphorus from wastewater using microalgae immobilized on twin layers: an experimental study. J Appl Phycol. 19:417–23.

Silva-Benavides AM, Torzillo G. 2011. Nitrogen and phosphorus removal through laboratory batch cultures of microalgae *Chlorella vulgaris* and cyanobacterium *Planktothrix isothrix* grown as monoalgal and as co-cultures. J Appl Phycol. 24:267–76.

Singh M, Reynolds DL, Das KC. 2011. Microalgal system for treatment of effluent from poultry litter anaerobic digestion. Bioresour Technol. 102:10841–8.

Sinha SN, Biswas M, Paul D, Rahaman S. 2011. Biodegradation potential of bacterial isolates from tannery effluent with special reference to hexavalent chromium. Biotechnol Bioinf Bioeng. 1 (3):381–6.

Song Z, Williams CJ, Edyvean RGJ. 2000. Sedimentation of tannery wastewater. Water Res. 34:2171–6.

Tadesse I, Isoaho S, Green F, Puhakka J. 2003. Removal of organics and nutrients from tannery effluent by advanced integrated wastewater pond systems technology. Water Sci Technol. 48:307–14.

Thanikaivelan P, Rao JR, Nair BU, Ramasami T. 2005. Recent trends in leather making: Processes, problems, and pathways. Critical Reviews in Environmental Science and Technology. 35:37–79.

Tiwari DP, et al. 1989. Removal of toxic metals from electroplating industries. Indian J Environ Health. 31:122–6.

Tunay O, Kabdasli I, Orhon D, Cansever G. 1999. Use and minimization of water in leather tanning processes. Water Sci Tech. 40:237–44.

Varadarajan DB, Krishnamoorthy S. 1993. Environmental Implications of Leather Tanneries APH Publishing. p. 2–3.

Xu WH, Liu UG, Zeng GM, Li X, Song HX, Peng QQ. 2009. Characterization of Cr(VI) resistance and reduction by *Pseudomonas aeruginosa*. Trans Nonferrous Met Soc China. 19:1336–41.

Zahoor A, Rehman A. 2009. Isolation of Cr (VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing wastewater. J Environ Sci. 21:814–20. doi:10.1016/S1001-0742(08)62346-3.