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Bioremediation and Sustainable Technologies for Cleaner Environment

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Dedicated to our respective families and our students

Preface

Rapid industrialization towards better economic development has resulted in environmental degradation globally. Major contamination sources from industry, agriculture and urban areas are a threat to the natural environment, the impacts of which are severe in both developed and developing countries. The accumulation, mobilization and toxicity of various contaminants in soils and groundwater are degrading the natural environment and are limiting the sustainable development of the nation as well. There is an urgent need for the conservation and effective utilization of natural resources; looking towards adoption of low cost techniques, low waste generation and environmentally friendly approaches to remediate contamination.

Increased need for the remediation of the diverse and ever-increasing classes of waste and waste dumping sites has created a demand for improved and newer remediation methods that are applicable at low cost and for a wider scope of waste management. A dramatic increase in the use of biological methods of waste remediation has been evidenced showing a positive inclination in environmental conservation. In addition to the generic studies benefited through waste remediation, there are some current factors that make biological approaches more attractive today. Recent researches focus on bioremediation, which is mainly concerned with usage of microbes and biological agents to improve the quality of environment and prevention of pollutant discharge into the environment, remediating the contaminated sites and also generating the valuable products to the society.

This book showcases the effective identification, delineation and remediation of contamination through simple ecofriendly methods using organisms or products for all aspects of land-, air- and water-based pollution. The recent issues related to environment deterioration and also biological approaches towards restoring the ecosystem is dealt with focusing on the role of bacteria, fungi and biosynthesized nanoparticles in pesticide degradation, dye degradation, dairy effluent treatment, food waste management, paper cup degradation, nitrate removal and heavy metal removal. In most cases, bioremediation can lead to simple organic constituents through reduction of wastes. Some chapters emphasize on bioenergy and bioproducts such as biohydrogen, bioethanol and bioplastics as derived products.

In short, this book discusses the scope of bioremediation and the assessment of various biological approaches to the remediation in itself with much attention being paid to the biological products derived from the waste processes.

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Part I
Bioremediation of the Environment

Biological Perspective and Role of Bacteria in Pesticide Degradation

S. Umadevi, P.M. Ayyasamy and S. Rajakumar

Abstract Use of pesticide is increasing day by day and the positive aspect renders enhanced crop/food productivity and drastic reduction of vector-borne diseases. However, its unregulated and indiscriminate applications have raised serious concerns in environmental pollution and health effects. Bioremediation methods use naturally occurring microorganisms to detoxify these pollutants and make soil fertile in the process. Bacterial degradation is one of the most environmentally sound, cost effective and quick process for the pesticide contaminated environment. This study includes the usage and the effects of pesticides, bacterial degradation, genetically modified bacteria, enzymatic immobilization approach and few limitations of biodegradation.

Keywords Pesticides · Bacteria · Biodegradation · Enzyme

1 Introduction

World food production is in demand due to rapidly growing population and it necessitates the use of chemical pesticides. About 45% of annual food production was lost due to the pest attack. One of the best ways to increase the crop productivity is effective pest management by using chemical pesticides (Odukkathil and Vasudevan 2013). The term pesticide includes algaecides (control algae in bodies of water, including swimming pools), antimicrobials (kill microorganisms

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that produce disease), attractants (attract specific pests using natural insect chemicals called pheromones that confuse the mating behavior of insects), avicides (control pest birds), biopesticides (naturally occurring substance with pesticidal properties), defoliants (cause foliage to drop from a plant, typically to aid in the harvesting process), desiccants (aid in the drying process of plants or insects, usually for laboratory purposes), fumigants (produce vapors or gases to control air or soilborne insects and diseases), fungicides (destroy fungi that infect plants, animals or people), herbicides (control noxious weeds and other vegetation that are growing or competing with a desired species), insect growth regulators (accelerate or retard the rate of growth of insects), insecticides (control or eliminate insects that affect plants, animals or people), mitticide/acaricides (kill mites that live on plants, livestock and people), molluscicides (kill snails and slugs), nematicide (kill nematodes, which are microscopic wormlike organisms that live in the soil and cause damage to food crops), ovicides (control insect eggs through the application of low-sulfur petroleum oils to plants and animals), piscicides (control pest fish), plant growth regulators (accelerate or retard the rate growth of plant), predacides (control vertebrate pests), repellents (repel pests such as mosquitoes, flies, ticks and fleas), rodenticides (control mice, rats and other rodents) (Gavrilescu 2005).

About two million tonnes of pesticides are consumed per year throughout the world and among the 2 million tonnes, 24% is consumed in USA, 45% in Europe

Table 1 Types of pesticides and their effects

Pesticide	Class	Health effect
Insecticides	Organophosphates	Neuropathy, myopathy, tremors, irritability, convulsions, inhibiting the enzyme acetylcholinesterase, paralysis
	Carbamates	Inhibition of acetylcholinesterase enzyme, paralysis
	Organochlorines (dichlorodiphenyl methane and cyclodienes)	Stimulation of the nervous system by disrupting the sodium/potassium balance of the nerve fiber, tremors, irritability, convulsions, hyperexcitable state of the brain, cardiac arrhythmias and reproductive problems
Herbicides	Phenoxy, benzoic acids, riazines, ureas and chloroacetanilides	Dermal toxicity, carcinogenic effect, damage to liver, thyroid, nervous system, bones, kidneys, blood and immune system
Fungicides	Substituted benzenes, thiocarbamates, thiophthalimides, organomercury compounds etc.	Damage to liver, thyroid, nervous system, bones, kidneys, blood and immune system, carcinogenic property also

Source Niti et al. (2013)

and 25% in rest of the world. The usage of pesticide in India is about 0.5 kg/ha of which major contribution is from organochlorine pesticides and the consumption of herbicide is the highest (Odukkathil and Vasudevan 2013). The Indian Pesticide Industry with 82,000 MT of production for the year 2005–2006 is ranked second in Asia (behind China) and ranks twelfth in the world for the use of pesticides with an annual production of 90,000 tons (Boricha and Fulekar 2009). Pesticide residue in environment ultimately affects the health of man and other organisms. In man it triggers acquired immunodeficiency (immunosuppression), autoimmunity, hypersensitivity reactions, like eczema, dermatitis, allergic respiratory diseases and recurrent infections. Many pesticides are known to cause mutations in chromosomes of man and animals which may lead to carcinoma, neuropathy, nephropathy, hepatotoxicity and reproductive disorders (Chauhan and Singhal 2006). Table 1 represents the different classes of pesticides and their effects in human health.

2 Intra Multiple Properties of Pesticides

Most of the pesticide groups have dual/multiple properties. Based on the classified list of pesticides (http://www.alanwood.net/pesticides/class_pesticides.html), the present study analysed the different purposes of the chemicals as pesticides. The chemicals viz. bifujunzhi, binapacryl, dinobuton, dinocap, dinocton, dinopenton, dinosulfon, dinoterbon, disulfiram, flufenoxystrobin, sulfur and thioquinox have acaricides and fungicide properties. In addition to act as acaricides, some are effective against insects. Some algicides are used for additional purpose as fungicides and herbicides. Particular fungicides are used as bactericides, herbicides, insecticides and growth regulators. As well few insecticides have the properties of molluscicides and nematicides. Besides, multiple properties of a particular chemical are represented graphically as network model in Fig. 1.

Based on these data, this study suggested to use single compound with multiple properties based on the requirement of the particular agricultural field, so as the pollution may control. The major component of chemicals involved in these pesticides are chlordime form, hexachlorophene, coumaphos, diazinon, methiocarb, DNOC, sanguinarine, carvacrol, benomyl, carbaryl, arsenous oxide, fentin, dichlorophen, copper sulfate, endothal, guazatine, chloralose, endrin, fenthion, strychnine, probenazole, xinjunan, copper oxychloride, thiram, ziram, trimethacarb, diflubenzuron, penfluron, dichlofluanid, pentachlorophenol, allicin, zinc naphthenate, sodium pentachlorophenoxide, carvone, cyprosulfamide, zengxiaoan, sodium tetrathiocarbonate, cloethocarb, copper acetoarsenite, lead arsenate, tributyltin oxide, tribufos and lindane.

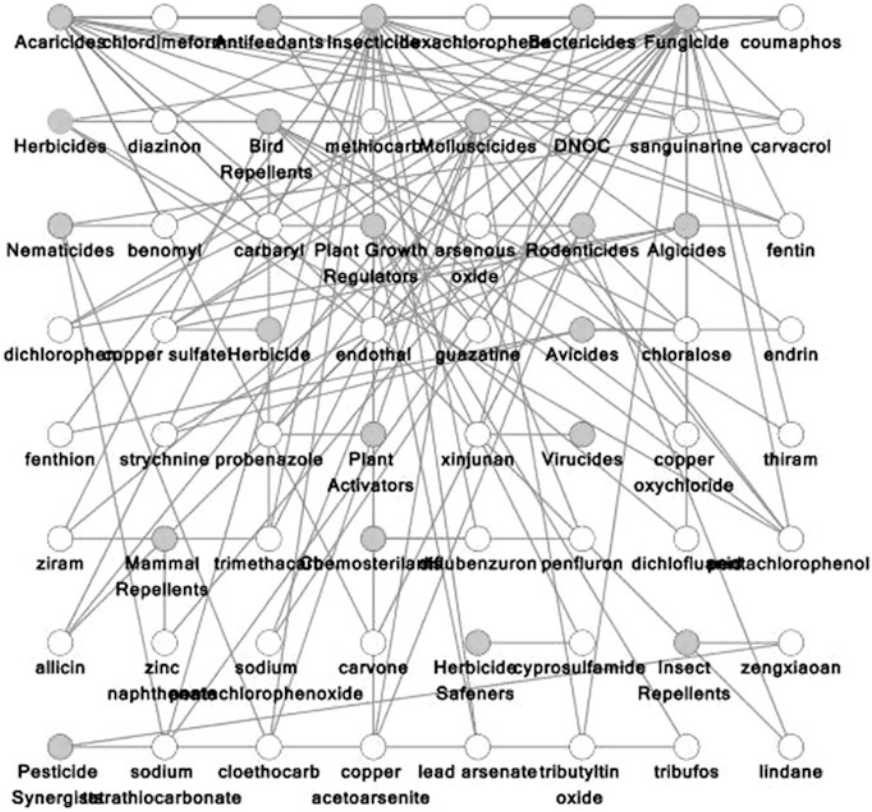

Fig. 1 Multiple properties of pesticide groups

3 Biodegradation Process

All the organic compounds that occur naturally and most of those synthesized by man can be decomposed in soil by a combination of chemical and biological action. The pesticide degradation process includes biodegradation, chemical degradation, hydrolysis, oxidation-reduction (redox), ionization and photo-degradation (Zhang and Qiao 2002). Biodegradation is the ecofriendly way of converting pesticides into simple non-toxic compounds by means of enzymes generally produced by microbes (Fig. 2).

The factors related degradation include the concentration of appropriate microbes, their interaction with pesticide, pH, temperature, salinity, nutrients, light intensity, available water, oxygen tension, redox potential, surface binding, presence of alternative carbon substrates, alternative electron acceptor. The second group of factors includes chemical structure, molecular weight, functional groups of the applied pesticides, their concentration, toxicity and their solubility in water.

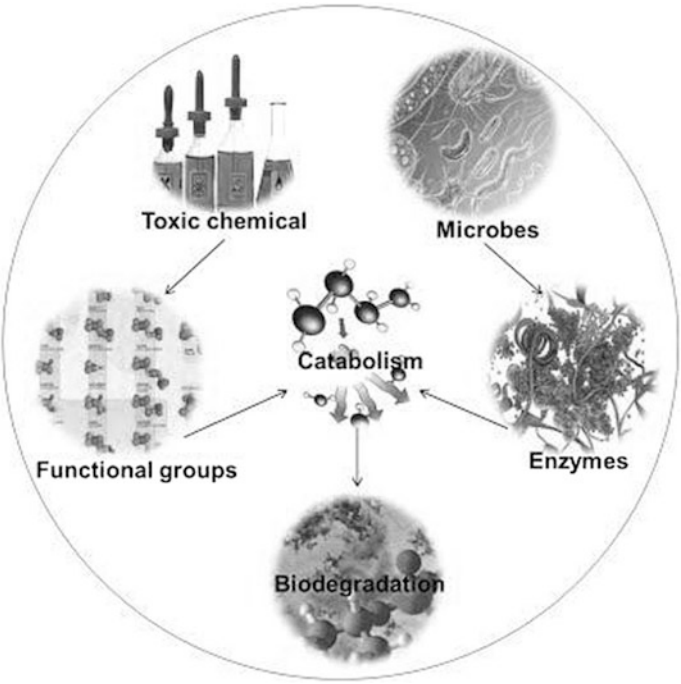

Fig. 2 Biodegradation process of pollutants. *Source* Umadevi et al. (2015)

Different techniques employed for bioremediation comes under in situ and ex situ processes. In situ remediation includes bioventing, biosparging, biostimulation, bioaugmentation and ex situ techniques includes land farming, biopiling, composting, bioreactors, precipitation/flocculation microfiltration and electro dialysis (later three not applicable for biodegradation). There is a significant role of metabolic activity of bacteria, fungi, actinomycetes and plants (including rhizosphere bacteria) in the degradation process.

4 Bacterial Degradation of Pesticides

Bacteria are the potential degraders of complex molecules and uses pesticides for their own metabolism and growth. Recent studies were reviewed by Hussain et al. (2009) and Sinha et al. (2009) and have indicated that different bacterial species apparently have different sensitivity to pesticides (Table 2).

Table 2 Microorganisms capable of degrading pesticides

Pesticide group	Microorganism
2,4-D	*Pseudomonas* sp. and *Alcaligenes eutrophus*
Alachlor	*Streptomyces capoamus*, *Streptomyces galbus* and *Streptomyces bikiniensis*
Aldrin and lindane	*Bacillus* sp., *Exiguobacterium aurantiacum*, *Pandoraea* sp., *Pseudomonas pseudoalcaligenes* and *Micrococcus luteus*
Atrazine, simazine, s-triazine	*Agrobacterium tumefaciens*, *Alcaligenes xylosoxidans*, *Escherichia coli*, *Pseudomonas* sp., *Pseudaminobacter* sp., *Providencia rustigianii*, *Ralstonia basilensis*, *Rhodococcus* sp. and *Erythropolis* sp.
Benomyl and carbendazim	*Rhodococccus erythropolis*
Cadusafos, diazinon, dichlorovos, ethoprophos, fenamiphos, fenitrothion, isazofos, isofenphos, isoxathion, malathion, methylparathion, monocrotophos, paraoxon, parathion, phosphomidon and quinolphos	*Acinetobacter radioresistens*, *Arthrobacter* sp., *Aulosira fertilissima*, *Flavobacterium* sp., *Nostoc muscorum*, *Pseudomonas putida* and *Sphingomonas paucimobilis*
Carbamate, aldicarb	*Methylosinus* sp.
Carbofuran	*Novosphingobium* sp.
Chlorpyrifos, fonofos, terbufos	*Alcaligenes faecalis*, *Enterobacter* sp., *Klebsiella* sp. and *Pseudomonas diminuta*
Chlorsulfuron, metsulfuronmethyl	*Pseudomonas fluorescens*
DDT	*Serratia marcescens* and *Dehalospirilum multivorans*
Dicamba	*Pseudomonas maltophilia*
Diclofop-methyl	*Sphingomonaspaucimobilis*
Diuron, chlorotoluron, isoproturon, monolinuron and linuron	*Arthrobacter* sp., *Methylopila* sp., *Pseudomonas* sp., *Sphingomonas* sp. and *Vibrio fischeri*
Endosulfan	*Bacillus* sp., *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Mycobacterium* sp., *Pandoraea* sp., *Pseudomonas* aeruginosa, Pseudomonas *spinosa* and *Pseudomonas cepacia*
Gramoxone and matancha	*Pseudomonas putida*
HCH	*Pandoraea* sp., *Pseudomonas aeruginosa* and *Pseudomonas putida*
Isoxaben	*Microbacterium* sp.
Mefenacet	*Sphingobacterium* sp.
Oxadiazon	*Pseudomonas fluorescens*
Pentachlorophenol	*Pseudomonas* sp.
Phenylurea	*Arthrobacter globiformis*

(continued)

Table 2 (continued)

Pesticide group	Microorganism
Propoxur	*Pseudomonas* sp.
Pyrethroidallethrin, beta-cyfluthrin, bifenthrin,cypermethrin, flumethrin and permethrin	*Acidomonas* sp., *Aeromonas sobria*, *Erwinia carotovora*, *Pseudomonas stutzeri*, *Serratia* sp., *Stenotrophomonas acidaminiphila* and *Yersinia Frederiksenii*
Trifluralin	*Bacillus* sp., *Brevundimonas diminuta*, *Herbaspirillum* sp., *Klebsiella* sp. and *Pseudomonas* sp.
Vinclozolin	*Pseudomonas putida*

4.1 Genetics for Pesticide Degradation

The recent advances in metagenomics and whole genome sequencing technologies explore the novel pollutant degradative genes and their regulatory enzymes. Mobile genetic elements such as plasmids and transposons have been shown to encode enzymes responsible for the degradation of several pesticides. These genes encoding for enzymes capable of degradation were reported and these plasmids are known as catabolic plasmids. The organisms containing them have the ability to degrade specific compounds (Nawaz et al. 2011). The majority of the pesticide groups are degraded by *Agrobacterium radiobacter*, *Agrobacterium tumefaciens*, *Alcaligenes* sp., *Aminobacter aminovorans*, *Arthrobacter crystallopoietes*, *Burkholderia cepacia*, *Caulobacter crescentus*, *Chelatobacter heintzii*, *Flavobacterium oryzihabitans*, *Bosea* sp., *Nocardia* sp., *Nocardioides* sp., *Pseudaminobacter* sp., *Pseudomonas putida*, *Ralstonia picketti*, *Rhizobium* sp., *Rhodococcus* sp., *Sphingomonas yaniokuyae*, *Stenotrophomonass maltophilia* and *Variovorax paradox* by secreting the enzymes hydrolase, urease, hydrolase and cytochrome P-450, the genes encoding these enzymes are *atzABCDEF*, *trzND* and *psbA1*. The bacterial species *Brevundimonas* and *Stenotrophomonas* secrete dioxygenase, isomerases, reductases, glutathione, transferases and degrades the chemical groups which are encoded by the genes *tfdA*, *tfdB*, *tfdC* and *ORF1-ORF6* (Hussain et al. 2009).

The genes *mpd*, *opd*, *phnE*, *glpT*, *tfdA*, *tfdB*, *tfdC* and *ORF1-pdeA* are responsible for the degradation of pesticides in *Ochrobactrum* sp. by secreting the bioenzymes *viz*. hydrolase, dioxygenase, isomerases, reductases, glutathione and transferases. *Pseudomonas* sp. is known for its detoxifying capacity of many toxic chemicals, it break down the chemical pesticides through the enzyme (hydrolase, urease, cytochrome P-450, deaminase, dioxygenase, isomerases, reductases, glutathione and stransferases) production and the genes responsible for that are *mpd*, *pcd*, *atzABCDEF*, *trzND*, *psbA1*, *atzB*, *atzC*, *trzD*, *triA*, *tfdA*, *tfdB*, *tfdC* and *ORF1-ORF6*. Bacteria namely *Sphingobium francense*, *Sphingomonas paucimobilis*,

Sphingomonas taejonensis and *Microbacterium* sp. involve in biodegradation process which secrete dehalogenase, dehydrogenase, dehydro-chlorinase, hydrolase and the genes activating these enzymes are *lin*, *linA*, *linB*, *linC*, *linD*, *linE* and *linX*. It is noted that most of the pollutants were degraded by the enzyme hydrolase, some of the other bacteria consistent to this are *Arthrobacter* sp. (genes: *atz*B, *atz*C, *trz*D, pRC1, pRC2 and pRC3), *Achromobacter* sp. (*mpd*, *mcd*, *opd*, *phnE*, *glpT* and *pdeA*), *Brucella* sp., *Delftia acidovorans*, *Enterobacter asburiae*, *Escherichia coli*, *Flavobacterium balustinum* and *Pseudomonas aeruginosa* (*mpd*, *opd*, *phnE*, *glpT* and *pdeA*) *Methylomonas methylovora*, *Methylobacteria organophilum* (*atzB*, *atzC* and *trzD*) (Hussain et al. 2009).

In recent years enzyme immobilization techniques are used to remediate the pesticides. Ghanem and Raushel (2005) reported that the enzyme OpdA is responsible for detoxification of organophosphates. This enzyme has broad substrate specificity, being able to cleave P–O, P–CN and P–F bonds in organophosphate compounds, and a very high catalytic activity. Based on this, Gao et al. (2014) covalently immobilized OpdA on highly porous non-woven polyester fabrics and degraded organophosphate pesticide. With the recent advances in biomolecular engineering, the bioremediation of persistent organic pollutants by using genetically modified microorganisms has become a rapid growing area of research. It allows the combination of different degradative activities within a single organism, and catabolic enzymes can be engineered to enhance the degradation of pesticides or to broaden their specificity for substrate (Ang et al. 2005). Strong et al. (2000) conducted first field-scale atrazine remediation by stabilized whole-cell suspension of genetically engineered *Escherichia coli* encapsulating overexpressed atrazine chloro hydrolase, *AtzA*. The inaccessibility of some pesticides across the cell membrane of the bacteria is one of the disadvantages. With the recent progress in biotechnology, a surface anchor system was developed to localize enzyme onto the surface of bacteria (Lee et al. 2003). The display of heterologous proteins or peptides on the surface of prokaryotic or eukaryotic cells, especially bacterial and yeast cells enabled by means of recombinant DNA technology has become an increasingly used strategy in the use of environmental bioadsorbents. Cao et al. (2013) successfully displayed organophosphorus hydrolase (OPH) on the cell surface of a hexachlorocyclohexane (HCH)-degrading *Sphingobium japonicum* UT26. The microbe could potentially be used for removing the two classes of pesticides that may be present in mixtures at contaminated sites. A surface anchor system derived from the truncated ice nucleation protein from *Pseudomonas syringae* was used to target OPH onto the cell surface of UT26 that stimulated the process. Table 3 depicts the potential use of cell surface-displayed enzymes for accelerated biodegradation of pesticides and their more persistent metabolites in soil and water environments.

Table 3 Surface cell display of enzymes in recombinant strains for biodegradation of pesticides

Strain	Fusion system	Comments
Escherichia coli	Truncated ice nucleation protein	Hydrolysis of methyl parathion occurred 25 fold faster than in wild type
Escherichia coli	Ice nucleation protein	Biodegradation of paraoxon occurred at rate of 0.65 mM/min/g of cells (dry weight) and retained almost 100% efficiency over a period of 45 days
Escherichia coli	Ice nucleation protein	More than 80% of the malathion was degraded and was highly promising for detoxification of organophosphorus pesticides
Moraxella sp.	Ice nucleation protein	Hydrolysis of methyl parathion, parathion, and paraoxon occurred at rate of 0.6 micromol/h/mg dry weights, 1.5 micromol/h/mg dry weights, and 9.0 micromol/h/mg dry weights, respectively
Pseudomonas putida	Ice nucleation protein anchor	The whole cell activity increased up to 10 times higher, which could be helpful in accelerated biodegradation

Source Hussain et al. (2009)

5 Limitations of Bioremediation

There are several limitations to implement bioremediation technology. One major limitation is that microorganisms are active only under their favorable environmental condition. The organism need to produce the degradative enzyme even in low level polluted area at over a period of time, in this case biostimulation is required for in situ remediation. The long-term effects of introducing naturally occurring non-native bioremediation organisms into an area and the impact of genetically altered bioremediation organisms are not fully understood. In spite of limitations, bioremediation is promising, economic way to solve public health problems (Singh 2008).

6 Conclusion

The removal of wide range of pesticide groups from the environment requires knowledge of its concentration in particular environment. It requires further exploration in relation to total microbial population and their biochemical activities, environmental limiting factors, physiology and genetics of degradation of bacterium. It is concluded that the knowledge of physiology, biochemistry and genetics of bacteria may further enhance degradation of pesticide groups. Genes encoding for the active enzyme were identified for several pesticides, which will provide better understanding and inputs to develop a super strain to achieve the desired effect of bioremediation.

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Nanobioremediation Technologies for Sustainable Environment

A. Sherry Davis, P. Prakash and K. Thamaraiselvi

Abstract Hybrid technologies like nanobioremediation are significant in transforming and detoxifying pollutants which harm the ecosystem. In situ or ex situ it has the potential for large scale clean- up activities with reduced cost and minimal harmful byproducts. Here five nanoscale metallic biosynthesized particles formed the topic of study. Zn, Ag, Au, Fe and Cu particles can be biosynthesized using plant extracts, bacteria, fungi and algae. The potential of these metallic nanoparticles to degrade and remedy various contaminants in the environment has been researched widely. A combination of biosynthesis and remediation lead to sustainable development and ultimately a sustained environment.

Keywords Biosynthesis · Nanoparticles · Bioremediation · Environment

1 Introduction

Environmental sustainability is defined as responsible interaction with the environment to avoid depletion or degradation of natural resources and allow for long term environmental quality. However the world's definition of sustainability is sustainable development which results in environmental degradation. The advancement in science and technology contribute directly or indirectly to the increase in waste and toxic materials in the environment. Environmental sustainability programs include protection and restoration of the natural environment.

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One of the restoration strategies used nowadays is bioremediation which makes use of microorganisms. The advantages of bioremediation over conventional treatments is cost effectiveness, high competence, minimization of chemical and biological sludge, selectivity to specific metals, no supplementary nutrient requirements, regeneration of biosorbent, and the possibility of metal recovery (Kratochvil and Volesky 1998). However this treatment method is not feasible for sites contaminated with toxic substances as it is harmful to microorganisms.

Nanoparticles however have the unique capability to remediate such toxic environments and also provide a healthy substrate for microbial activity thus speeding up the process of environment clean-up. Nanoparticles can be prepared by physico-chemical methods (Masala and Seshadri 2004; Swihart 2003) but the use of hazardous chemicals, high cost and toxic byproducts has given biological nanoparticle synthesis an advantage (Konishi et al. 2006). Nanobioremediation is the use of nanoparticles to remove pollutants by enhancing microbial activity.

Nanoparticles (NPs) may be either metallic or nonmetallic and differently shaped. NPs are of the following types-single metal NPs, bimetallic NPs, carbon based NPs, modified NPs etc. Metal nanoparticles have applications in different fields like medical imaging (Lee et al. 2008a), drug delivery (Horcajada et al. 2008), electronics (Lipovsky et al. 2008), nanocomposites (Seager et al. 2007), biolabeling (Liang et al. 2006), biocide or antimicrobial agents (Sanpui et al. 2008), sensors (Jiang et al. 2008), non-linear optics (Ebothe et al. 2006), hyperthermia of tumors (Pissuwan et al. 2006), intercalation materials for electrical batteries (Klaus-Joerger et al. 2001), optical receptors (Dahan et al. 2003) etc. The wide range of applications of nanoparticles is due to their unique optical, thermal, electrical, chemical, and physical properties (Panigrahi et al. 2004).

Nanoscale materials has the following characteristics—Larger surface area per unit mass, show quantum effect and hence is more reactive, exhibit plasmon resonance and can diffuse or penetrate contaminated sites easily. Selective sample extraction can be achieved by modifying the surface functionality of the nanoparticles.

In this review we have focused on the biosynthesis of five nanoparticles (Zn, Ag, Au, Fe, Cu) using plant extracts, bacteria, fungi and algae. We have also compiled the different degrading and remediation activities of these nanoparticles for possible large scale restoration programs.

2 Biosynthesis of Zinc Nanoparticles (ZnNP's)

In the last two decades ZnNP's have been given significant attention owing to its applications in varied fields like piezoelectric films (Martin et al. 2000), piezoelectric sensor (Wang et al. 2004), ceramics (Grigorjeva et al. 2008), photo catalysis

Table 1 Biosynthesis of ZnNPs

Materials	Organism	References
Plant	*Aloe barbadensis miller*	Sangeetha et al. (2011)
	Calotropis giganta	Vidya et al. (2013)
	Corriandrum sativum, Acalypha indica	Gnanasangeetha and Thambavani (2013a, b)
	Parthenium hysterophorus	Rajiv et al. (2013)
	Azadirachta indica	Gnanasangeetha and Thambavani (2013c)
Bacteria	*Lactobacillus*	Prasad and Jha (2009)
	Lactobacillus plantarum	Ameer et al. (2010)
	Aeromonas hydrophila	Jayaseelan et al. (2012)
Fungi	*Candida albicans*	Mashrai et al. (2013)
Algae	*Cystophora moniliformis*	Vigneshwaran et al. (2006)

(Pal and Sharon 2002) solar cells (Gordillo 2002), bio-imaging, drug delivery (Xiong 2013) actuator, biosensors (Yang et al. 2012) and water purification specifically arsenic removal (Singh et al. 2013b).

Owing to the wide applications of ZnNP's it has been synthesized by methods such as wet chemical method (Lee et al. 2009; Mehta et al. 2012), organic solvent method (Mezni et al. 2012) and microwave method (Nehru et al. 2012). However biological synthesis using bacteria, fungi, algae and plant extracts is the method of choice and many researchers have been successful in synthesizing ZnNPs (Table 1).

3 Biosynthesis of Silver Nanoparticles (AgNP's)

Nanobiotechnological developments have led to the development of environmentally benign nanoparticles. AgNP's have applications in non-linear optics, as intercalation materials for electrical batteries, optical receptors, as a catalyst and as an antibacterial. The antimicrobial activity of silver nanoparticles has many uses like the production of AgNP coated blood collecting vessels, coated capsules, bandaids etc. (Geoprincy et al. 2011). Biosynthesis of AgNp's using biological entities is tabulated in Table 2.

Table 2 Biosynthesis of AgNP's

Materials	Organism	References
Plant	*Euphorbia hirta, Nerium indicum*	Priya et al. (2011)
	Cleome viscosa	Yamini et al. (2011)
	Trigonella foenumgraecum	Singh et al. (2011)
	Cycas	Jha and Prasad (2010)
	Ocimum	Mallikarjuna et al. (2011)
	Sinapis arvensis	Khatami et al. (2015)
	Iresine herbstii	Dipankar and Murugan (2012)
	Lantana camara	Kumar et al. (2015)
	Citrus limon	Prathna et al. (2011)
	Artemisia nilagirica	Vijayakumar et al. (2013)
	Pithophora oedogonia	Sinha et al. (2015)
	Butea monosperma	Chaturvedi and Verma (2015)
	Nerium oleander	Subbaiya et al. (2014)
Bacteria	*Morganella psychrotolerans*	Ramanathan et al. (2010)
	Bacillus licheniformis	Kalimuthu et al. (2008)
	Streptomyces sp. LK3	Karthik et al. (2014)
	Staphylococcus aureus	Nanda and Saravanan (2009)
	Brevibacterium casei	Kalishwaralal et al. (2010)
	Streptomyces rochei	Selvakumar et al. (2012)
Fungi	*Aspergillus fumigates*	Bhainsa and D'souza (2006)
	Aspergillus tamarii	Kumar et al. (2012)
	Schizophyllum commune (mushroom fungus)	Arun et al. (2014)
	Trichoderma reesei	Vahabi et al. (2011)
	Cladosporium cladosporiodes	Balaji et al. (2009)
	Fusarium oxysporum	Birla et al. (2013)
	Aspergillus clavatus	Verma et al. (2010a, b)
Algae	*Navicula atomus, Diadesmis gallica, Stauroneis sp. Sargassum wightii, Fucus vesiculosus*	Asmathunisha and Kathiresan (2013)
	Turbinaria conoides	Rajeshkumar et al. (2012)
	Sargassum longifolium	Devi et al. (2013)
	Caulerpa racemosa	Kathiraven et al. (2015)
	Cystophora moniliformis	Prasad et al. (2013)
	Sargassum muticum	Azizi et al. (2013)

4 Biosynthesis of Gold Nanoparticles (AuNP's)

Gold nanoparticles show high chemical reactivity on comparison with bulk gold. It exhibits surface plasm on oscillations which can be used in fields like labeling, imaging and sensing. AuNPs are biocompatible and hence can be used in disease diagnosis and therapy. AuNPs have been biosynthesized from plant extracts, bacteria, fungi and algae. Table 3 focuses on the biological synthesis of AuNP's.

Table 3 Biosynthesis of AuNP's

Materials	Organism	References
Plant	*Sorbus aucuparia*	Dubey et al. (2010)
	Chenopodium album	Dwivedi and Gopal (2010)
	Maduca longifolia	Fayaz et al. (2011)
	Rosa hybrida	Noruzi et al. (2011)
	Ocimum sanctum	Philip and Unni (2011)
	Magnolia kobus, Diopyros kaki	Song et al. (2009)
	Sesbania drummondii	Sharma et al. (2007)
Bacteria	*Rhodopseudomonas capsulate*	He et al. (2007)
	Escherichia coli DH5a	Du et al. (2007)
	Thermomonospora sp.	Kasthuri et al. (2009)
	Rhodococcus sp.	Park et al. (2011)
	Delftia acidovorans	Johnston et al. (2013)
	Pseudomonas aeruginosa	Narayanan and Sakthivel (2010)
	Geobacillus sp. strain ID17	Correa-Llantén et al. (2013)
	Klebsiella pneumonia	Malarkodi et al. (2013)
	Marinobacter pelagius	Sharma et al. (2012)
Fungi	*Candida albicans*	Chauhan et al. (2011)
	Fusarium semitectum	Sawle et al. (2016)
	Epicoccum nigrum	Sheikhloo et al. (2011)
	Cylindrocladium floridanum	Narayanan and Sakthivel (2011)
	Aspergillus clavatus	Verma et al. (2010a, b)
	Neurospora crassa	Castro-Longoria et al. (2011)
	Aspergillus oryzae	Binupriya et al. (2010)
	Hormoconis resinae	Mishra et al. (2010)
	Penicillium brevicompactum	Mishra et al. (2011)
	Saccharomyces cerevisiae	Sen et al. (2011)
Algae	*Sargassum wightii*	Singaravelu et al. (2007)
	Shewanella oneidensis	Suresh et al. (2011)
	laminaria japonica	Ghodake and Lee (2011)
	tetraselmis kochinensis	Senapati et al. (2012)
	Klebsormidium flaccidium	Dahoumane et al. (2012)
	Centella asiatica	Das et al. (2010)
	Lemanea fluviatilis	Sharma et al. (2014)
	Sargassum muticum	Namvar et al. (2015)
	Padina gymnospora	Singh et al. (2013a)

5 Synthesis of Iron Nanoparticles (FeNP's)

The three major forms of iron oxides found in nature are magnetite (Fe_3O_4), maghemite (γ-Fe_2O_3), and hematite (α-Fe_2O_3) (Cornel and Schwertmann 1996). Of all the different kinds of iron oxides, magnetite have aroused the interest of many researchers owing to the fact that it can be easily synthesised, can be modified or coated and has superparamagnetic characteristics (McHenry and Laughlin 2000). This property makes it easy to separate these supermagnetic particles from aqueous solution and complicated matrices by applying an external magnetic field. Some problems associated with using FeNP's are its intrinsic instability resulting in the formation of agglomerates and its high chemical activity which promotes oxidation, subsequent loss of magnetism and dispersion. Hence for application in various fields these nanoparticles have to be coated with either inorganic substances like silica, carbon etc., or with organic species like surfactants and polymers (Wu et al. 2013). Iron nanoparticles have been synthesized by various chemical and physical methods (Afonso et al. 2011). Some emerging methods of synthesis of iron nanoparticles are the use of microorganisms and plant extracts which exclude the use of harmful chemicals and toxic byproducts (Table 4).

Table 4 Biosynthesis of FeNP's

Materials	Organism	References
Plant	*Sorghum sp.*	Njagi et al. (2010)
	Green tea	Shahwan et al. (2011)
	Musa paradisiaca	Herlekar et al. (2014)
	Dodonaea viscosa	Daniel et al. (2013)
	Musa paradisiaca	Venkateswarlu et al. (2013)
	Terminalia chebula	Kumar et al. (2013)
	Eucalyptus tereticornis, Melaleuca nesophila, and *Rosemarinus officinalis*	Wang et al. (2014)
	Aloe vera	Phumying et al. (2013)
Bacteria	*Bacillus subtilis*	Sundaram et al. (2012)
	Shewanella oneidensis	Perez-Gonzalez et al. (2010)
	E. coli	Lee et al. (2008a, b)
	Klebsiella oxytoca	Arčon et al. (2012)
Fungi	*Fusarium oxysporum*	Bharde et al. (2006)
	C. globosum	Kaul et al. (2012)
	Plerotus Sp.	Mazumdar and Haloi (2011)
	Alternaria alternata	Mohamed et al. (2015)
Algae	*Sargassum muticum*	Mahdavi et al. (2013)
	Sargassum myriocystem	Sangeetha and Kumaraguru (2014)

6 Synthesis of Copper Nanoparticles (CuNP's)

Copper is one of the most widely used materials in the world owing to its usage in fields like electricity, optics, catalysis, biomedical and antimicrobial applications. Many researchers have been successful in the biosynthesis of copper nanoparticles using the seed, flower, leaves and fruit skin of plants (Table 5). The nanoparticles synthesized from plant extracts were found to be covered by the medicinal properties of the plant. CuNP is an antimicrobial agent used in food packaging and water treatment.

Table 5 Biosynthesis of CuNP's

Materials	Organism	References
Plant	*Magnolia kobus*	Lee et al. (2013)
	Pterocarpus marsupium	Sharma et al. (2015a)
	Malva sylvestris	Awwad et al. (2015)
	Calotropis gigantea	Sharma et al. (2015b)
	Tamarix gallica	Nasrollahzadeh et al. (2015)
	Aloe barbadensis miller	Kumar et al. (2015)
	Tabernaemontana divaricate	Sivaraj et al. (2014a, b)
	Pyrus pyrifolia	Sundaramurthy and Parthiban (2015)
	Acalypha indica	Sivaraj et al. (2014b)
	Nerium oleander	Gopinath et al. (2014)
	Tridax procubens	Gopalakrishnan et al. (2012)
	Calotropis procera L	Harne et al. (2012)
Bacteria	*M. psychrotolerans* and *M. morganii RP42*	Ramanathan et al. (2011)
	Pseudomonas stutzeri	Varshney et al. (2010)
	Pseudomonas fluorescens	Shantkriti and Rani (2014)
	Serratia	Saif Hasan et al. (2008)
	Streptomyces sp.	Usha et al. (2010)
	Escherichia coli	Singh et al. (2010)
Fungi	*Hypocrea lixii*	Salvadori et al. (2013)
	Penicillium aurantiogriseum, Penicillium citrinum, Penicillium waksmanii	Honary et al. (2012)
	Aspergillus Sp.	Cuevas et al. (2015)
Algae	*Bifurcaria bifurcata*	Abboud et al. (2014)

7 Nano Bioremediation

Population growth, rapid industrialization and long term droughts has resulted in the spread of wide range of pollutants in surface and ground water system (Chong et al. 2010). The major contaminants include heavy metals, inorganic compounds, organic pollutants and many other complex compounds (Li et al. 2011). It is imperative to remove these toxic substances as they are harmful not only to human beings but also to the ecological environment (Pang et al. 2011). Waste water treatment processes like photo catalytic oxidation, adsorption/separation processing and bioremediation (Huang et al. 2006; Zelmanov and Semiat 2008) have been tried and tested. But factors like efficiency, operational method, energy requirements and high cost have restricted their usability (Huang et al. 2006; Zelmanov and Semiat 2008). In the past two decades nano scale materials have been used as an alternative to existing treatment materials due to its efficiency, cost effectiveness and eco-friendly nature (Dastjerdi and Montazer 2010).

Iron NPs is considered to be the first nanoparticle to be used in environmental clean-up (Tratnyek and Johnson 2006). Current applications of iron-based technologies in contaminated land or groundwater remediation can be broadly divided into two groups, based on the chemistry involved in the remediation process: technologies which use iron as a sorbent (adsorptive/immobilization technologies) and as an electron donor to break down or to convert contaminants into a less toxic or mobile form (reductive technologies) (Cundy et al. 2008). However, it should be noted that many technologies utilize both these processes.

Zn NPs a semiconductor photo catalyst have been extensively studied by researchers around the globe owing to its capacity to degrade organic dyes. ZnNPs can photo catalyse and cause the complete degradation of a wide variety of compounds from dyes to phenols and pharmaceutical drugs (El-Kemary et al. 2010).

Among all nanoparticles noble metal nanoparticles like gold and silver have enormous applications in diverse areas. In recent times researchers have analyzed the potential of Au and Ag nanoparticles in the degradation of organic dyes. Copper nanoparticles also can be used in the degradation of organic dyes with good results. Table 6 focusses on the degradation and subsequent remediation of various environmental pollutants.

Table 6 Remediation by metal nanoparticles

Metal nanoparticles	Remediation	References
Iron	Pentachlorophenol	Kim and Carraway (2000)
	Chlorobenzene	Lee et al. (2011)
	Dissolved sulfides	Chaung et al. (2014)
	Pyrene	Chang and Kang (2009)
	Dibenzo-*p*-dioxins and furans	Kim et al. (2008)
	Nitrate	Ryu et al. (2011)
	RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine)	Naja et al. (2008)
	Cationic and anionic dyes	Shahwan et al. (2011)
	Alachlor, pretilachlor	Kim et al. (2006)
	Perchlorate	Moore et al. (2003)
	Brominated methanes	Lim et al. (2007)
	Chlorinated ethanes	Song and Carraway (2005)
	4,4′- dinitrostilbene-2,2′-disulfonic acid	Fan et al. (2007)
	Uranium	Fan et al. (2012)
	Lindane, atrazine	Joo and Zhao (2008)
	Alachlor and atrazine	Bezbaruah et al. (2009)
	Polychlorinated biphenyls (PCBs)	Choi et al. (2008)
	Atrazine	Satapanajaru et al. (2008)
	Cr(VI)	Cutting et al. (2010)
	Ni(II)	Li and Zhang (2006)
	As(V), Cr(VI)	Pradeep (2009)
	Cu(II), Pb(II)	Mahdavian and Mirrahimi (2010)
	Pb(II), Hg(II)	Ambashta and Sillanpää (2010)
	Cu(II), Cr(VI)	Huang and Chen (2009)
	Cr(VI) and Cd(II)	Li et al. (2013)
	Trichloro ethylene	Smuleac et al. (2011)
	Cd2+	Boparai et al. (2013)
	Metalachlor	Santornchot et al. (2010)
	Dichloroethane	Wei et al. (2012)

(continued)

Table 6 (continued)

Metal nanoparticles	Remediation	References
Zinc	4-chloro catechol	Kamat (2002)
	Cd(II)	Srivastava et al. (2013)
	Brown CGG dye	Islam (2015)
	Methylene blue	Srivastava et al. (2013)
	RhodamineB	Ali et al. (2013)
	Organic dyes	Sanna et al. (2016)
	Direct red 23	Kumar et al. (2014)
	Eriochrome black-T dye	Kazeminezhad and Sadollahkhani (2014)
	Methylene blue	Jain et al. (2014)
	Formaldehyde	Darvishi Cheshmeh Soltani et al. (2015)
	Phenol	Kruefu et al. (2012)
	Congo Red and Benzopurpurine 4B	Elaziouti and Ahmed (2011)
	Malachite green	Khezami et al. (2016)
	Resorcinol	Pardeshi and Patil (2009)
	Rhodamine B	Zhao and Wang (2011)
	fuchsine	Zhou et al. (2009)
Gold	Methylene blue	Suvith and Philip (2014)
	Tertiary dye effluent (Methyl orange, Acid red 88, Acid orange 10)	Sathishkumar et al. (2013)
	4-nitrophenol	Huang et al. (2009)
	Methylene blue	Gupta et al. (2010)
Copper	Methylene blue	Sinha and Ahmaruzzaman (2015)
	Methyl orange	Soomro and Nafady (2015)
	Dichloromethane	Huang et al. (2012)
Silver	Organic dyes (methyl violet, saffranin, eosin methylene blue, methyl orange)	Bhakya et al. (2015)
	Methylene blue	Morones et al. (2005)
	4-nitrophenol	Gangula et al. (2011)
	Congored	Modi et al. (2015)
	Coomassie Brilliant Blue G-250	Arunachalam et al. (2012)
	Textile Effluent	Corso and Almeida (2009)

8 Conclusions

Nanotechnology is revolutionizing the way we live. The unique characteristics of nanoparticles have made them the particle of choice in many fields including remediation of environmental pollutants. Ecofriendly synthesis of nanoparticles coupled with remediation can go a long way in promoting sustainability. Biosynthesis helps minimize the use of harmful chemicals and solvents and is simple, cost effective and time saving. Zn, Ag, Au, Fe and Cu nanoparticles have been synthesized by many researchers using various biological methods. Although there are many studies on the synthesis of Au, Ag, Cu and Zn nanoparticles, very few have concentrated on the biosynthesis of FeNPs using bacteria, fungi and algae. Green synthesis of metal NPs using plant extract seems to be the subject of choice of a majority of researchers, however only a few is documented in this review. On comparing the bioremediation properties of these NPs it was noted that FeNPs has a wider application, degrading pollutants like pesticides, dyes, hydrocarbons, TCE etc. Most of the other NPs find applications in the photocatalytic degradation of dyes. Considering the wide applications of FeNPs in remediation it is necessary to find novel methods to biosynthesize FeNPs on a large scale.

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Stabilization of Market Vegetable Waste through the Process of Vermicomposting by *Eisenia Foetida*

J. Girija, T. Roja Devi, S. Pavithra Devi, T. Soundharya and J. Pawlin Vasanthi

Abstract India produces around 3000 million tonnes of organic wastes annually. The disposal of ever increasing amount of organic wastes is becoming a serious problem in India. The hygienic disposal of organic wastes by composting is an environmentally sound and economically viable technology resulting in the production of organic fertilizer which is a basic and valuable input in organic farming. The waste collected from the market was grouped into vegetable waste, tapioca waste and sugarcane bagasse. The waste was subjected to initial decomposition under shadow. After that it was allowed for stabilization (24 h) in mud pots before inoculation with Eisenia foetida. Physico chemical analysis was performed on the 51st day. It is found that tapioca generates a rich vermicompost with an acidic pH, low electrical conductivity, high TKN%, phosphorus and potassium. This is followed by sugarcane bagasse with neutral pH, low electrical conductivity, high TKN % and low total organic carbon. Vegetable waste has high phosphorous and potassium and low total organic carbon.

Keywords Organic waste · Organic fertilizer · Organic farming · Stabilization · Vermicompost

1 Introduction

India and many other countries are suffering from problems due to urbanization, which is a very rapid process and a worldwide phenomenon. Deteriorating quality of urban environment is one of its important impacts. Solid waste is the major contribution and its volume is greatly increasing due to increase of living standards and population density. Hence the importance of efficient "solid waste management" is increasingly recognized.

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India produces around 3000 million tonnes of organic wastes annually (Alok Bhardwaj 2010). The disposal of ever increasing amount of organic wastes is becoming a serious problem in India. The hygienic disposal of organic wastes by composting is an environmentally sound and economically viable technology resulting in the production of organic fertilizer which is a basic and valuable input in organic farming. In India, about 350 million tonnes of agricultural wastes are generated annually, of which vegetable waste alone is in major portion.

The biological treatment of these wastes appears to be most cost effective and carry a less negative environmental impact (Paraskeva and Diamadopoulus 2006). Paddy straw and cane straw are produced in the largest quantity in the country. In many countries the traditional management practice of postharvest residues is the elimination by open air burning which leads to release of greenhouse gases and the production of particulate matter are obvious.

Vermicomposting from sugarcane waste (bagasse) provides one of the best examples of renewable based cogeneration yet it remains largely exploited. Until now, the potential for bagasse generation of vermicomposting has been largely unquantified. This report was compiled to highlight the advantages and main issues of bagasse generation and the potential it offers for vermicomposting production.

Vermicomposting has been reported to be a viable, cost effective and rapid technique for the efficient management of organic solid wastes (Logsdon 1994). Vermicomposting, utilizing earthworms, is an eco-biotechnological process that transforms energy-rich and complex organic substances into a stabilized humus-like product (Benitez et al. 2000). Vermicomposting is an important aspect, as it converts waste to wealthy by using cheap eco-friendly option with activity of earthworm (Mall et al. 2005).

The objective of the present study is to analyse the Physico-Chemical parameters of the vermicompost of three different market vegetable wastes and to find out the most suitable substrate on the basis of the nutrient content, for the production of quality manure.

2 Materials and Methods

Vegetable market waste was collected from the Ukadam market, Coimbatore. The waste collected was grouped into vegetable waste, tapioca waste and sugarcane waste as bagasse (after the extraction of juice). Vegetable waste comprised of cauliflower, beetroot, beans, tomatoes, lady's finger, cabbage etc. The waste was subjected to initial decomposition under shadow. After that it was allowed for stabilization (24 h) in mud pots before inoculation with earthworms. Eisenia foetida was collected from the Vermicompost pit of the Department of Zoology, Nirmala College for women, maintained under the Career Oriented Program of UGC. The species was identified in the Department.

Vermicomposting studies were carried out with 15 days pre-decomposed market waste. The waste was mixed with cowdung in the ratio of 1:1. After stabilization,

10 earthworms (Eisenia foetida) were introduced into each mud pot. The vermicomposing trials were performed under controlled environmental conditions where the temperature was 26 ± 1 °C. The mud pots were maintained for 50 days.

Physico chemical analysis was performed on the 51st day.

2.1 pH: Electrometric Method (Standard Method)

A sample suspension was made by taking 1.0 g of air dried sample with 5.0 ml distilled water in a flask to prepare a suspension (1:5 w/v). The suspension was kept over a shaker for 30 min. The shaking period was not allowed to exceed 30 min otherwise various biological processes may start in suspension which may change actual pH of suspension. The pH of the suspension was estimated by pH meter. The pH meter was calibrated with buffer solution.

2.2 Electrical Conductivity (EC)

A sample suspension was made as for pH and used. The conductivity meter was adjusted to know the conductivity with standard KCL solution (0.1 N) by cell constant. The conductivity is measured by dipping the electrode into the solution.

2.3 Total Organic Carbon (Nelson and Sommers Method, 1982)

Organic carbon was determined by dry combustion (Nelson and Sommers, 1982). A sample of 500 mg of dried ground (<2 mm) was put into pre-weighed china crucibles. The sample was ignited in a muffle furnace at 600 °C for 1½ h. The furnace was allowed to cool and the ash produced was weighed. Organic C was calculated from the following relationship:

$$\text{Organic C(percent)} = (\text{100-ash percent})/1.724.$$

2.4 Total Kjeldahl Nitrogen (Bremner and Mulvaney Method, 1982)

500 mg of dry material was taken in digestion tube. To each tube, 1 g of digestion mixture and 10 ml of sulphuric acid was added. It was then digested on Kjeldahl digester till bluish green colour appeared in tube. Then 10 ml of boric acid indicator

solution was taken in 100 ml Erlenmeyer flask that was marked to indicate a volume of 50 ml and flask was placed under the condenser of steam distillation apparatus. The digested mixture was taken in distillation flask and flask was attached to steam distillation colour turned black. Distillation was started by closing stop cork on steam by pass tube of distillation apparatus. When the distillate reached 50 ml mark of receiver flask, the distillation was stopped by opening stop cork on steam by pass tube and end of condenser was rinsed. The total nitrogen in distillate was determined by titration with N/50 HCL. The colour change at end point was from green to permanent faint pink. From the following formula, N (per cent) was estimated as:

$$\mathrm{N}\% = \mathrm{V} \times 0.00014 \times \mathrm{D} \times 10 \div \mathrm{W} \times \mathrm{A}$$

where,

V = Volume of N/50 HCL used, D = Dilution factor (Volume made in volumetric flask), W = Weight (g) of sample, A = Volume of liquid taken.

2.5 Phosphorus (Garg et al. 2005)

This was determined by spectrophotometric method (Garg et al. 2005). 0.5 g sample was taken in a 250 ml conical flask and to it was added 100 ml of 0.002 N H_2SO_4. Solution was shaken for half an hour and then 2 ml of ammonium molybdate solution was added. 50 ml of filtered solution was taken and 5 drops of $SnCl_2$ was added into it. A blue colour appeared and the reading was taken at 690 nm on a spectrophotometer using a blank with the same amount of reagents. Reading was taken after 5 min but before 12 min of the addition of last reagent. The concentration was found out with help of standard curve.

The amount of TAP was estimated by using the following formula:

$$\text{Available phosphorus (percent)} = P \times V/W \times 100$$

where,

P = Phosphate content in extract (mg/I), V = Total volume of extract (ml) prepared,

W = Weight of air dried sample (g).

2.6 Potassium (Flame Photometric Method)

This was determined by flame photometric method where, 0.5 g of sample was weighed and to it 100 ml of ammonium acetate solution was added and kept for overnight. The solution was filtered and during filtration the first few ml of filtrate was discarded. Potassium content in extract was determined by using potassium

filters after necessary settings and calibration of the instrument. Readings for sample were noted. Potassium content in sample was determined with the help of standard curve.

The level of potassium was estimated by using the following formula:

$$\text{Available potassium} = K \times V/1000 \times \times S$$

where,

K = amount of potassium (mg) per litre in sample, V = Total volume of sample extracted prepared, S = weight of sample taken in gms.

3 Results and Discussion

The pH value is significantly low ($p < 0.05$) with tapioca waste (6.24 ± 0.181) and neutral in sugarcane bagasse (7.1 ± 0.15) and vegetable waste (7.44 ± 0.288) (Fig. 1). At the end of the composting process, pH in all the treatments was found to be nearer to neutral pH(7) which is optimum according to Beulah and Partheeban (2001), who conducted composting with municipal solid waste. pH increase is due to degradation of short-chain fatty acids and ammonification of organic nitrogen (Tognetti et al. 2007) (Table 1).

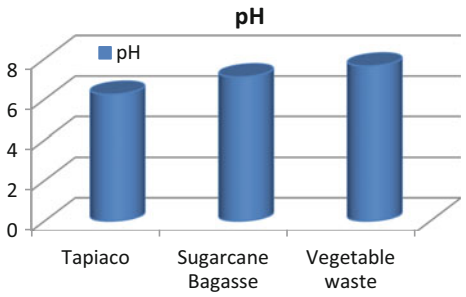

Fig. 1 pH of the vermicompost produced from tapioca, sugarcane bagasse and vegetable waste

Table 1 Physico-chemical parameters of the vermicompost produced from tapioca, sugarcane bagasse and vegetable waste

Physico-chemical parameters	Tapioca	Sugarcane bagasse	Vegetable waste
pH	6.24 ± 0.18*	7.1 ± 0.15*	7.64 ± 0.28*
Electrical conductivity (siemen units)	9.54 ± 0.04*	8.22 ± 0.03*	19.35 ± 0.03*
TKN (%)	0.91 ± 0.02*	1.02 ± 0.01*	0.9 ± 0.02*
Phosphorus (mg/kg)	679.37 ± 0.62*	466.46 ± 0.47*	484.33 ± 0.47*
Potassium (mg/kg)	4141.03 ± 0.92*	1867.39 ± 1.32*	8816.14 ± 1.59*
Total organic carbon (%)	11.05 ± 0.90*	8.34 ± 0.87*	9.97 ± 0.84*

*Indicates $p < 0.05$

The decrease in pH was caused by the voltalization of ammonical nitrogen and H^+ released due to microbial nitrification process by nitrifying bacteria. This might be due to production of carbon dioxide and organic acids during decomposition of organic residues. The near neutral pH of vermicompost may be attributed by the secretion of NH^{4+} ions that reduce the pool of H^+ ions and the activity of calciferous glands in earthworms containing carbonic anhydrase that catalyzes the fixation of CO_2 as $CaCO_3$, thereby preventing the fall in pH (Pattnaik and Reddy 2010).

According to Sangwan et al. (2008), shifting the pH to lower levels could be attributed to mineralization of nitrogen and phosphorus and bioconversion of organic materials into intermediate species of organic acids.

Electrical conductivity is significantly low ($p < 0.05$) in sugarcane bagasse (8.226 ± 0.033) and tapioca (9.54 ± 0.044) and high in vegetable waste (19.35 ± 0.037) (Fig. 2). The EC values also showed a decreasing tendency when compared to initial values indicating that the vermicompost is ideal for land application. Organic carbon was found to be lower at the end of the experimental period.

The type of mutualism between the earthworms and the microorganisms is very much the cause for the degradation of organic matter leading to the release of CO_2, which is nothing but mineralization of organic carbon. The increase in EC might have been due to release of different mineral salts in available forms. As the composting process further progressed the available salts are converted into insoluble salts which may be the reason for the reduction of EC at the latter stage.

TKN is significantly high ($p < 0.05$) in sugarcane bagasse (1.022 ± 0.0119) than vegetable waste (0.968 ± 0.028) and tapioca (0.914 ± 0.020) (Fig. 3).

The earthworms accelerate the microbial mediated nitrogen transformation during the process of vermicomposting. However, the nitrogen mineralisation or the increase in the N levels in the final substrate may be attributed to the addition of excretory products of the earthworms, along the mucous, body fluids and enzymes into the substrate. The nitrogen mineralisation pattern is very much dependent on the initial nitrogen content of the substrate and the activity of the earthworms in the waste composting system (Suthar 2007; Sivakumar et al. 2009).

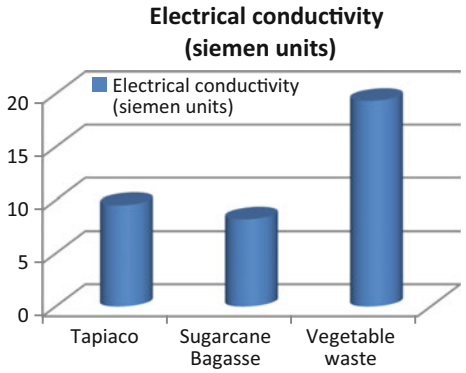

Fig. 2 Electrical conductivity of the vermicompost produced from tapioca, sugarcane bagasse and vegetable waste

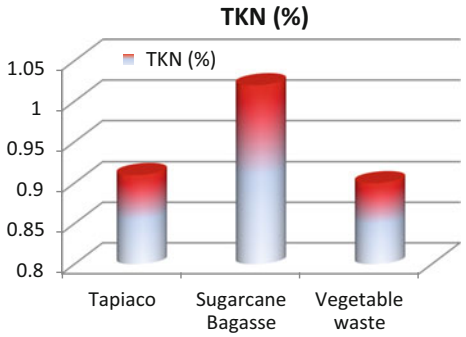

Fig. 3 Total Kjeldahl nitrogen of the vermicompost produced from tapioca, sugarcane bagasse and vegetable waste

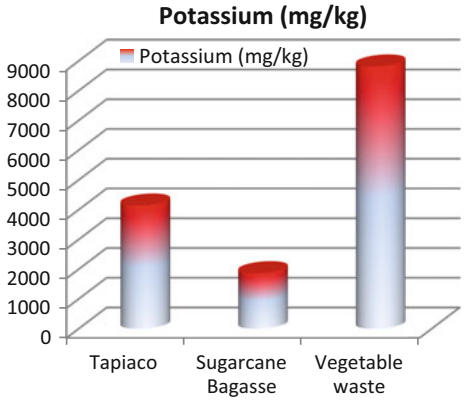

Fig. 4 Potassium content of the vermicompost produced from tapioca, sugarcane bagasse and vegetable waste

Potassium concentration in the vermicompost is high for vegetable waste (8816.14 ± 1.59) than tapioca (4151.03 ± 0.925) and sugar cane (1867.39 ± 1.32) (Fig. 4).

Total organic carbon is lowest for sugarcane (8.346 ± 0.879), followed by vegetable waste (9.977 ± 0.848) and Tapioca (11.005 ± 0.900) (Fig. 5). The observed results are supported by those of other researchers who have reported 20–45% and

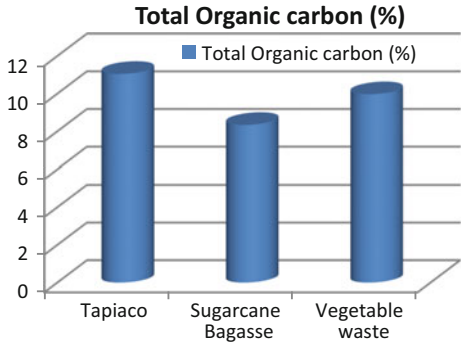

Fig. 5 Total organic carbon of the vermicompost produced from tapioca, sugarcane bagasse and vegetable waste

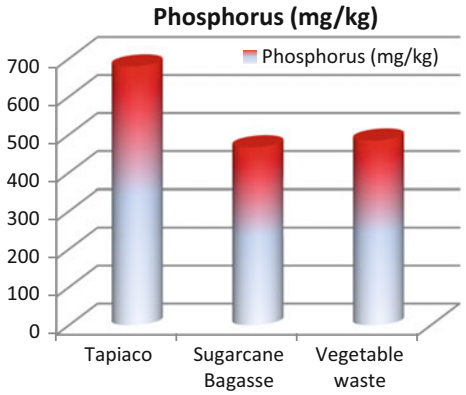

Fig. 6 Phosphorus content of the vermicompost produced from tapioca, sugarcane bagasse and vegetable waste

40–50% reduction of TOC as CO_2 during vermicomposting of municipal or industrial wastes and filter mud respectively (Khawairakpam and Bhargava 2009; kaviraj and Sharma 2003).

The phosphorus content is high ($p < 0.05$) in tapioca (679.37 ± 0.620) when compared to sugarcane (466.46 ± 0.472) and vegetable waste (484.33 ± 0.476) (Fig. 6). The amount of total phosphorus increased gradually with increase in composting period because of the gradual mineralization of organic matter.

When the organic matter passes through the gut of earthworm, the phosphorous is converted to more available forms. Similarly, further release of P is aided by phosphate solubilising microorganisms in the vermicasts. These findings were similar to that reported by Delgado et al. (1995) in sewage sludge vermicomposts.

According to Curry and Schmidt (2007), earthworms prime the symbiotic microflora with secreted mucus and water to increase the degradation of ingested organic matter and releases of metabolites.

Earthworm activity enriches the nitrogen profile of vermicompost through microbial mediated nitrogen transformation, through addition of mucus and nitrogenous wastes secreted by earthworms. Decrease in pH may be an important factor in nitrogen retention as N_2 is lost as volatile ammonia at high pH values.

From the above experiment it is inferred that tapioca generates a rich vermicompost with acidic pH, low electrical conductivity, high TKN%, phosphorus and potassium. This is followed by sugarcane bagasse with neutral pH, low electrical conductivity, high TKN% and low TOC. Vegetable waste has high phosphorous and potassium and low TOC%.

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Bioremediation—A Ecosafe Approach for Dairy Effluent Treatment

A. Punnagaiarasi, A. Elango, G. Rajarajan and S. Prakash

Abstract The dairy industry involves processing of raw milk into products like consumer milk, butter, cheese etc. The quantity of water required in a milk processing plant depends upon the size of the plant, generally expressed in terms of the maximum weight of milk handled in a single day, and the processes involved. The daily volume of water required may vary widely, depending mainly on the availability of water and the control of all water using operation in the amount of water needed for the operations which involves continuous flow, for rinsing and washing and is not necessarily proportional to the amount of product processed. Most of the waste water discharged into water bodies, disturbs the ecological balance and deteriorates the water quality. The casein precipitation from waste decomposes further into highly odorous black sludge. Effluent from milk processing unit contains soluble organics, suspended solids, trace organics which releases gases, causes bad taste and odour, impart colour and turbidity, and promote eutrophication. Bioremediation is a ecosafe approach for treating the dairy effluent without disturbing the environment. This review deals about characteristic of dairy effluent and Microorganisms used in the bioremediation of the dairy industry wastes.

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1 Introduction

1.1 Bioremediation

"Remediate" means to solve a problem, and "bio-remediate" means to use biological organisms to solve environmental problems such as contaminated soil or groundwater. In a non-polluted environment, bacteria, fungi, parasites, and other microorganisms are constantly at work breaking down organic matter. What would occur if an organic pollutant such as oil contaminated this environment? Some of the microorganisms would die, while others capable of eating the organic pollution would survive. Bioremediation works by providing these pollution-eating organisms with fertilizer (dairy residues like proteins, salts, fatty substances and lactose), oxygen, and other conditions that encourage their rapid growth. These organisms would then be able to break down the organic pollutant at a correspondingly faster rate. In fact, bioremediation is often used to help clean up oil spills. Bioremediation of a contaminated site typically works in one of two ways. In the case described above, ways are found to enhance the growth of whatever pollution-eating microbes might already be living at the contaminated site. In the second, less common case, specialized microbes are added to degrade the contaminants. Bioremediation provides a good cleanup strategy for some types of pollution, but as you might expect, it will not work for all. Nonetheless, bioremediation provides a technique for cleaning up pollution by enhancing the same biodegradation processes that occur in nature. Depending on the site and its contaminants, bioremediation may be safer and less expensive than alternative solutions such as incineration or land filling of the contaminated materials. It also has the advantage of treating the contamination in place so that large quantities of soil, sediment or water do not have to be dug up or pumped out of the ground for treatment.

The bioremediation is divided into two broad categories that includes in-situ bioremediation and ex-situ bioremediation.

1.2 In Situ Bioremediation

In situ bioremediation is a natural process taking place ever since the first microbes and excess organic substance were both present in the soil (Litchfield, 1993). In this process there is no need to excavate or remove soils or water in order to accomplish remediation. This method exploits natural ways of recycling nutrients through the cycles of nitrogen and carbon. These cycles nowadays are utilized by man to augment the degradation and recycling of wastes and the similar cycles are employed by in situ bioremediation to hygienic contaminated soils (Nelson et al. 1996). In this process, the disintegration of the contaminants is carried out by the indigenous microorganisms which grow on this contaminated soil and can only endure in that environment by using the contaminating substances as a source of

energy (Aelion et al. 1987). In in situ bioremediation, organic pollutants are completely distressed; therefore no secondary waste stream is produced (Dott et al. 1995).

The main advantage of in situ treatment is that no excavation is needed and no exceptional equipment is required. In the specific methods used for bioremediating contaminated soil and water, land farming, composting, intrinsic bioremediation and slurry bioreactor are included.

1.3 Ex Situ Bioremediation

Ex situ bioremediation techniques can be faster, easier to control and used to treat a wider range of contaminants and soil types than in situ techniques. This process requires excavation of contaminated soil or pumping of groundwater to facilitate microbial degradation. One of the main advantages of ex situ bioremediation is that it requires less time than the in situ treatment. Common ex situ treatments include land farming, windrows and bio-piling. Bioremediation of organic compounds has been successfully employed at many sites, however physical factors can be rate limiting. Biopiling enhances aerobic catabolism of creosote by inoculation of air into piles of contaminated soil.

2 Dairy Industry

The composition and volume of dairy wastewaters depends primarily on the sort of products made, measures taken to minimize the amount of waste, water economy and cleansing agents used. The pH of dairy wastewaters depends on the nature of the end product and can assortment from 6.6 to 12.2 (Alvarez-Manteos et al. 2000; Carrasco et al. 2004).

The main characteristics of dairy waste can be summarized as follows:

- High organic freight (fatty substances, etc.)
- Large variations in waste supply (waste residues in dairy industry)
- Considerable variations in pH (4.2–9.4)
- Relatively large load of perched solids (SS) (400–2000 mg/L)

Introduction of products such as milk solids into waste streams also represents a loss of important product for the dairy facilities (Baskaran et al. 2003), in addition to environmental problems that can result from discharge of dairy wastewaters. These effluents may cause serious problems, in terms of organic load on the local municipal sewage treatment systems (Perle et al. 1995) because dairy waste streams contain high concentrations of organic matter. Large fractions of dairy wastewater may restrain proteins, salts, fatty substances, lactose and various kinds of onslaught

chemicals are derived from organic ingredients and nutrients in the waste streams from dairy milk and dairy products. Nitrogen originates mainly from milk proteins in industrial dairy wastewaters and is present in different forms; either organic nitrogen ions (proteins, nucleic acids and urea), or such as NH_4+, NO^3 and NO_2.

Suspended solids in the wastewater originate from gelatinous milk and the curd fines or flavorings (Brown and Pico 1979). Mostly high Na concentrations point out the utilization of huge quantity of alkaline cleaners at dairy plants. Both nitrogen and phosphorus components amounts to a global pollution load per annum of 850–1788 tonnes of phosphorus and 3337–5217 tonnes of nitrogen by the cleaning and disinfection of dairy installations. A partial gentrification and some uptake of phosphorus (from 40 to 70%) can be attained in the activated sludge process. The application of chemical phosphate precipitation also increases because it allows an abolition of 80–90% of phosphorus (Schöberl and Huber 1988) (Table 1).

Table 1 Microorganisms used in the bioremediation of the dairy industry wastes

Microorganisms or biological component used	Products	Potential industrial uses or applications	Reference
Geotrichum candidum	Biomassdegradation of phenol-derived compounds	Bioremediation	Aouidi et al. (2010)
Kluyveromyces marxianus var *marxianus*	Unicelular protein	Food	Páez et al. (2008)
Bacillus megaterium SRKP-3, *Brevibacterium casei SRKP*2	Polyhydroxyalkanoates (PHA)	Plastics	Pandian et al. (2010) and (2009)
Kluyveromyces fragilis	Etanol, butanol, electricity, H_2	Bioenergy or biofuels	Parrondo et al. (2000)
Propionibacterium shermanii, *Propionibacterium acidipropionici*	Citric acid, lactic acid, succinic acid, propionic acid, gibberellic acid	Pharmaceuticals, food industry, agriculture	Anderson et al. 1986; Woskow and Glatz (1991)
Candida bombicola ATCC 22, 214, *Bacillus licheniformis M*104	Biosurfactants	Food industry, medicine, pharmaceutics	Daverey et al. 2009; Gomaa 2013
Proteolytic enzymes of *Maclura pomifera*-papain and hemispherecine	Bioactive peptides	Pharmaceutics, food industry,	Corrons et al. 2012; Reyes-Nava et al. 2006

3 Conclusion

Bioremediation is an efficient process for such organic pollution of soils. It is possible to humiliate about 50% of the initial pollution load in 200 days by simply aerating the soil layer. Importantly, it has been shown that pollutant biodegradation could be valuably followed by appraisal of carbon dioxide evolution during the process which provides a simple way for characterizing the kinetics of degradation. Bioremediation has been the used for biological treatment in a number of locations around the world, including Europe, with varying degrees of success. Techniques have been improved and gained with more knowledge and experience. There is no doubt that the biological treatment has great potential to deal with many types of pollution including soil pollution.

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Application of Bioremediation on Food Waste Management for Cleaner Environment

A. Punnagaiarasi, A. Elango, G. Rajarajan and S. Prakash

Abstract In the past few years, there has been a tremendous increase in food waste generation due to rapid urbanization and industrialization. These food wastages have put an emphasis to employ novel techniques for management of waste generated so that waste generation could be reduced to a minimum or these wastes could be converted into some valuable products. Food waste consists of high levels of sodium and moisture and is usually mixed with other types of waste during its collection. Amount of waste generated is largely determined by two factors—population in a given area and its consumption patterns. In order to cope with this huge waste production, advanced and effective waste management systems are to be adopted that can overcome the gap between production and management of waste disposal. Therefore, in this view much technological advancement has occurred in the recent past which has proved to be useful for combating this problem. In this review, a brief introduction to bioremediation for various food industry waste management and advantages and limitation of bioremediation has been discussed.

Keywords Bioremediation · Food waste management · Cleaner environment

1 Introduction

Food industry is a source of an untapped energy which is mostly dumped in landfills whereby it releases greenhouse gases into an atmosphere (Zafar 2012). It is very difficult to treat and recycle food waste due to its composition. Food waste consists of high levels of sodium and moisture and is usually mixed with other

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types of waste during its collection. Amount of waste generated is largely determined by two factors- population in a given area and its consumption patterns (Mavropoulos 2011). In order to cope with this huge waste production, advanced and effective waste management systems are to be adopted that can overcome the gap between production and management of waste disposal. Bioremediation is a naturally occurring process in which microorganisms either immobilize or transform environmental contaminants to innocuous end products (Thassitou and Arvanitoyannis 2001). Biotreatment is well accepted by industry as it goes along with the current popularity of maintaining nature's harmony. Bioremediation has become a widely accepted option for the clean up of contaminated soils and aquifers although it does not have a fully credible reputation within the regulatory community (NRC 1993).

2 Characteristics and Treatment of Various Food Industries

2.1 Fruit and Vegetable Processing Industry

Industries that process fruits and vegetables are a very important part of the food industry especially in the Mediterranean countries where agriculture still remains one of the main sources of income. The fruit and vegetable canning industry, frozen vegetable industry, vegetable dehydration industry, fruit and vegetable drying industry, fruit pulping, tomato juice concentrate and fruit concentrate belong to this category. These industries may operate seasonally since operation time depends on the production of the fruits and vegetable that they process. That means that the environmental pollution from those industries' waste will also be seasonal. According to the processing stage, different types of waste may be produced thus contributing with different percentages to the formation of the final process waste. The wastes from fruit and vegetable processing industries generally contain large amounts of solid suspensions and a high biochemical oxygen demand (BOD). Some other parameters usually of interest to the waste treatment are pH, chemical oxygen demand (COD), dissolved oxygen and total solids. Indicative values for BOD, COD, suspended solids (SS) and pH for the processing of some fruit and vegetables are summarized. As has already been described, fruit and vegetable industry wastes consist of various by-products with an acidic pH (Riggle 1989), and a moisture content of 80–90% (Grobe 1994). The chemical composition of the wastes varies and depends on the processed fruit or vegetable. In general, the wastes consist of hydrocarbons and relatively small amounts of proteins and fat. The hydrocarbons are mainly sugars and nitrogen and cellulose fibers. The water wastes contain dissolved compounds, pesticides, herbicides and cleaning chemicals. These differences in the nature of the wastes require their separate treatment. Although the solid waste is mainly treated with composting, because of superior results slurry

bioreactors and land farming may also constitute two further options. A pretreatment is necessary to remove the water and neutralize the pH to ensure the best conditions for microbial growth and development. Bulking agents are also added to improve the porosity of the sludge and decrease the bulk density (Schaub and Leonard 1996). The increased porosity may help in the drainage of water, which can be carried out either by gravity or by exerting pressure on the sludge. In some investigations the waste was left in open air so that the excess water is evaporated (Grobe 1994). Aerated piles are more frequently used for the treatment of solid waste from fruit and vegetable industries (Nakata 1994) because they allow the best mixing of the sludge while it is easy to add moisture, nutrients or more waste for processing if necessary. However, if static piles are initially used, then later the compost has to be moved to an aerated pile for further cure.

2.2 *Fermentation Industry*

The fermentation industry is divided into three main categories: brewing, distilling and wine manufacture. Each of these industries produces liquid waste with many common characteristics, such as high BODs and CODs, but differ in the concentration of the organic compounds that determine the biological treatment that will be selected. The difficulty in dealing with fermentation wastewaters is in the flows and loads of the waste. Since the fermentation industry's wastewater contains high concentrations of tannins, phenols and organic acid, anaerobic treatment results in higher performance. Mayer (1991) attempted to compare aerobic with anaerobic treatment of the wastewaters in a German brewery. Anaerobic treatment achieved 91% COD reduction at loading rates up to 20 g COD/L day, whereas the aerobic treatment resulted in a 76% reduction at a loading rate of 69 g COD/L day. In order to optimize the conditions of anaerobic treatment, Suzuki et al. (1997) conducted several experiments for the optimization of acidity and temperature of highly concentrated brewery wastewater by applying the upflow anaerobic sludge blanket. These experiments showed that the optimal conditions for the particular treatment were 40 °C and 5–6 pH. The amount and load of distillery waste varies according to the raw materials used. In winery, the treatment methods are based on principles similar to the previous fermentation industries. Experiments conducted both in the laboratory and on industrial scale showed that with the use of a full-scale, modular, multi-stage activated sludge treatment plant could reduce the COD level up to 98% when the influent COD varies between 2000 and 9000 mg/L (Fumi et al. 1995). One of the main problems in winery waste treatment is the presence of vinasse (It is like a molasses, a hone like dark brown syrup), which needs to be treated biologically for 4–8 days in order to reduce 90% of the COD (Boudouropoulos and Arvanitoyannis 2000).

2.3 Dairy Industry

Dairy industries contribute substantially to the pollution of surface water and soil. The main wastes from these industries are chemically modified liquid wastes. The main characteristics of dairy waste can be summarized as follows: high organic load (fatty substances, etc.), large variations in waste supply, considerable variations in pH (4.2–9.4), relatively large load of suspended solids (SS) (400–2000 mg/L). The dairy wastewater may contain proteins, salts, fatty substances, lactose and various kinds of cleaning chemicals. Detergents represent the biggest portion of chemicals used in dairies. The detergents may be alkaline or acid and are used for different purposes. Hydroxides or alkaline salts are responsible for the alkalinity of the detergent. They are mainly added to dissolve and remove proteins, but they also help to eliminate fats through saponification. Sodium hydroxide is the most widely applied alkaline detergent but for special applications it may be replaced or mixed with other strong bases. Acids are used to remove the inorganic deposits or so-called milkstone. For that purpose, nitric acid or phosphoric acid are used separately or in combination. Both alkaline and acid detergents often contain additives to improve their cleaning capability. These are phosphates, sequestering agents, surfactants and some minor components like dispersing agents, anti-foaming agents and inhibitors (Romney 1990). The presence of detergents and their additives in dairy waste water hardly influences the total COD in contrast to milk, cream or whey. However, detergents also present difficulties in their treatment. Wildbrett (1990) reported that sodium carbonate passed a two-stage effluent unchanged and was discharged into the river.

2.4 Meat and Poultry Industry

Meat, poultry and fish industries produce the highest loads of waste within the food industry. The meat industry contains slaughterhouses and processing units where meat is prepared, cut in pieces and is either frozen, cooked, cured, smoked or made into sausages. Slaughterhouses are more important than the other units in terms of environmental pollution. The wastes coming from these units contain various quantities of blood, fats and residues from the intestine, paunch grass and manure (Cournoyer 1996). The wastes are best separated into wastewater and solid waste. Solid waste, like intestines, pieces of meat or bones have been used as animal feed after further processing. Slaughterhouse wastewater is typically high in both moisture (90–95%) and nitrogen, has a high BOD and is odorous. Pretreatment is also necessary because the sludge derived from processing of wastewater contains pathogens. Therefore proper management is a prerequisite to ensure that potentially high levels of pathogens are eliminated (Cournoyer 1996). Poultry wastes are equally problematic to meat wastes because the main source of wastewaters is the slaughtering process. Starkey (1992) reviewed the considerations for selection of a

treatment system for poultry processing wastewater, including land availability, previous site history, conventional waste treatment systems, and land application systems. The performance of anaerobic treatment systems, including lagoons, contact processes, sludge beds, filters, packed beds, and hybrid reactors were out lined (Ross and Valentine 1992). Pretreatment is also regarded as necessary for poultry waste to reduce the moisture and increase the porosity with the addition of bulking agents, which also increase the aeration and carbon level in wastewater. Proper treatment is needed to eliminate the pathogens.

3 Advantages of Bioremediation

- It conserves financial resources by the virtue of reduced cleanup times
- Capital expenditure is also less as compared to other remediation technologies (Geo Environmental 1998)
- This technology is widely accepted by industry as it maintains nature's harmony
- It destroys contaminants rather than transferring them from one medium into another (Thassitou and Arvanitoyannis 2001).

4 Limitation of Bioremediation

- If the process is not controlled it is possible the organic contaminants may not be broken down fully resulting in toxic by-products that could be more mobile than the initial contamination.
- In the great majority of cases, an inoculation with specific microorganisms is neither necessary nor useful. Besides all these some other factors are also effect the bioremediation such as solubility of waste, nature and chemical composition of waste, oxidation—reduction potential of waste and microbial interaction with this. Hence the researchers should search genetically different type of microbes which can also work on slightly adverse condition. Therefore, bioremediation is still considered as a developing technology to regulate the day to day environmental problems faced by man residing in an area.

5 Conclusion

Increase in the production of waste disposal has resulted in massive land degradation. This may lead to an increase in the demand for most promising techniques which will result in reducing the pollution level. Much of the technological advances occurred in the field of waste management have proven to be a great asset

for managing food waste. Despite several benefits which they offer, they have their downsides too, which needs to be looked into or find some alternative technologies to overcome the problem of land degradation. Although researcher had found the variety of the ways by which we can degrade the food waste. But bioremediation also making its leap to tackle the problem associated with different categories of waste with the help of microorganism. Even with the obstacles discussed above, there are tremendous market opportunities for bioremediation and also the trend is slowly changing by using bioremediation in order to reduce the food wastage.

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The Role of Decomposer Animals in Bioremediation of Soils

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Abstract Soil animals are useful indicators of soil contamination, both before and after the bioremediation. Many toxicity and bioavailability assessment methods utilizing soil animals have been developed for hazard and risk-assessment procedures. Not only the survival of the animals, but also more sensitive parameters like growth, reproduction and community structure have often been taken into account in the assessment. Microorganisms are degrading the contaminants in bioremediation processes, soil animals can also have an important but usually an indirect role in these processes. Soil animals, especially the large ones, can also actively take part in the ecological recovery processes through their own activity. The potential risk of transfer of contaminants accumulated in soil animals to the above-ground food webs should be borne in mind. Earthworms are one of the most important soil animal's which plays a role in bioremediation of soil for cleaner environment due to their biological, chemical and physical actions. Earthworms can be directly employed within bioremediation strategies to promote biodegradation of organic contaminants. Earthworms have been shown to aerate and bioturbate soils and improve their nutritional status and fertility, which are variables known to limit bioremediation. Earthworms have also been shown to retard the binding of organic contaminants in soils, release previously soil-bound contaminants for subsequent degradation, and promote and disperse organic contaminant degrading microorganisms. This review discusses the earthworm actions upon the soil environment and how they might influence the fate and behaviour of soil associated organic contaminants, subsequently improving bioremediation potential.

Keywords Decomposer animals · Bioremediation and ecosystem engineers

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1 Introduction

Bioremediation is usually recognized as an inexpensive, effective, and environmentally safe technology to clean up hazardous wastes or chemicals. It involves the use of either naturally occurring or deliberately introduced microorganisms to consume and break down environmental pollutants, in order to clean a polluted site. At the moment, the lack of complete understanding of biodegradation processes and the absence of diverse engineering techniques required for applications are regarded as major constraints in the development of bioremediation (Pritchard et al. 1996). Soil organisms can also be exploited in remediation and reclamation processes, principally in 2 ways. First, they can take part in the process, increasing microbial, metabolic activity of the soil. Second, soil creatures can be used as indicators of non fertile soil, either to assess the toxicity or risk of the contaminated soil or to evaluate the remediation process, that is, to evaluate the toxicity or risk of the end product. Earthworms is a one of the important soil creature having role in the bioremediation of soil and due to their biological, chemical and physical actions, earthworms can be directly employed within bioremediation strategies to promote biodegradation of organic soil contaminants. These burrows create pores through which oxygen and water can enter and carbon dioxide can leave the soil. Earthworm casts (their faeces) are also very important in soils and are responsible for some of the fine crumb structure of soils. Earthworms have been shown to aerate and bioturbate soils and improve their nutritional status and fertility, which are variables known to limit bioremediation. Earthworms play an important role in breaking down dead organic matter in a process known as decomposition. This is what the earthworms living in the compost bin are doing and earthworms living in soils also decompose organic matter. Decomposition releases nutrients locked up in dead plants and animals and makes them available for use by living plants. Earthworms do this by eating organic matter and breaking it down into smaller pieces allowing bacteria and fungi to feed on it and release the nutrients. So these earthworms are called as “Ecosystem Engineers”.

2 Importance of Soil Animal (Earthworm) in Bioremediation of Soil

- Due to their biological, chemical and physical actions, earthworms can be directly employed within bioremediation strategies to promote biodegradation of organic contaminants.
- Earthworms have been shown to aerate and bioturbate soils and improve their nutritional status and fertility, which are variables known to limit bioremediation.

- Earthworms have also been shown to retard the binding of organic contaminants to soils, release previously soil-bound contaminants for subsequent degradation, and promote and disperse organic contaminant degrading microorganisms.

2.1 *Earthworm Abiotic and Biotic Effects Upon the Soil Environment*

2.1.1 Abiotic Effects

Environmental factors like moisture, temperature, pH and soil texture also affect the beneficial effect of earthworms. Bhadauria and Ramakrishnan (1989) determined that regional earthworm biodiversity and species dispersal pattern was influenced by a variety of biotic and abiotic forces such as soil properties, surface litter inputs, surface vegetation type, dynamic land management history, local or regional climate and human pressure. The significance of diverse soil habitats is one of the most influencing factors affecting the overall earthworm distribution and its overall performances (Rajkhowa et al. 2015). Changes in land use patterns have also directly affected the composition and population structure of earthworm communities in different agro-climatic regions (Lalthanzara et al. 2011). Endogeic earthworm appears a key feature of soil functioning in the urban context through their roles on organic matter transformation, the formation and maintenance of soil structure (Amosse et al. 2015). Martin (1991) observed earthworm (Millsoniaanomala) degradation of soil organic matter determined a size reduction of 25–30%. Such research highlights the major role that earthworms have in repartitioning soil litter into smaller aggregate sizes (Martin 1991; Barois et al. 1993). It could be hypothesised that here in might exist potential for earthworm assisted release of soil associated (aged) organic contaminants (Barois et al. 1993). In addition, such activities have also been shown to increase soil organic matter surface area (Edwards and Bohlen 1996), which will have a bearing upon the fraction of material exposed for microbial interaction (Dominguez 2004), potentially resulting in increased contaminant destruction. These aspects are worthy of

serious research and consideration with respect to increasing degradation potential and offsetting the residual concentration problem, which often limits final bioremediation success. During the digestion and excretion (as earthworm casts) of the now reworked ingested soil mineral and organic matter fractions, it is known that there are many subsequent positive effects upon the improvement of the soil environment and soil structure.

2.1.2 Biotic Effects

Earthworms act on with soil microorganisms (fungi, bacteria and actinomycetes species) on three broad spatial places (Brown and Doube 2004). Earthworms have a additive good effect on bacteria and fungi in soils. Where earthworms are present there are more bacteria and fungi and they are more participative in soil fertility. They release nutrients from organic matter and making them available to plants and for other soil creatures. Earthworms were associated with decreased incidence of field diseases of clover, grains, and grapes incited by *Rhizoctonia* spp. and *Gaeumannomyces* spp. Mucus and cast deposition on burrow walls, and other organic carbon sources transferred through the burrow systems promote the increase and distribution of microorganisms in earthworm burrows (Farenhorst et al. 2001), whilst mucus, urine and glucose can result in increases in microbial biomass (Scheu 1987). This is of relevance because increases in microbial biomass is linked to increased microbial catabolic activity (Meharg 1996), and as Gevao et al. (2001) stated, such increased activity, linked with potential increases in bioavailability due to earthworm actions upon the soil within earthworm guts could potentially increase compound losses via microbial mineralisation. Higher microbial numbers, diversity and activity are also known to be related to passage of microorganisms through the earthworm gut, as well as the promotion therein, and the 'awakening' of dormant gut flora this is, however, very much related to gut passage time (Brown and Doube 2004). Importantly, the increased gut associated microflora are then excreted throughout the media within earthworm casts and via microbial adheration to earthworm skin (Edwards and Bohlen 1996), whilst the transit and dispersal mechanisms associated with the water flow (Kretzshmar 2004) also help to further dissipate microorganisms. Such actions are clearly relevant to any bioremediation methodology desiring the spatial incorporation of microorganisms capable of organic contaminant degradation.

2.1.3 Bioremediation Limitation

- Movement and burrow creation
- Ingestion
- Digestion and excretion
- Casts Lack of oxygen, anaerobic conditions Soil heterogeneity Compacted soil

- Inappropriate C:N ratio
- Insufficiency of available nutrients
- Low bioavailability of bound residues Presence,
- Number and activity of degrader microorganisms Contaminant concentrations too low to induce catabolic ability Toxic levels of contaminants, or presence of co-contaminants restricting induction of catabolic ability
- Inappropriate Temperature, pH and moisture conditions
- Increased soil porosity (Bolan and Baskaran 1996)

increased oxygenation, moisture retention (Edwards and Bohlen 1996) improved soil fertility via accelerated organic matter decomposition, and improvement of the availability of nutrients (Edwards and Bohlen 1996).

3 Soil Animals as Indicators of Environmental Contamination

Contaminated soils are usually characterized by complex chemical mixtures rather than single chemicals. Moreover, soils may contain compounds that have not been expected and not looked for in chemical analyses. Transformation of the original harmful substances may further complicate the situation. To determine the environmental impact associated with complex contaminations, a toxicity-based approach rather than a chemically-based approach should be adopted in risk assessment (Callahan and Linder 1992). It has been emphasized that direct measurements of, e.g. heavy metal concentrations in soils, has relatively little value, whereas uptake into e.g. earthworms is a much more sensitive and meaningful criterion (Morgan 1986). Thus, relevant biological information needs to be incorporated into the toxicity and risk-assessment procedures.

Animal group	Species	Comments
Earthworms	*Eisenia fetida/Andrei*	Standardized survival (ISO, 1993) and reproduction (ISO, 1998) tests in artificial soil, tests also with contaminated soils
	Aporrectodea caliginosa Lumbricus terrestris	Common species in field soils, used in several ecotoxicological studies as above

Decomposer animals frequently used (the procedure has been established or is under development) in the evaluation of toxicity of chemicals and contaminated soils

4 Potential Utilization of Soil Animals in Bioremediation

Usually, abiotic conditions in the soil under bioremediation process are hostile for soil decomposer animals. The physical and chemical soil conditions may be outside the tolerance limits of most species, or animals may have been unable to colonize the soil due to limited dispersal. In addition, because animals do not harbor significant metabolic ability to degrade the chemicals to be treated, one may argue that decomposer animals are not important in remediation processes. It should be noticed, however, that when different kinds of organic wastes are composted, many soil animals, like earthworms, enchytraeids and mites, do have positive effects on the process by utilizing organic compounds in their own metabolism, and more importantly, by increasing metabolic activity of soil microbes (Haimi and Huhta 1987; Edwards 1998). Stabilization of household wastes may even be faster in vermicomposting (utilizing effectively the earthworms) than in conventional composting process (Haimi and Huhta 1986). Soil animals can also be exploited in remediation and reclamation processes, principally in two ways. First, they can take part in the process, increasing overall, and especially microbial, metabolic activity of the soil. Second, soil animals can be used as indicators of soil contamination, either to assess the toxicity and risk of the contaminated soil or to evaluate the efficacy of the remediation process, that is, to evaluate the toxicity and risk of the

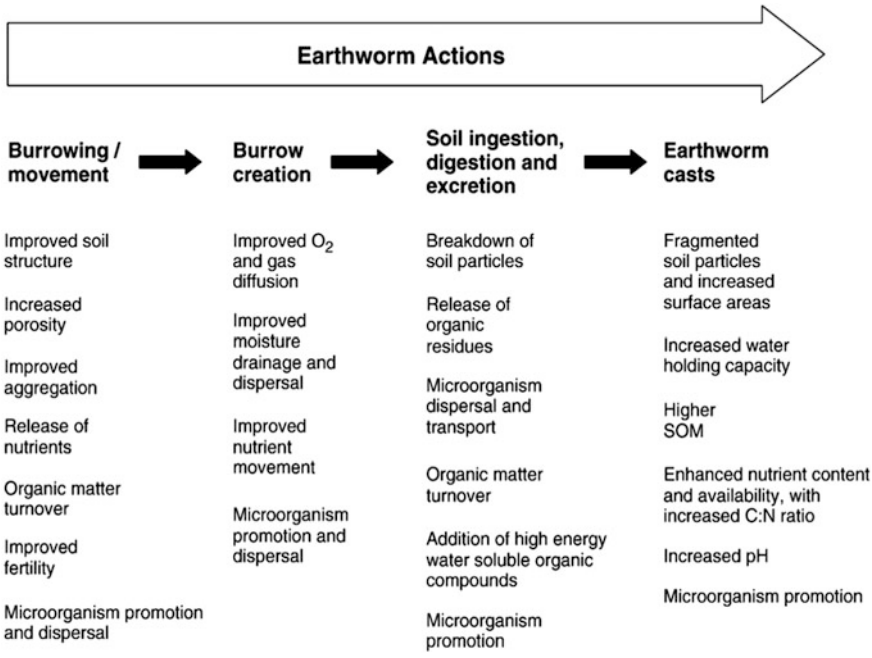

Fig. 1 Positive earthworm actions upon the soil environment

end product. Indeed, a number of methodologies with respect to pre-application testing, post application bioassays, monitoring, waste management and risk assessment have been proposed and also already utilized in environmental management (Fig. 1).

5 Conclusion

Earthworm potential for remediation of contaminated soils has been noted by a number of authors, with the main beneficial areas being the mechanical development of the abiotic systems in terms of burrowing, ingestion, grinding, digestion and excretion of casts, and upon the biotic systems in terms of promoted microorganisms. Additionally, following ingestion and re-working of contaminated soils, it is plausible that the sequestered or sorbed fraction of contaminants would undergo physical release, thus making them available for subsequent microbial degradation; an area for further research. There would appear to be a number of advantages in the utilisation of earthworms within organic contaminant bioremediation they have been shown to both retard the binding of compounds and increase compound availability, whilst increasing microbial degradation and promoting favourable microbial conditions. Their mechanical actions further optimise soil conditions, offsetting known remediation limitations. Furthermore, earthworms have the potential to be employed not only in the recovery of contaminated soils as part of a bioremediation strategy, but also in the subsequent improvement of that soil, in terms of structure and nutritional status. Regardless of this, earthworm inclusion as a biotechnology has potential, and is worthy of future research if a number of obstacles could be overcome, such as toxicity issues and appropriate remediation strategies.

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The Role of *Eudrillus eugenia* in the Degradation of Paper Cup Waste and the Morphological, Physiological and Histological Changes in the Organism

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Abstract Earthworms play an important role in the monitoring of environmental pollution. Hence when exposed to pollution (or) stress condition, physical and deleterious changes are reported to be set in the metabolism of the exposed organism. Karthika et al. (2014) reported that earthworms degrade the paper cup wastes by separating the plastic lining material coated inside the paper cup during the process of vermicomposting. During the degradation process, the organisms take in the plastic resulting in toxicity of the organism (Karthika et al. 2014). Hence attempt was carried out to observe the physiological and morphological modification in the earth worm during the degradation of paper cup wastes. Fortunately there were some abnormal changes noted in the morphology and physiology of earthworm during the initial, middle and final stages of vermicomposting process In the present study, the morphological changes (length, weight) and physiological changes including the antioxidative enzymes (CAT, SOD, GST, GPX), DNA damage and tissue damage occurred during the degradation were investigated in earthworms. However, the overall antioxidant activity was normal during the 1st week of process, later there was increase in its activity during the 12th week and at the 19th week of the process, a drastic reduction in the antioxidant enzyme was noted. However the enzyme activities were observed to be normal in the control. Meanwhile the DNA damage to the earthworm was observed by comet assay where the high level of DNA damage was noted in test animal compared to control. Also the histological examination of the earthworm shows the changes in the external

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barrier of the tissues of the test animal when compared to the control. The results demonstrate that the earthworm employed for the degradation of paper cup waste undergoes oxidative stress, DNA damage and also tissue damage. However the earthworms have the capable of regaining its metabolic activity when inoculated into the new substrate.

Keywords Earthworm · Paper cup · Antioxidant · Comet assay · Histopathology

1 Introduction

Paper cups are laminated with a plastic resin called polyethylene terephthalate (PET). This helps to keep beverages warm and prevents liquid from leaking. PET is a very lightweight material, moisture barrier, strong, impact-resistant and naturally colorless with a high transparency. Paper cups are not specially recycled. They come under regular waste and are burnt or discarded in the landfill. Also the decomposition process in the landfill releases methane, a greenhouse gas which is estimated to possess 23 times the heat-trapping power of carbon dioxide (Kennedy 2012). The composition of paper cups and plastic makes them complicated to recycle. It needs about 150 years (same as plastics) to degrade because of the plastic foil (Bijayani et al. 2013). There is no advanced technology to separate the plastic from the paper cup. To overcome this problem vermicomposting technology was found to be an effective method for the conversion of the paper cup waste material into nutrient rich manure and at the same time earthworms involved in the process separate the plastic material from the paper cup (Karthika et al. 2014).

Any kind of disturbance in the activity of animal during chemical toxicities was reported to leads to the changes in the metabolic activity pattern of the enzymes. Therefore the enzyme studies are very important to know about the biochemical indices of normal metabolism. Sabatini et al. (2009) reported that Reactive Oxygen Species (ROS) can be generated in living organism when it is exposed to environmental contaminants. To prevent the damage caused by oxygen-free radicals, tissues have developed an antioxidant defense system that includes enzymatic activities such as that of Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPX) and the non-enzymatic antioxidant glutathione S-transferase (GST). The antioxidant enzyme is a mutually supportive team of defense against ROS and was reported to reduce ROS to stable compounds. SOD is a metalloprotein and is the first enzyme involved in the antioxidant defense by lowering the steady-state level of O_2^-. CAT is a hemeprotein, localized in the peroxisomes or the microperoxisomes and catalyses the decomposition of H_2O_2 to water and oxygen thus protecting the cell from oxidative damage by H_2O_2 and OH^-. GPX is a seleno-enzyme, two third of it is present in the cytosol and one third in the mitochondria. It catalyses the reaction and reduces hydroperoxides. GPX can

also terminate the chain reaction of lipid peroxidation by removing lipid hydro peroxides from the cell. The GPX and GST activities cause a reduction in the GSH level and thus decrease the cellular antioxidant status too (Flohe 1982). Additionally the ROS directly oxidize the cellular components like DNA leading to DNA damage. Singh (1988) developed comet assay, a rapid technique for measuring the DNA damage. Many scientific studies have justified that comet assay is a simple and reliable method for the detection of genotoxins in a wide variety of eukaryotic cells (Song et al. 2009; Li et al. 2011). Histopathology refers to the microscopic examination of tissue in order to study the changes that occur in the morphology of the animal. During environmental stress condition the animal will face both morphological and physiological changes. Merel et al. (2014) studied the histology of earthworm after the treatment with silver nano particles. The histological section of the earthworms showed whether some hyperplasia occur in the epidermis and some lipofusion like deposits occurred in the circular muscle of the earthworm. Also the histological and cytological changes in epidermal and non epidermal tissue during early wound healing have been reported by some authors too (Burke 1974; Moment 1974). Hence the histological study would be helpful to know about the damage in the tissue of the animal due to environmental stress.

During the degradation of the paper cup waste, physiological and morphological changes have been noted in earthworms. In the present study we made an attempt to examine the changes in the anti oxidative enzymes, DNA content, tissue damage and growth parameters in earthworm throughout the vermicomposting process.

2 Materials and Methods

2.1 Preparation of Experimental Media

Two 5 L circular plastic tub (diameter 40 cm, depth 9 cm) were filled with the feed mixture as given below and were subjected for vermicomposting process, **Control** (1:1 ratio): 1000 g of cow dung slurry +20 nos earthworms; **Test** (1:1 ratio): 500 g of shredded paper cup wastes +500 g of cow dung slurry +20 nos earthworms.

2.1.1 Extract of Earthworm

Earthworms were taken from both control and test for 1st, 12th and 19th week of the process. Worms were crushed in a prechilled mortar and pestle under ice cold conditions in 590 mM phosphate buffer (pH 7). The homogenate was centrifuged at 9000 rpm for 30 min. The supernatant was collected and used for the assay to determine the enzyme activity.

2.1.2 Antioxidative Enzyme Assay

The activity of catalase (CAT) was determined (Song et al. 2009) by using H_2O_2 as a substrate for the enzyme. The enzyme activity was calculated from the reduction in ultraviolet absorption at 250 nm, following the degradation of H_2O_2 by CAT present in the sample. One unit of activity was defined as the enzyme quantity required consuming half of H_2O_2 in 100 s at 25 °C. The activity of SOD inhibits the photochemical reduction of Nitrobluetetrazolium chloride (NBT) measurement as described by Giannopolitis and Ries (1997). One unit of SOD activity was considered to be the amount of the enzyme required to inhibit NBT reduction by 50% and the result was expressed as units per milligram of Fresh Mass (FM). Absorbance of the reduction mixture was read at 560 nm. The activity of GPX was also measured using reduced hydrogen per oxide substrate (Günzler and Flohe 1985). Sodium azide (1 mM) was added to the reaction mixture in order to inhibit remnant CAT activity. One unit of activity of enzyme is defined as the amount of enzyme decomposing 1 µmol H_2O_2 per min. The GST assay was measured using 1 chloro 2, 4-dinitro-benzene as its substrate. One unit of GST activity is defined as the amount of the enzyme catalyzing the formation of 1 µmol of the product per min under the assay conditions. The concentration of protein was quantified using Bradford method (Bradford 1976).

2.1.3 Comet Assay

Earthworm coelomocytes were obtained using the non invasive extrusion method as described by Eyambe et al. (1991). Earth worm coelomocytes were spontaneously secreted in the medium and washed with phosphate buffer saline for 3 min. An alkaline comet assay was performed according to Singh et al. (1988). The cell suspension was mixed with 65 µl of 0.7% low melting agar. About 75 µl was pipetted out and was pre-coated with normal melting agar. The slides were immersed into a lysis solution for 1.5 h (4 °C, 2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, (pH10), 10% dimethyl sulfoxide and 1% Triton X-100 just before use). Slides were then incubated in an electrophoresis tank containing 300 mM NaOH with 0.2 mM Na_2EDTA, pH 10 for 20 min at 25 V (300 mA). The slides were then neutralized (0.4 M Tris, pH 7.5) thrice at 5 min intervals and were stained with 40 µl ethidium bromide (20 µl m/l) for fluorescence microscopy analysis (BX50/BX-FLA fluorescence microscopy) using a digital imaging system.

2.1.4 Histopathological Study

Fresh specimens of earthworm had been fixed in alcoholic bouin's fixative, dehydrated in ethyl alcohol, cleared in Xylene and finally embedded in paraffin wax. A serial Section (6–8 µ) has been prepared stained with haematoxylin and

eosin, dehydrated in ethyl alcohol, cleared in cedar wood oil and mounted in Canada balsam. Finally photographs were taken using a microscope attached with an automatic camera (Abdel-Haleem et al. 2014).

3 Results and Discussion

In animals poor nutrient would reduce the supply of energy to cells, thereby decreases their intracellular concentration and directly limit the growth and affect the reproduction too. During the process of vermicomposting of paper cup wastes, the earthworm consume only the paper and separate the plastic lining material coated inside the paper cup. This may caused both the physiological and morphological modification in the earthworm. This is evident from Fig. 1. During the process, growth and reproduction of the earthworm gets affected. Figure 2 shows the changes in the biomass of earth worm during the vermicomposting process at the 1st week of the process equal number (20 nos) of earthworms is maintained. In the middle stage (12th week) the number of earthworm (260 nos) gets increased and at the final stage (19th week) a drastic decrease in the number of the

Fig. 1 Morphological changes in the earthworm during the vermicomposting process

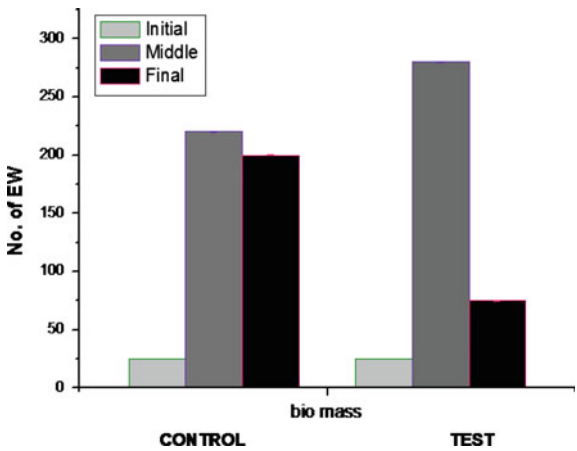

Fig. 2 Changes in the Bio mass of earthworm during the vermicomposting process

Table 1 Variation in weight and length of the earthworm

Stages	Weight (g)	Length (cm)
Control	0.72 ± 0.14	8.1 ± 0.24
1st week	0.79 ± 0.24	15.5 ± 0.24
12th week	1.66 ± 0.17	23.2 ± 0.14
19th week	0.34 ± 0.09	5.2 ± 0.07

earthworms (75 nos) in the vermin bin was noticed (Fig. 1) and Table 1. shows the variation in the weight and length of the earthworm during the process. Also the reproduction rate of earthworms gets affected.

3.1 Antioxidative Assay

3.1.1 Variation in CAT

Catalase enzymes eliminate free radicals thus protecting the cell from damage. It is reported that the reduction in the activity of the enzyme may be due to stress. The defensive effect of antioxidative enzymes would be lost with the increase in stress. As shown in Fig. 3, the CAT activity changed during the initial, middle and final stages of vermicomposting. The CAT is normal during the 1st week of the vermicomposting process in both control and test (15 U/mg/pro). Later during the 12th week of the process there is an increase in the activity of CAT (45 U/mg/pro). But during the final stages (19th week) of composting a drastic decrease in the activity of CAT enzyme (10 U/mg/pro) was noticed. In the control group the activity of enzyme is maintained throughout the process and statistically significant differences ($P < 0.05$) were observed on 1st–9th week of the period.

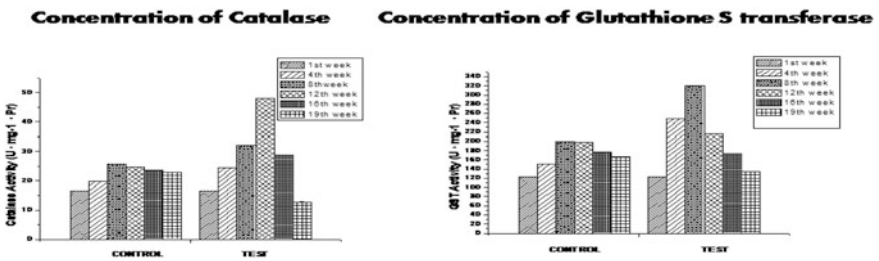

Fig. 3 Biochemical responses CAT and GST activity of *Eudrillus eugenia* exposed to the vermicomposting of paper cup waste

3.1.2 Variation in GST

GST involved in Xenobiotic metabolisms in living organism (Canesi et al. 2007). It is one of the important detoxification enzyme and its activity has been used as a potential biomarker in earthworms exposed to stress. GST activity has also increased up to the middle of the process (320 U/mg/pro) and shows decrease at the end of the process (122 U/mg/pro) (Fig. 3). As suggested by the data obtained from the study after 1st week of exposure, the activity of the GST is stimulated in earthworm which catalyzes the conjugation of glutathione with both endogenous and exogenous substrates.

3.1.3 Variation in GPX

GPX terminates the chain reaction of lipid peroxidation by removing lipid hydro peroxides from the cell. In the present study GPX activity has increased during the middle stages (30 U/mg/pro) of waste decomposition following which the enzyme has got drastically reduced (7.5 U/mg/pro) in its quantity (Fig. 4).

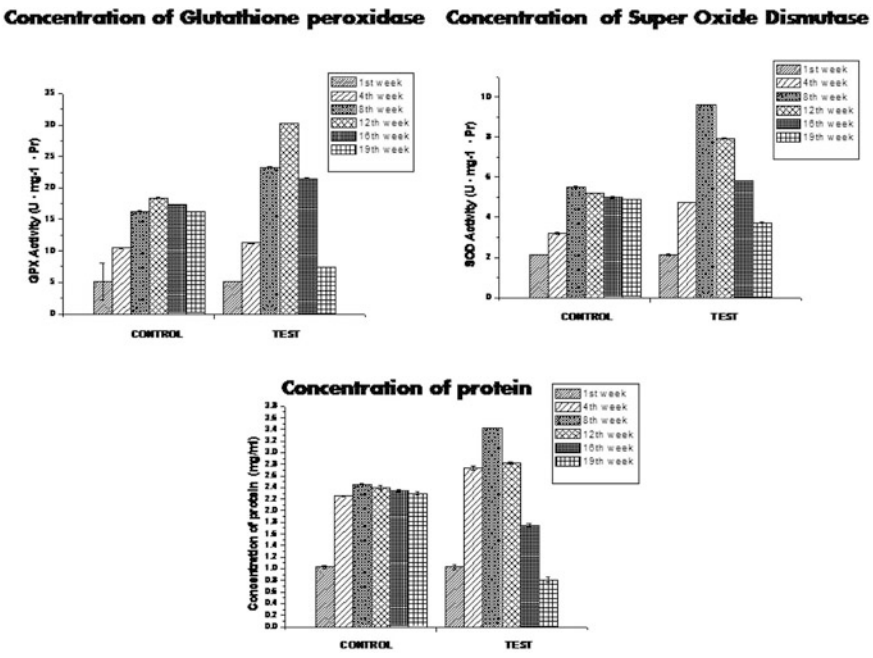

Fig. 4 Biochemical responses GPX, SOD and protein activity of *Eudrillus eugenia* exposed to the vermicomposting of paper cup waste

3.1.4 Variation in SOD

SOD activity is considered an important biomarker for determining the effects of pollutants on the ecosystems.SOD removes the O_2^- produced during biological oxidation of the waste. The reduction in SOD is due to the reason that O_2^- may be converted to OH^- ions by GSH and GST. The SOD activity in earthworm after different days of vermicomposting process is shown in Fig. 4. The SOD activity was initially lesser during the 1st week (2 U/mg/pro). It increased during the middle stages (12th week) and has drastically decreased (4 U/mg/pro) at the end of the process (19th week).

3.1.5 Variation in Protein

Protein content in the earthworm shows the same result as that of the anti oxidative enzymes. Protein estimation by (Bradford 1976) reveals that the protein concentration has increased and then decreased (0.8 mg/ml) (Fig. 4). Xiangyu et al. (2014) reported that the anti oxidative enzymes of earthworm in the test group were significantly higher than those observed in control group after the 12th week of vermicomposting process. During the early stage of the stress, earthworm has the capacity to tolerate the oxidative stress and activate the antioxidant enzymes. During later stages, the activity of the enzymes decreases with an increase in toxic stress. This demonstrated that the defensive effect of antioxidative enzymes might be lost.

In our study the stimulation of enzyme activity has occurred till the middle of the process and the reduction occurred in its activity during the final stages of decomposition. This may be due to nutrient based stress. Earthworm starts the separation of plastic from the paper cup during the 8th week of the process which means it does not feed on the plastic after that period, therefore during the 19th week of the process the earthworm had almost separated all the plastic from the paper cup and there is no available paper to feed. Hence there might have been a decrease in the activity of the anti oxidative enzymes.

3.2 Comet Assay

The comet assay is a rapid, simple and sensitive method for measuring DNA damage bio monitoring and determining genotoxicity (Singh et al. 1988). The comet assay result is shown in Fig. 5. The earth worm exposed to vermicomposting of paper cup had a significant increase in tail Length (TL) at the 19th week of vermicomposting process. The analysis of comet assay in earthworm shows the positive responses for the separation of plastic. Therefore it was concluded that as the plastic got separated from the paper cup. Simultaneously DNA damage in the

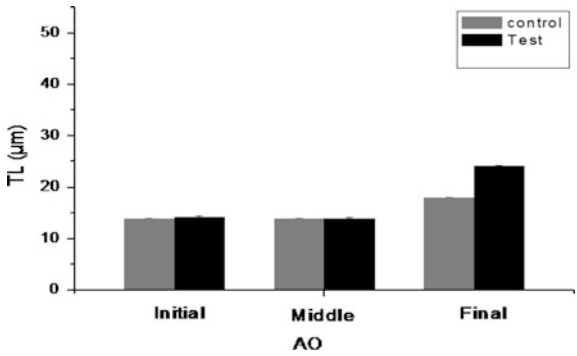

Fig. 5 DNA damage responses of *Eisenia fetida* exposed to vermicomposting of paper cup

Eudrillus eugenia is noted. The DNA damage observed in earthworm exposed to vermicomposting of paper cup waste might have been due to the nutrient based stress and the consequent production of ROS that leads to cell damage and death (Lin et al. 2010).

3.3 Histological Analysis

The histopathological examination of earthworm tissue indicates that vermicomposting of paper cup may affect the external barrier of the earth worm. Damages to the outer skin and the intestine may seriously affect the health of the earth worm because this damage interferes with the correct functioning of the tissues and cells of the earth worms (Lapied et al. 2010). Figure 6 shows some erosion of epithelium, inflammation and has granular changes in the coelom occurred in the earth worm exposed to the vermicomposting of paper cup waste compared to that of the control. This also suggests that some fibrotic changes in the circular muscle and longitudinal muscle were noted. The result indicates that the immune system is trying to respond to the exposure of stress during the separation of plastic from the paper cup. Later it fails to control the normal metabolism leading to tissue injury. Similar result is reported by Lapied et al. (2010) that the external barrier of the earth worm *L. terrestris* was affected by AgNP exposure.

3.4 Regaining the Metabolic Process

The *Eudrillus eugenia* employed to degrade paper cup wastes separated the plastic lining that was coated inside the cups which was settled around the rim of the tub as a layer. It was curious to observe that the tiny worm recognized and rejected the non biodegradable material.

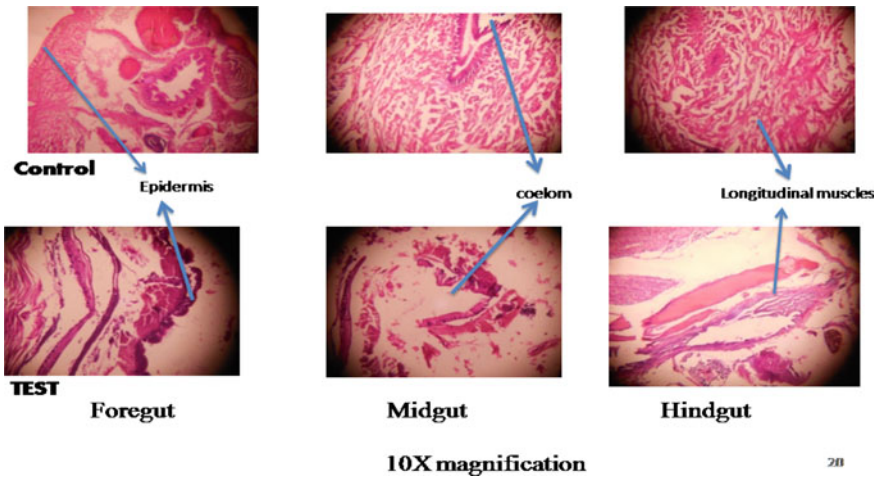

Fig. 6 Transverse section of segments from the anterior region of *Eudrillus eugenia* (Control and Test)

This was the reason for the loss in the body weight and length of the organism during the final stages, when compared to the initial stage. Fortunately this earthworm has the ability to regain the metabolic rate when those organisms are re inoculated into fresh waste material. Figure 7 shows the regaining of the length and weight of the earthworm after the 1 week period of the completion of the process.

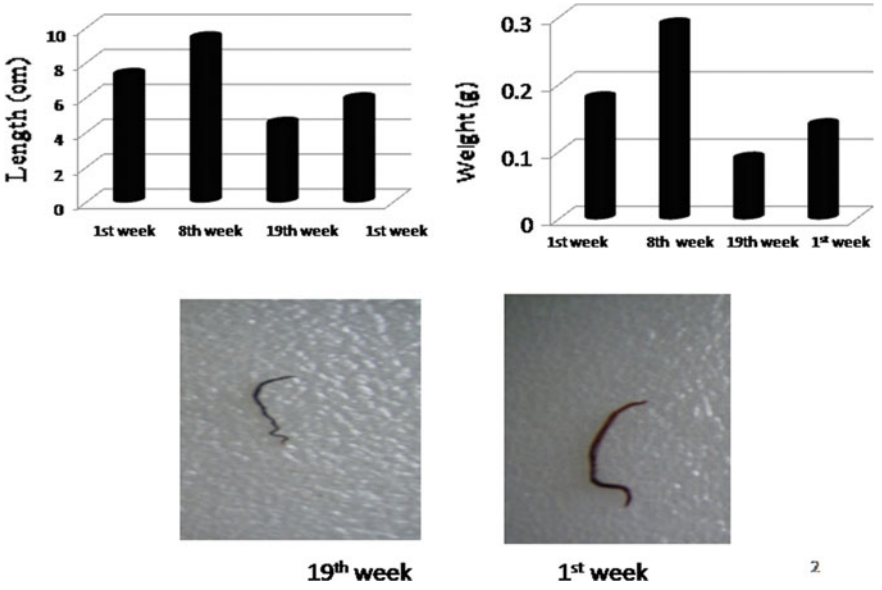

Fig. 7 Regaining the length and weight of the earth worm after the vermicomposting of paper cup waste

4 Conclusion

During the vermicomposting of PET coated paper cups, morphology and physiology of the earthworm was affected. The reduction in the antioxidative enzyme indicates the changes in the normal metabolism of the earthworm which leads to the DNA damage as understood by comet assay. Histopathology test confirms the tissue damage in the earthworm. Earth worm has the capacity to regain the body mass when inoculated into a new environment. Therefore, the loss of weight and height may be due to the nutrient based stress during the vermicomposting process.

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Part II
Biodegradation and Bioremediation of Hazardous Compounds

Nitrate Removal from Ground Water Through Lab Scale Bioreactor Using Dissimilatory Nitrate Reducer *Bacillus weihenstephanensis* (DS45)

R. Seenivasagan, P.M. Ayyasamy, R. Kasimani, A. Karthika, S. Rajakumar and Olubukola Oluranti Babalola

Abstract The study has been attempted as a combined process to treat groundwater contamination with nitrate through lab scale bioreactor using dissimilatory denitrifying bacterium *Bacillus weihenstephanensis* (DS45) and chemical treatments by alum and lime. Nitrate removal was attained up to 80.04% at 156 h by bioreactor process. The residual nitrate, nitrite, ammonium and bacterial growth were effectively removed by addition of lime at 150 mg/L as active coagulants. The treated sample was applied to various disinfectants such as boiling, membrane filtration, UV radiation and chlorination in order to remove bacterial biomass. The bacterial cells of 17×10^4 CFU/ml was completely removed by various disinfectant methods.

Keywords Nitrate · Bioremediation · Bioreactor · Coagulation · *Bacillus weihenstephanensis*

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1 Introduction

The main source of drinking water is groundwater, but it is contaminated by nitrate. Nitrite contamination is reported on many countries such as India, United Kingdom, China, United States of America, Saudi Arabia, Japan and some parts of Europe (Potter et al. 2010). Groundwater is contaminated with pesticides and surface water by nitrogen compounds etc., due to the extensive use of agricultural chemicals. Nitrate is the principal N-bearing constituent of ground water (Pauwels and Talbo 2004). Nitrate is highly water soluble; it imposes a serious threat causing ecological disturbances such as eutrophication of rivers, deterioration of water sources, as well as hazards to human health (Liu et al. 2005; Fernandez-Nava et al. 2010). Nitrate exposure to infants, causes the condition known as methemoglobinemia, also called as blue baby syndrome. The US Environmental Protection Agency (US EPA) has set a maximum concentration level (MCL) of 45 mg/L of nitrate or 10 mg/L of nitrate-nitrogen (NO_3–N) in drinking water (Ogata et al. 2015).

Owing to various health hazards created by nitrate/nitrite, convenient remediation is needed to solve the problem in ground water. Physical and chemical methods to remove nitrates include ion exchange, reverse osmosis and electrochemical methods showed efficient reduction; however these methods are relatively costly and produce concentrated waste brines which require further treatment or disposal (Ergas and Rheinheimer 2004; McAdam and Judd 2006). Biological denitrification is considered to be the most economical strategy for denitrification because it does not require post-treatment and does not produce by-products (Zhang et al. 2012). Biological denitrification is most versatile and widely using technology, due to nitrate removal limitation from water and/or wastewater (Kesseru et al. 2002). Barana et al. (2013) used biological approach to remove nutrients containing nitrate through nitrification and denitrification. Mainly, nitrate to gaseous nitrogen and ammonia is reduced via respiratory chain by denitrifying bacteria. Under aerobic conditions the nitrate respiration has been observed in a number of bacteria, including *Pseudomonas* sp. (Zhang et al. 2011), *Rhodococcus* sp. (Chen et al. 2012), *Bacillus methylotrophicus* (Zhang et al. 2012), *Agrobacterium* sp. (Chen and Ni 2011), *Pseudomonas stutzeri* (Guo et al. 2013), *Desulfovibrio* (Shen et al. 2013). Investigation of aerobic metabolism of *Pseudomonas* and *Bacillus* species has revealed many interesting features of dissimilatory nitrate reduction to ammonia (Chen et al. 2003; Chitra and Lakshmanaperumalsamy 2006).

Denitrification is microbial mediated process and subsequently reduces nitrogenous oxides. This knowledge might help to optimize the design of reactors for different conditions (Metcalf and Eddy 2003). Bioreactor is a device that supports biologically active environment. Several types of bioreactors have been used in wastewater treatment (Espinosa and Stephenson 1999). In bed bioreactors the commonly used supporting materials such as granular activated carbon (GAC), anthracite and sand that allow the growth of microorganisms (Choi et al. 2007). For the treatment of wastewater, coagulating agents have been widely applied to remove chemical ions and colloidal particles such as mineral colloids, microbial

colloids, virus particles, alum, lime, ferric sulfate and ferric chloride (Jiang and Graham 1998). The removal of microbes from the effluents with high microbial is possible by chlorination or ultraviolet treatment (McAdam and Judd 2007). In this study, an experiment was made to remove nitrate from ground water using biological lab scale bioreactor and the trace amount of nitrate and microbes were effectively removed by conventional purification process, i.e., coagulation (alum and lime), sand filtration and disinfectant method like boiling, membrane filter and UV treatment.

2 Materials and Methods

2.1 Water Sample Collection

Water samples were collected in pre-cleaned, acid washed 15 L plastic (Tarson) containers from Soundapuram of Salem District, Tamil Nadu, India. Prior to collection of samples, the containers were thoroughly rinsed, four to five times with the sample and then filled till the mouth avoiding air space. The samples were kept in ice-box and transported to laboratory.

2.2 Estimation of Physico-Chemical Parameters

Physico-chemical parameters such as electrical conductivity, total suspended solids, pH, total solids, dissolved oxygen, total dissolved solid, total hardness, BOD, COD, phosphate, alkalinity, sulphate, calcium, chloride, potassium, sodium, magnesium, ammonium, total nitrogen, nitrite and nitrate in the water samples were analysed by standard methods (APHA 2005).

2.3 Bacterial Isolation and Identification

Soil samples were collected from Dharmapuri district and bacterial strain isolated through serial dilution methods. 74 isolates were screened by two ways such as assimilatory and dissimilatory nitrate reducer. *Bacillus* sp. (DS45) was more efficient for dissimilatory nitrate reduction in comparison with isolated strains and it is sequenced by 16S rRNA. The sequence was analysed with (Basic Local Alignment Search Tools) to identify the species as *Bacillus weihenstephanensis* (DS45) and in the construction of phylogenetic tree by MEGA software. The partial 16S rRNA sequence was deposited in GenBank and received the accession number KF926418 and this work published in my previous paper (Seenivasagan et al. 2014).

2.4 Nitrate Reduction in Ground Water Through a Bioreactor

For preparation of inoculum, pre sterilized 100 ml nutrient broth was inoculated with a loopful culture and kept in rotator shaker (120) for 12 h at 35 °C. Broth culture was centrifuged at 4000 rpm for 20 min and the cell suspension was adjusted to 1OD (10^4 CFU/ml) using sterile distilled water by UV-Visible Spectrophotometer (Cyberlab UV-100, USA). One ml containing 10^4 CFU/ml of the cell suspension was used as inoculum for the nitrate reduction.

In the previous study, effect of various carbon sources, starch concentration, pH and incubation temperature on nitrate removal was studied in mineral salt medium amended (MSM) with 100 mg/L of nitrate. Rapid nitrate removal efficiency was obtained in MSM amended with 0.5% of starch at an optimum pH 7 and temperature of 35 °C. Hence, 0.5% starch concentration was selected as optimum for ground water treatment. As shown in Fig. 1, the lab-scale experimental set-up was used in this study. It consisted of three reservoirs, reactor tank, settling tank, filtration tank and collection tank. All the tanks are 10 L capacities and made up of tarson. The reactor and settling tanks were fitted with mechanical stirrer for agitation process. The bottom of the settling tank and sand filtration tank were closed with a stopper and were packed with large stones (18.0–20.0 cm) followed by small stones (7.0–7.5 cm), gravel (4.0–6.0 cm), coarse sand (3.0–5.1 cm) and fine sand (1.1–4.3 mm) diving the packing zone into 5 portions.

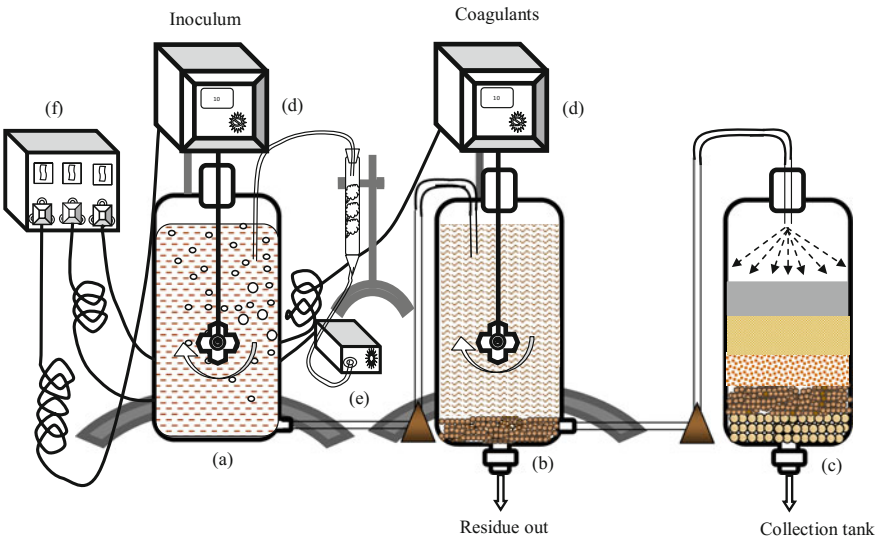

Fig. 1 A model of bioreactor design used in this study

The reactor tank was filled with sterilized contaminated ground water containing 82 mg/L of nitrate and 0.5% of starch. The water sample was inoculated with DS45 (10^4 CFU/mL) and kept for mechanical stirring at 800 rpm in room temperature for 156 h. The bacterial growth were analysed at regular intervals 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144 and 156 h using UV-Vis spectrophotometer (Cyberlab UV100, USA) at 600 nm. The estimation of r nitrate reduction (phenol disulphonic acid method); nitrite (NEDA method) and ammonium formation (Nessler's reagent) in the ground water was done by standard methods. To remove the suspended particles and microorganism the water sample were subjected for primary treatment (bioreactor), secondary (coagulation and sand filtration) and tertiary (disinfection) treatment respectively. During the primary process; the samples were drawn out and physico-chemical parameters such as pH, electrical conductivity, total solids, total suspended solids, total dissolved solid, dissolved oxygen, BOD, COD, alkalinity, total hardness, calcium, magnesium, chloride, sulphate, phosphate, sodium, potassium, total nitrogen, ammonium, nitrite and nitrate were analyzed (APHA 2005).

2.5 *Secondary Treatment for the Removal of Nitrate and Microbial Biomass*

Since there is presence of microbial biomass (1.66 OD) and 16.36 mg/L (19.96%) of nitrate in the bacterially treated water, secondary treatment of water sample was carried out using chemical coagulants such as lime ($CaCO_3$) and alum ($Al_2(SO_4)_3$) respectively. Bacterial treated samples were taken in coagulation tank under aseptic condition. Lime and alum with various concentrations (50, 100, 150, 200, 250, 300, 350 and 400 mg/L) were added to coagulation tank containing bacterial treated sample. The samples were slightly agitated for 10 min with the help of a stirrer and then allowed to settle for 30 min (Ayyasamy et al. 2007). After filtering, the samples physico-chemical parameters and bacterial population were analyzed by the standard methods.

2.6 *Effect of Various Disinfectants on the Removal of Bacteria*

The treated samples were subjected for different disinfection study such as boiling the sample at 100 °C, membrane filtration at various pore size such as 0.5, 0.4, 0.3 and 0.2 µm and exposing the sample to UV radiation at a distance of 20 cm for every 10, 20, 30, 40, 50 and 60 min time intervals. For chlorination about 100 ml of the sample (sample treated with lime at 150 mg/L) was taken in separate conical flasks and 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L of commercial bleaching powder was

added. At the intervals of 15, 30, 45, 60, 75, 90, 105 and 120 min the bacterial population was estimated by standard plate count method on nutrient agar medium. The percentage of NO_3^- reduction was determined in lab scale bioreactor at the equilibrium points as follows (Nameni et al. 2008; Okeola and Odebunmi 2010):

$$\mathrm{NR}\,(\%) = \frac{(\mathrm{A_i} - \mathrm{A_f})}{\mathrm{A_i}} \times 100 \tag{1}$$

where NR is the total amount (mg/L) of nitrate removal in percentage (%), A_i and A_f were the initial and final concentration of nitrate respectively (mg/L). Finally, the data were interpreted using analysis of correlation (R^2 score) for nitrate removal through lab scale bioreactor.

3 Results and Discussion

3.1 Nitrate Removal from Ground Water Using Pilot Lab Scale Bioreactor Model

The results showed that bioreactor treatment had reduced the nitrate to about 80.04% (82–16.36 mg/L) at 156 h (Fig. 2). The bacterial growth in treated water sample with and without starch was (1.66 and 0.57 OD) respectively. The amount of nitrite formation was higher (6.24 mg/L) at 36 h in ground water sample amendment without starch. In the case of 0.5% starch, the formation of nitrite found to be less (4.2 mg/L). Our results are good agreement with the various previous studies like the removal of nitrate from industrial wastewater were done by using Membrane Bioreactor (MBR) with suspended biomass (Shen et al. 2009; Ravnjak et al. 2013); the removal of carbon, nitrogen and phosphorus from synthetic wastewater (Fu et al. 2009) as well as nitrate removal from polluted surface water (Buttiglieri et al. 2005). Heterotrophic denitrification combined with a well-type reactive barrier system using solidified molasses namely slowly Released Molasses (SRM system) has been developed as a long-term in situ remedial option for treating nitrate in groundwater (Lee et al. 2010). Similarly the maximum level of ammonium (22.78 mg/L) was recorded in both water samples with and without starch. After bacterial treatment the physicochemical parameters of the water sample were analysed and found to be less (Table 1).

3.2 Physico-Chemical Parameter of Ground Water Samples

The ground water samples collected from Soundapuram village of Salem districts shows high concentration of TS, TDS, alkalinity, chloride pH, DO, hardness, magnesium, sulphate, nitrate (82 mg/L), total nitrogen (129 mg/L) and phosphate

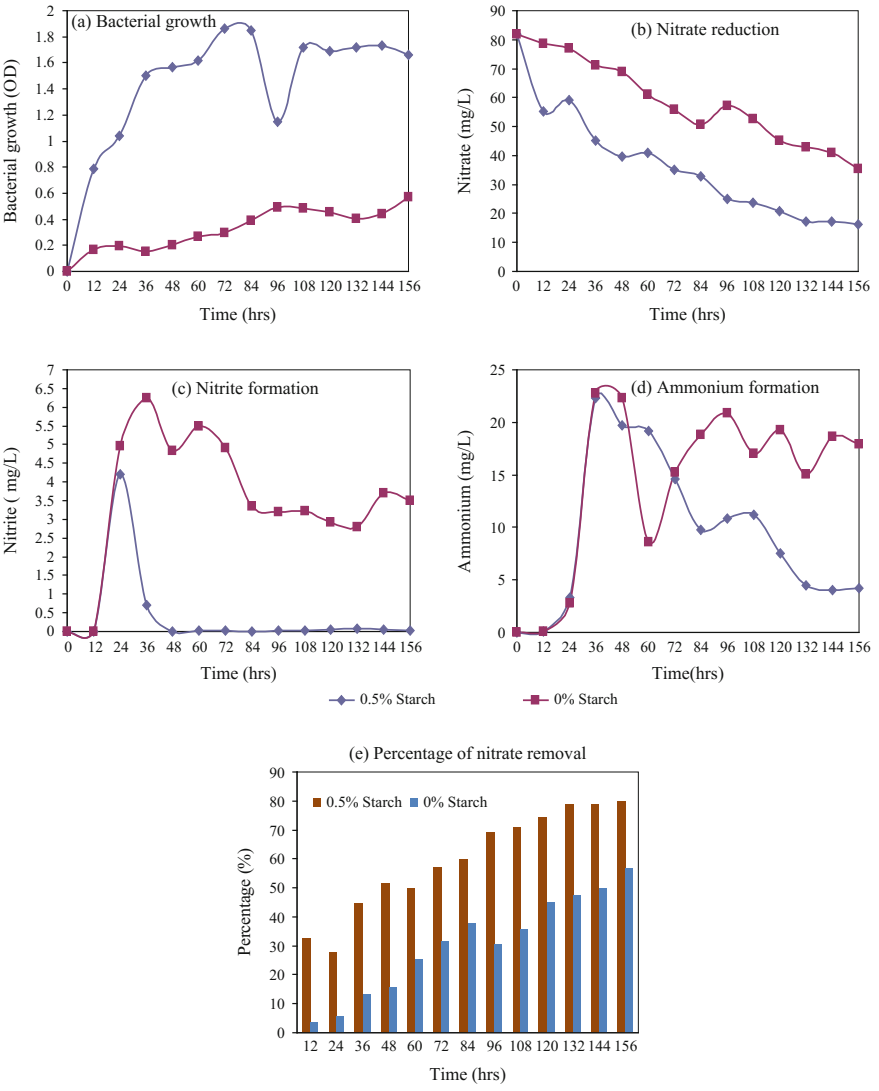

Fig. 2 Effect of starch at 0.5% on bacterial growth (DS45), nitrate removal, nitrite and ammonium formation in ground water sample containing 82 mg/L of nitrate

(329 mg/L). The high nitrate, phosphate and nitrogen contamination in ground water may be due to enormous application of nitrogenous fertilizers, organic manures, industrial effluents, human and animal wastes (Hem 1992; Kross et al. 1992). The physico-chemical parameters of ground water initial and after bioreactor treatment are showed in Table 1. In the experiment, numerous parameters like pH (7.4–7.6), TS (1514–1785 mg/L), TSS (91–1636 mg/L) and BOD (35–118 mg/L) slowly increased in water samples. EC (15–7 mV), TDS (1413–164 mg/L),

Table 1 Physico-chemical parameters of before, after bacterial and lime treated water samples collected from Soundapuram village of Salem district

S. No	Parameters	Before treatment	After bacterial treatment	After lime treatment
1	pH	7.4	7.6	7.6
2	EC μS cm^{-1}	15	7	9
3	Total solids	1514	1785	47
4	Total suspended solids	91	1636	19
5	Total dissolved solid	1423	149	28
6	Dissolved oxygen	6.9	3.1	3
7	BOD	35	118	165
8	COD	259	203	142
9	Alkalinity	250	221	218
10	Total hardness	489	189	514
11	Calcium	20	4	4
12	Magnesium	46	6	9
13	Chloride	280	206	16
14	Sulphate	212	41	29
15	Phosphate	329	155	44
16	Sodium	49	47	5
17	Potassium	112	121	18
18	Total nitrogen	129	23	21
19	Ammonium	10	4.25	0.11
20	Nitrite	8	0.036	0.012
21	Nitrate	82	16.36	0.23

All the values are expressed in mg/L except pH and EC

DO (6.9–3.1 mg/L), COD (259–203 mg/L), alkalinity (250–221 mg/L), total hardness (489 –189 mg/L), calcium (20–4 mg/L), magnesium (46–6 mg/L), chloride (280–206 mg/L), sulphate (212–41 mg/L), phosphate (329–155 mg/L), sodium (49–47 mg/L), total nitrogen (129–23 mg/L), ammonium (10–4.25 mg/L), nitrite (8–0.036 mg/L) and nitrate (82–16.36 mg/L) decreased after bacterial treatment.

3.3 Secondary Treatment for the Removal of Trace Nitrate and Microbial Biomass

After bacterial treatment, the coagulant treatments were optimized by various concentrations. In this experiment; the lime was more effective when compared to alum (Figs. 3 and 4) on nitrate and ammonium removal. In various lime concentration

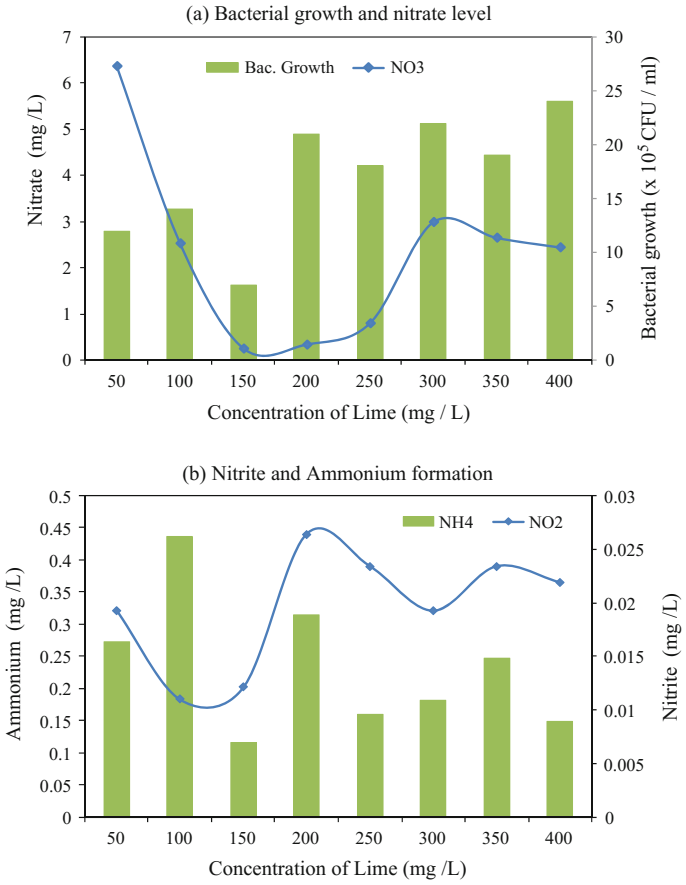

Fig. 3 Coagulation process on the removal of bacterial biomass, nitrate, nitrite and ammonium by various concentration of lime

applied to the bacterial treated water, 0.5 mg/L lime concentration was found to be more effective in removal of nitrate (16.36–0.23 mg/L), nitrite (0.036–0.012 mg/L) and ammonium (4.25–0.11 mg/L). An attempt was made to remove the remaining concentration of nitrate using chemical coagulants alum and lime. The removal of microbial biomass was also significant. The use of bacterial cultures before chemical treatment was not only useful in reducing nitrate but also the bacterial biomass was useful for the attachment of chemical coagulants and quicker precipitation. This may be due to negative charge of cell wall. Since, the bacterial cell wall possesses negative charge they would bind on surface of the calcium. Hence, the bacterial rate was decreased in the coagulant treatment. Similar mechanisms occurred in removal of nitrite and ammonium (Ayyasamy et al. 2007).

As WHO permissible limit of nitrate in drinking water is 45 mg/L (Lunkad 1994) the filtered water sample was analyzed for the physico-chemical parameters.

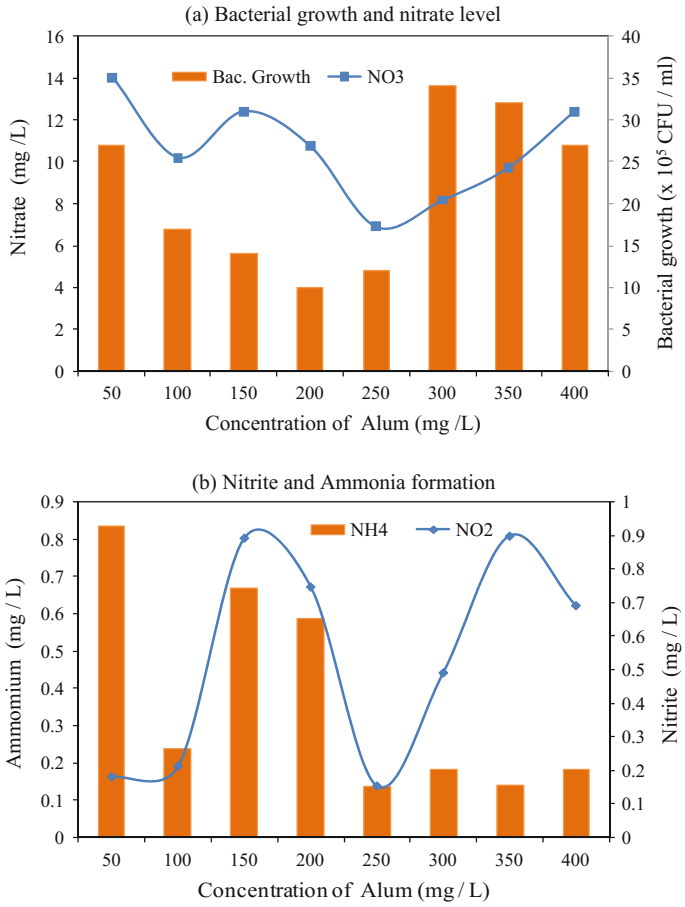

Fig. 4 Coagulation process on the removal of bacterial biomass, nitrate, nitrite and ammonium by various concentration of alum

All the parameters of treated samples showed lesser values compared to that of untreated water samples (Table 1).

3.4 Disinfectant Study

The treated water samples by the strain were used to find out the efficiency of various disinfectants on the removal of microbial cells, turbidity, coagulated and flocculated particles and to purify the water samples. After sand filtration, the samples (samples treated with lime at 150 mg/L) were subjected to physical and

Table 2 Effect of disinfectant on bacterial population of treated water sample

S. No	Disinfection methods	Water sample treated by DS45 ($\times 10^4$ CFU/ml)
1	Initial population	17
2	Boiling for 30 min at 100 °C	0
3	Membrane filtration (0.2 μm)	0
4	UV for 50 min	0

chemical disinfectant process. The disinfectant like boiling, membrane filter, UV and chlorination were used for the study which proved to be very effective.

3.4.1 Effect of Boiling Technique on Bacterial Population

Water sample that was boiled at 100 °C for 30 min showed complete reduction of bacterial colonies in both ground water and treated samples by DS45 (Table 2).

3.4.2 Effect of Membrane Filter Method and UV Radiation on Bacterial Population

The bacterial population was completely eliminated in both the samples by using membrane filter (0.2 μm) technique (Table 2). The bacterial populations in the treated water samples gradually decreased when the UV exposed time increased from 10 to 60 min (Fig. 5) and were completely eliminated (17×10^4 CFU/ml) when exposed to ultra violet (UV) radiation up to 50 min. UV disinfectant is particularly efficient against viruses, which are major agents of water borne diseases in ground water (Craun 1986). Rice and Hoff (1981) and Wolfe (1990) have studied disinfection of water with ultra violet radiation at a range of 380–5500 μW s/cm^2 for bacteria, 3600–8000 μW s/cm^2 for viruses and 35,000–82,000 μW s/cm^2 for protozoan cysts. The UV wavelength ranging from 240 to 280 nm is an optimum dosage to kill microorganisms effectively by damaging their DNA thereby preventing the DNA replication in organism (Harm 1980).

3.4.3 Effect of Chlorine Treatment on Bacterial Population

The commonly used disinfectants for water treatment is chlorine because it has high oxidizing potential with less residual and is effective in killing bacteria and deactivating viruses (Trussell 1993). It also prevents the microbial recontamination (Sadiq and Rodriguez 2004). Chlorine dioxide is consider to be a strong water disinfectant over a wide pH range.

After sand filtration, the samples (samples treated with lime at 150 mg/L) were exposed to chlorine (Commercial bleach) of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L. At intervals of 15, 30, 45, 60, 75, 90, 105 and 120 min, cells were counted by plate

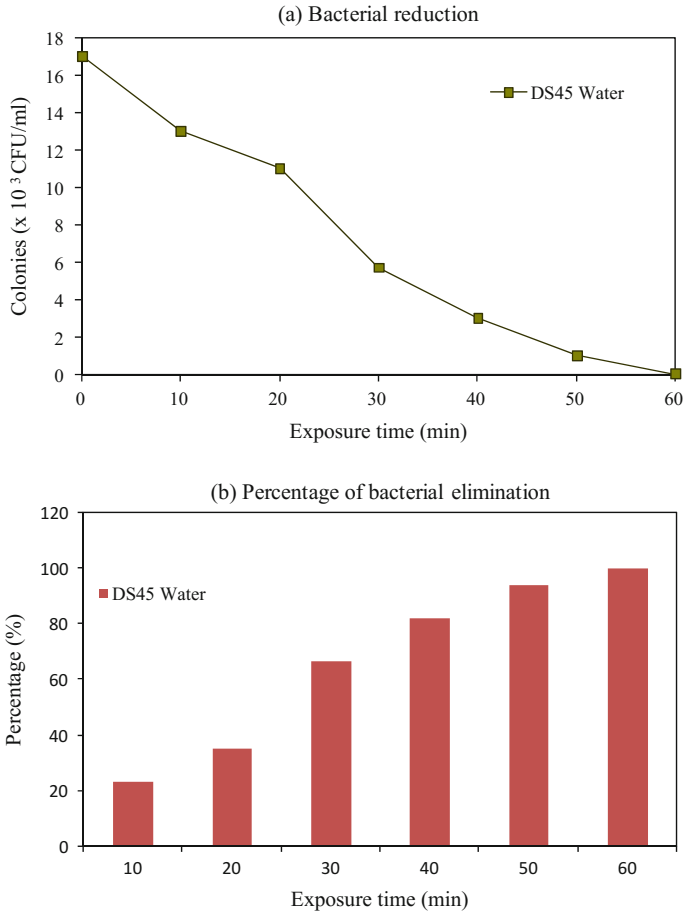

Fig. 5 Disinfection of microorganisms from water samples by UV treatment method

count method on nutrient agar medium and the susceptibility of bacterial population in each concentration were plotted (Fig. 6). Initially 17 × 10^4 CFU/ml bacterial population were observed. The bacterial population were completely eliminated at 0.5 mg/L chlorine concentration after 105 min of exposure, whereas in the concentration of 0.1 mg/L the population reduced only 21.43%. Hence it was observed that the populations were gradually eliminated the chlorine concentration is increased from 0.2 to 0.5 mg/L. Among the disinfectant methods boiling, UV treatment and membrane filter method proved to be very effective. But in chlorination, there was less removal of bacteria at lower concentration (<0.4 mg/L). Savithamani (2002) reported that chlorination at a dosage of 0.5 mg/L for 150 min is optimum for 100% elimination of *Aeromonas hydrophila*. But at normal dosage rates, it does not kill all viruses, cysts or worms. Likewise the present study exhibited the same. In DS45 treated water samples, the entire bacterial population

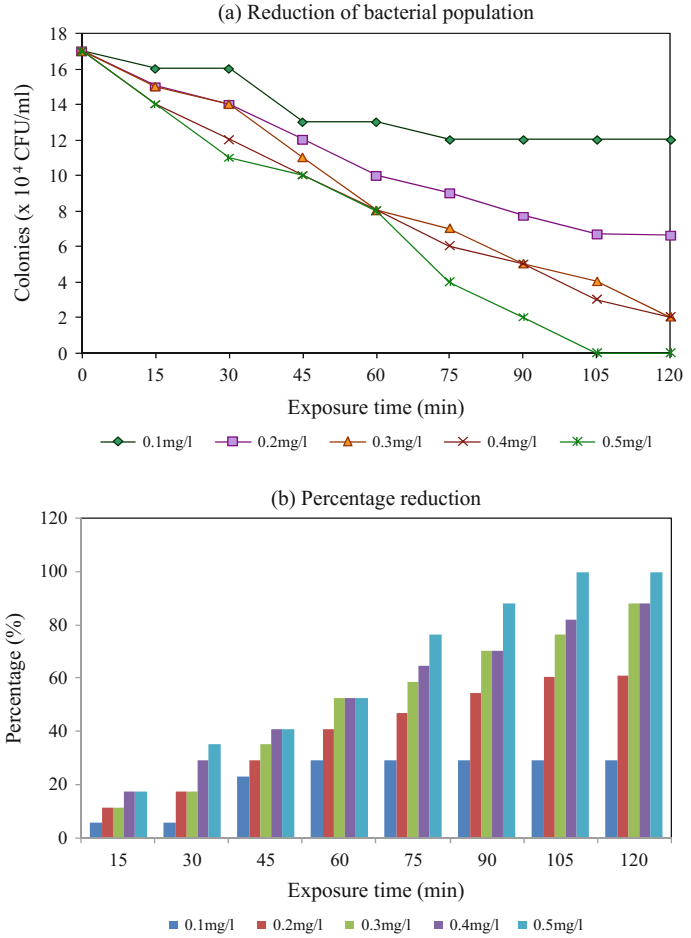

Fig. 6 Chlorination of treated water samples using various concentrations of residual chlorine

was removed (from 17 to 0 × 10^4 CFU/ml) when samples were treated with 0.5 mg/L of chlorine.

3.5 Analysis of Correlation (R^2 Score) for Nitrate Removal Through Lab Scale Bioreactor

The first order reaction occur were correlation coefficient (R^2) < 1.0. It was found that the nitrate reduction was not so efficient and it does not undergo to first order. Thus, the nitrate removal efficiency of the isolates is not of first-order relationship. The two step liner relationship was obtained by the bacterial species

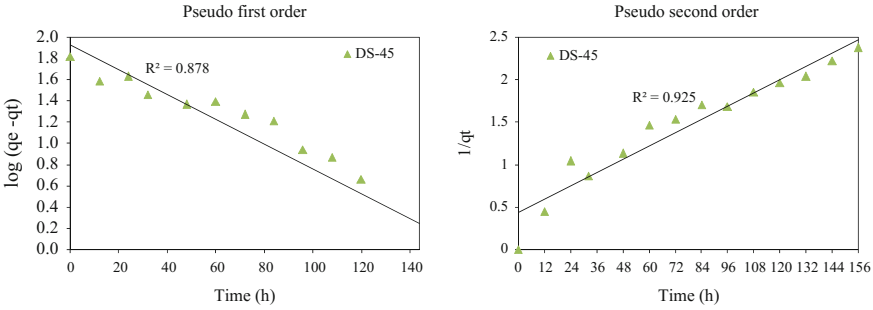

Fig. 7 Analysis of correlation (R^2 score) for nitrate removal through lab scale bioreactor

in pseudo-second-order equation. Thereby, a good agreement of experimental data with the second-order kinetic model was obtained 0.93 (Fig. 7). Our finding was similar to Song et al. (2013) that the determination of kinetic models were obtained with the relatively high correlation coefficients ($R^2 > 0.97$).

4 Conclusion

The present study concludes that, *Bacillus weihenstephanensis* (DS45) was identified by16S rRNA sequence. Results represented that about 80.04% nitrate was removed at 156 h by bioreactor treatment. Lime at 150 mg/L was more effective on nitrate (19.96%), nitrite, ammonium and bacterial removal. After lime treatment, the bacterial growth 17×10^4 CFU/ml was completely removed through disinfectant methods such as boiling (30 min for 100 °C), membrane filtration (0.2 µm) and UV radiation for 50 min. It would be concluded that the strain DS45 more effectively removed nitrate within the permissible limit after 48 h.

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Catalytic Degradation of Reactive Red 120 by Copper Oxide Nanoparticles Synthesized by *Azadirachta indica*

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Abstract An easy, green and economically viable approach for synthesis of copper oxide nanoparticles (CuO NP's) has been used. CuO NP's were synthesized using neem extract as reducing agent and were characterized by UV-visible spectroscopy, Transmission electron microscopy (TEM) with SAED pattern, and Fourier transform infrared spectroscopy (FTIR). Its effect in catalytic degradation of Reactive red 120 dyes was determined in presence and absence of $NaBH_4$ using sunlight as main source. The result showed excellent catalytic activity of nanoparticles in the presence of $NaBH_4$.

Keywords Green synthesis · CuO NP's · Reactive red 120 dye · Photocatalytic degradation

1 Introduction

The synthesis of nanoparticles under environmentally benign condition is one of the greater importances to address growing concerns for medical and biotechnological applications (Tamuly et al. 2013). Interestingly, synthesis of nanoparticles by green method approach is the utilization of various plants and their parts. The various biomolecules present in the plant extract such as enzymes, proteins, flavonoids, terpenoids and cofactors act as both reducing and capping agents (Tavakoli et al. 2015). The plant-mediated synthesis of nanoparticles is relatively fast as there is no need of maintaining definite media and culture conditions, unlike microbial synthesis. Also, extracellular synthesis of nanoparticles by plants may be advantageous over chemical methods that meet the requirements of industrial applications (Shankar et al. 2003).

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Azo dyes are the largest and most versatile class of dyes, which are currently used in dyeing various materials such as textiles, leather, plastics and cosmetics. During these dyeing processes some amount of particularly soluble reactive dyes are being released into the treatment systems or to the environment (Sahoo et al. 2014). Tiruppur, the knit capital of India, is undergoing an unprecedented crisis. There are about 9000 knitting, dyeing/bleaching, manufacturing processing units in Tiruppur that offer employment for more than 2 lakh people. In textile processing, dyeing and bleaching are the two major activities that need a large amount of water. However, these activities are not consumed totally and most of the water used by these units is discharged as effluent after processing into the Noyyal (Daniel et al. 2014).

Azo dyes usually have a synthetic origin and also complex aromatic molecular structure, which make them more stable and more difficult to biodegrade them. They have inhibitory effect on the process of photosynthesis and, thus, affecting the aquatic life. Many dyes may also cause skin irritation, allergic dermatitis, dysfunction of kidney, liver, brain, reproductive and central nervous system. Azo dyes have been used increasingly in industries because of their ease and cost effectiveness in synthesis compared to natural dyes. However, most azo dyes are toxic, carcinogenic and mutagenic (Pinherio et al. 2004).

Therefore, the removal of Reactive red dyes from colored effluents of textile industries is one of the major concerns. Various techniques like chemical coagulation, foam flotation, electrolysis, chemical oxidation, photochemical degradation, membrane filtration, biological treatment, adsorption have been used in the past for the removal of dye from the textile effluents. Most of these techniques are rather very expensive and not very effective. Nanotechnology provides effective and inexpensive solution for dye removal (Velmurugan et al. 2011). Recently, Kumar et al. (2013) reported that silver nanoparticles synthesized by facile method from *U. lactuca* can able to degrade methyl orange dyes in the presence of visible light and paves way for environmental bioremediation. Similarly, Sinha and Ahmaruzzaman (2015) reported that copper nanoparticles synthesized by green method were used to degrade the methylene blue dye.

Thus, in this study, CuO NP's were synthesized using neem extracts as reducing agents and characterized nanoparticles used as catalyst for degradation of Reactive red dye 120 was studied.

2 Methods and Materials

2.1 Preparation of Extracts and Synthesis of CuO NP's

Neem leaves (*Azadirachta indica*) were collected, thoroughly washed and allowed to dry for 2–3 days at room temperature. Dried leaves were finely cut into pieces and powdered using blender. 5 g of the powder was added to 100 ml of sterile

distilled water in 200 ml beaker and boiled at 100 °C for 15 min. The solution was then filtered and stored at room temperature.

Kumar et al. (2015) method was adopted with some modification for synthesis.10 ml of neem leaf extract was added into 1 mM copper sulphite solution in 100 ml of deionized water and stirred for 6 h at 75–80 °C. The synthesized nanoparticles were further subjected for characterization.

2.2 Characterization of CuO NP's

The bioreduction of CuO NP's were monitored by UV-Vis spectroscopy on a Shimadzu (UV-1800). The formation of nanoparticles was recorded by using a quartz cuvette with water as blank. UV-Vis spectrometric readings were recorded at a scanning speed of 200–700 nm. The synthesized nanoparticles were centrifuged for 15 min at 10,000 rpm and the pellet was ultrasonicated and the purified suspension was subjected further characterization of TEM with SAED (Jeol/JEM 2100) pattern and FT-IR analysis (JASCO FT-IR4100).

2.3 Photocatalytic Degradation

5 mg of CuO NP's and in presence and absence of aqueous solution of $NaBH_4$ (2 ml, 1×10^{-4} M) were rapidly added one by one into an aqueous solution of Reactive red 120 (100 mg in 100 ml). All the experiments were carried out outdoor with sun as the source of light. The whole mixture was then subjected to UV–visible spectral analysis at room temperature at different time intervals. The concentrations of dyes were quantified by measuring the absorption at 540 nm.

Percentage of dye degradation was estimated by the following formula:

$$\%\,\text{of Degradation} = (C_o - C)/C_o \times 100$$

where C_o is the initial concentration of dye solution and C is the concentration of dye solution after photocatalytic degradation.

3 Results and Discussion

3.1 Synthesis and Characterization of Nanoparticles

The prepared neem leaf extract was added to 1 mM Copper Sulphite solution. The formation of CuO NP's from neem leaf extract was initially monitored by color change from light to dark brown, which indicate the formation of the CuO NP's

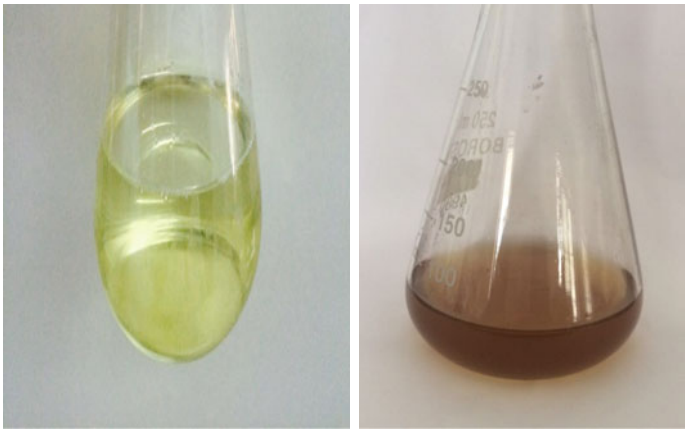

Fig. 1 Color changes showing the formation of copper nanoparticles

shown in the Fig. 1. Figure 2 shows the UV-visible spectra of the CuO NP's synthesized using neem leaf extract, which recorded the strong absorption peak at 340 nm. The incidence of the SPR absorption reflects the shape and size of the nanoparticles (Mulvaney 1996). Observation of this strong broad Plasmon peak has been well documented for various Me-NPs, with sizes ranging from 2 to 100 nm (Burygin et al. 2009).

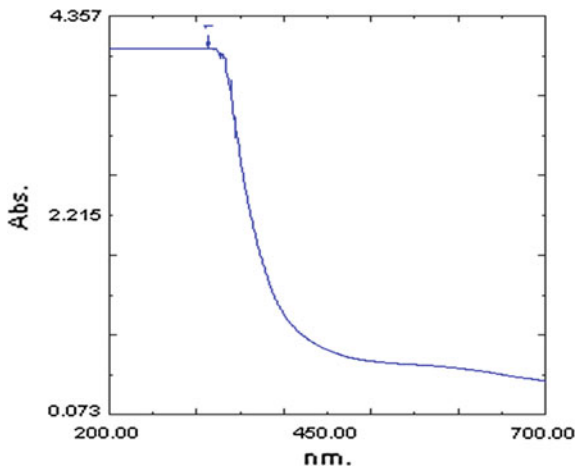

Fig. 2 UV-Visible spectra of CuO NP's absorbance at 340 nm

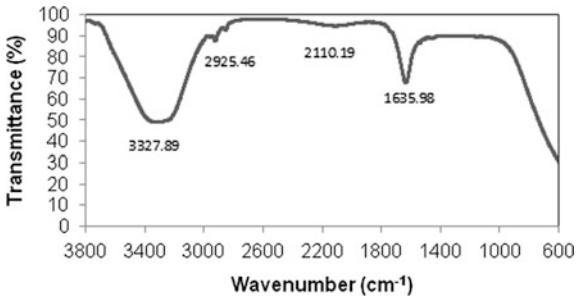

Fig. 3 FTIR absorption spectra of copper nanoparticles synthesized by *Azadirachta indica*

3.2 FT-IR Analysis

Figure 3 shows the FT-IR analysis of the copper nanoparticles. In this analysis the absorption peaks were observed at 1635.98, 2110.19, 2925.46 and 3327.99 cm^{-1}. The observed absorbance spectra band at 1635.98 is formed due to Normal polymeric OH, 2110.19 is formed due to the presence of Methylene C–H asymetric bond, 2925.46 is formed due to C=C bond, terminal alkyene (mono substituted) and 3327.99 is formed due to of C=C conjugated Ketone. In earlier studies, (Gopinath et al. 2014) observed their spectra at 3913.68, 3829.92, 3674.27, 3590.48, 3430.22, 3254.34, 3247.35, 2924.64, 1639.09, 1383.92 cm^{-1}. It revealed the presence of different groups like Alcohol (OH stretch H-bonded, free), Alkane (C–H stretch, –C–H bending) Alkene (=C–H bending, C=C stretch). So, obtained data confirms the formation of Copper nanoparticles. The constituents present in the neem leaf extract and the associated functional groups may be involved in the reduction and stabilization of the Cu NP's (Konwarh et al. 2011). Thus, the above findings indicated the dual role of *A. indica* extract in the synthesis of Cu NP's.

3.3 TEM with SAED Pattern Analysis of Copper Nanoparticles

A high magnification of TEM images were recorded for the Cu NP's by *A.indica* leaf extract was predominantly found to have circular and hexagonal with size ranging from 10 to 50 nm as shown in the Fig. 4a and b.

From the SAED pattern (Fig. 4c) with bright circular fringes shows the highly crystalline nature of the Cu NP's obtained, as reported by Philip (2009) for gold nanoparticles.

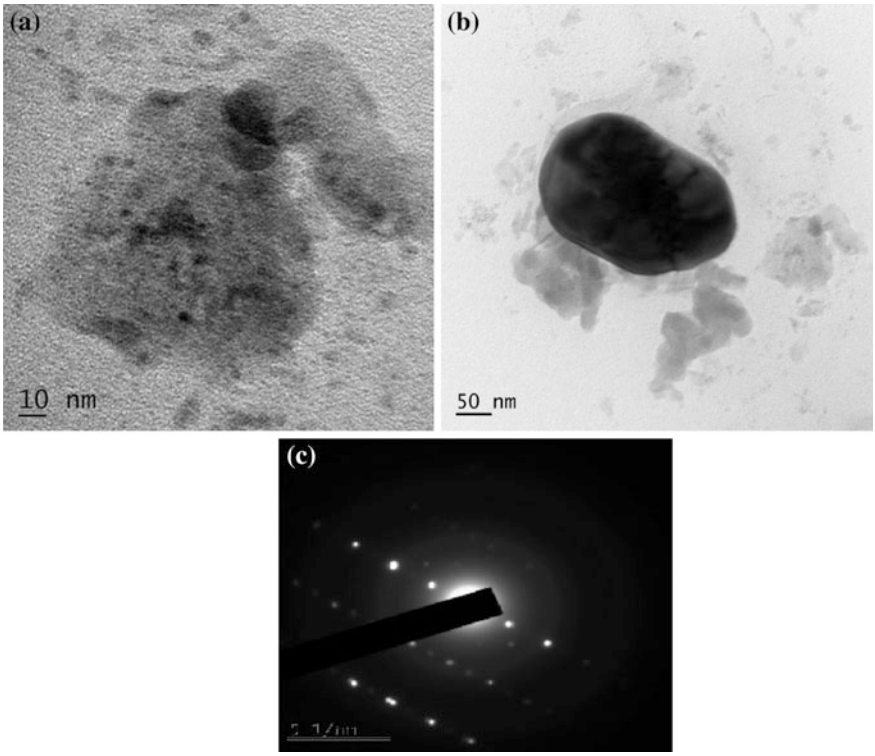

Fig. 4 TEM images of biologically synthesized of Cu NP's with various ranges of size

3.4 Photocatalytic Degradation of Reactive Red Dye

Photocatalytic degradation of Reactive red dye was carried out by using nanoparticles which are synthesized using neem leaf extract. Dye degradation was initially identified by color change. Initially the color of the dye showed dark pink color, after 1 h incubation of the dye with metallic nanoparticle solution under sun light the color change was observed from dark pink color to light pink color. The absorbance was taken at different time of intervals at 540 nm for 4 h incubation period. Figure 5 showing the degradation of reactive red dye by CuO NP's. The color dye degradation is mainly attributed to the size, morphology and surface charge property of the green synthesized copper oxide nanoparticles (Arunachalam et al. 2012). In earlier studies (Zhou and Srinivasan 2015) performed the photocatalytic degradation of organic dyes using immobilized silver nanoparticles, and achieved a similar degradation rate of dye. Similarly, CuO NP's has already been used for the photocatalytic degradation of coomassie brilliant blue R-250 dye (Sankar et al. 2014).

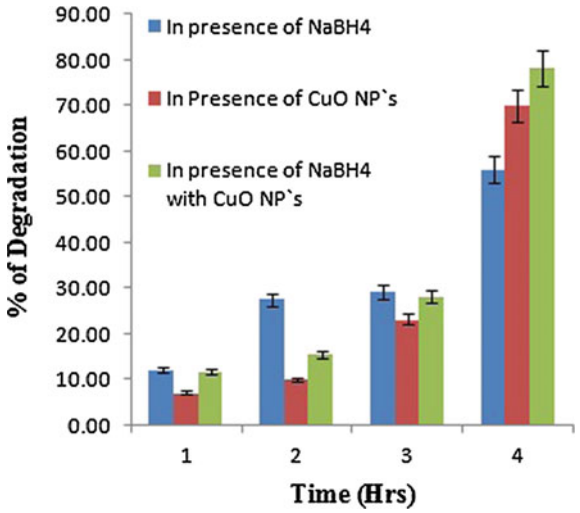

Fig. 5 Photocatalytic degradation of reactive red dye 120 using CuO NP's at different time intervals

4 Conclusions

Green Nanotechnology is gaining more importance due to the elimination of harmful reagents and provides effective synthesis of expected products in an Economical manner. Green synthesis of CuO NP's shows more compatible, low cost, ecofriendly and less time consuming process. Herein, CuO NP's were synthesized by using plant leaf extract of neem. Circular and hexaconal shape of nanaoparticles were confirmed by using TEM analysis. The functional groups responsible for formation of nanoparticles from ions were confirmed by FTIR spectroscopy. The photocatalytic activity of green synthesized CuO NP's that are doped over silica nanoparticles were evaluated by choosing Reactive red 120 dye. In the present study, it is found that CuO NP's synthesized by green methods showed excellent photocatalytic activities in dye degradation. Thus, it can be used as photocatalyst using sunlight as the source for the pollution remediation.

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Removal of Nickel from Synthetic Waste Water Using Gooseberry Seeds as Biosorbent

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Abstract The sorption of Ni (II) metal using Indian gooseberry (*Phyllanthus acidus)* seeds was studied. The main objective behind carrying out this study was to test the metal removal efficiency of gooseberry seeds for a heavy metal such as Ni (II). The main parameters (kinetic data) included in this study were effect of pH, of contact time, initial metal concentration and dosage concentration studies. The optimum equilibrium found was at 90 min. The equilibrium data was well agreed with Langmuir, Freundlich and Harkins–Jura isotherm models. The adsorption system was found to follow the pseudo-second kinetics. The presence of functional groups and its corresponding effect on Ni (II) metal was observed using FTIR spectroscopy. SEM analysis was carried out to compare the intraparticle pore size of the sorbent material before and after adsorption. The study indicated that gooseberry seed powder activated using sulphuric acid (H_2SO_4) proved to be a promising biosorbent for the removal of Ni(II).

Keywords Biosorption · Optimization · Isotherm · Kinetics · Nickel · Gooseberry seeds · Sulphuric acid · FTIR · SEM analysis

1 Introduction

The enormous rate of growth of industrial sectors in India practicing mining, tanning, steel production and several other resource extraction techniques have resulted in increased percentage of toxic deposition of heavy metals in the environment. Some of the commonly used conventional techniques of metal removal

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involve precipitation, oxidation-reduction, ion exchange and some electrochemical treatment using membrane purification (Fu and Wang 2011). But these removal techniques have certain drawbacks that hinder in making these techniques industrially applicable and commercially viable. Deposition of waste, high operating cost and environmental pollution are some of the limitations that exist. Biosorption proposes to be an efficient, economical and environment friendly technique for metal purification/removal in comparison to the conventional techniques (Rao and Ikram 2011). There are several criteria to be considered while selecting the materials to be used as a biosorbent directly or for the production of sorbent, the main criteria being the raw material availability (Oliveira et al. 2008). In our study we have taken Indian gooseberry seeds as a biosorbent. It belongs to the family of Phyllanthaceae, and is specific to the region of study. The seeds are generally discarded after the extraction of juice from the flesh of the fruit and hence can be tested for biosorbent properties.

Nickel a compound that occurs in very low levels in the environment has been used for many applications, its most common application being used as an ingredient of steel and other metal products. It is also be found in a number of food stuffs like chocolates and fats. These applications of nickel have made the metal a common agent in the effluents being discharged into the environment as well as a part of the air pollution affecting our environment. Nickel has also been found to be a major part of detergents. All the sources of Ni can be given in one sentence. With Nickel being such a consistent part of the pollution surrounding us, it affects the humans in a many ways, especially through drinking water. Nickel exposure may also occur through skin contact to contaminated soil or water. This exposure to nickel has been studied and was found that it could lead to various health issues such as lung embolism, birth defects, heart disorders, allergic reactions such as skin rashes and certain respiratory diseases and disorders like respiratory failures, asthma and chronic bronchitis. Nickel has also been found to be carcinogenic, increasing the chances of developing lung cancer, nose cancer, larynx cancer and prostate cancer. Exposure to nickel can cause severe effects like nausea, vomiting and migraine. There is a large quantity of nickel present in industrial plant waste waters; moreover these polluted waters are not purified up to the optimum levels. Thus an attempt to remove Ni (II) using the gooseberry seeds was made by studying the effect of physical parameters such as pH, contact time, adsorbent dosage and initial metal ion concentration on the biosorption of Ni (II) by gooseberry seeds. The adsorption isotherms such as Freundlich, Langmuir and Harkins–Jura isotherm models were studied to check which model suits the current adsorption studies. The mechanism of the reaction can be clearly understood by studying the kinetic data using the pseudo-first and pseudo-second order kinetic models.

2 Materials and Methods

2.1 Collection and Preparation of Adsorbent

Gooseberry seeds *(Phyllanthus acidus)* were collected from Coimbatore in Southern part of India. The gooseberry seeds obtained was washed under running tap water and collected in a separate vessel. Further it was subjected for drying at an optimum temperature of 40 °C for a time period of 1 h. The dried seeds were finely ground and sieved. The finely powdered biosorbent was treated with 0.1 N sulphuric acid (H_2SO_4). The mixture was further filtered and the powered residue was washed thoroughly with distilled water. The residue was dried at 50 °C for 45 min. The dried powder was used for the experiment.

2.2 Batch Adsorption Studies

The ability of the biosorbent material to remove Ni (II) was determined by varying important parameters such as metal ion concentration, pH, adsorbent dosage and contact time. One parameter was varied while all the other parameters were maintained constant. The volume of the metal solution was maintained at 100 mL throughout the study. The sample was agitated in an orbital shaker maintained at 120 rpm and filtered. The amount of Ni (II) was spectrometrically determined from the filtrate by the DMG method.

2.3 Preparation of Metal Solution

Nickel (II) ions were prepared using 1,000 mg mL^{-1} of stock solution using $NiCl_2$ salt. The pH of the solution was balanced by adding 1.0 N of HCl or NaOH. The process also involved preparation of fresh dilutions of 100 mg L^{-1} which were used for the study.

2.4 Nickel Analysis

DMG (Dimethylglyoxime) was used to analyze nickel (II) spectrophotometrically. A blank was prepared for nickel (II) solution. To different standard solutions containing less than 100 mg L^{-1} of nickel (II), 2 mL of 20% w/v potassium sodium tartrate solution, 10 mL of 4% w/v ammonium per sulfate, 2.5 mL of 5 M sodium hydroxide solutions, 15 mL of HCl and 0.5 mL of 1% DMG solutions were added. After an incubation period of 30 min, the absorbance was measured at 465 nm

using UV-visible spectrophotometer. The amount of nickel present in the sample was determined using the calibration curve.

2.5 Removal Efficiency

The percentage of nickel removed can be obtained using the formulae:

$$\text{Removal efficiency}(\%) = \frac{Ci - Co}{Ci \times 100} \quad (1)$$

where C_i and C_o represent the initial and final concentration of nickel metal.

3 Results and Discussion

3.1 Fourier Transform Infrared (FTIR) Spectroscopy

The changes in the vibrational frequencies of functional groups in the biosorbent were analyzed using FTIR. The spectra of the adsorbent before adsorption and after adsorption were measured within the range of 4,000–400 cm^{-1} (Fig. 1). The peak shifts showed that stretching of O–H group, bending of C–H groups, stretching of

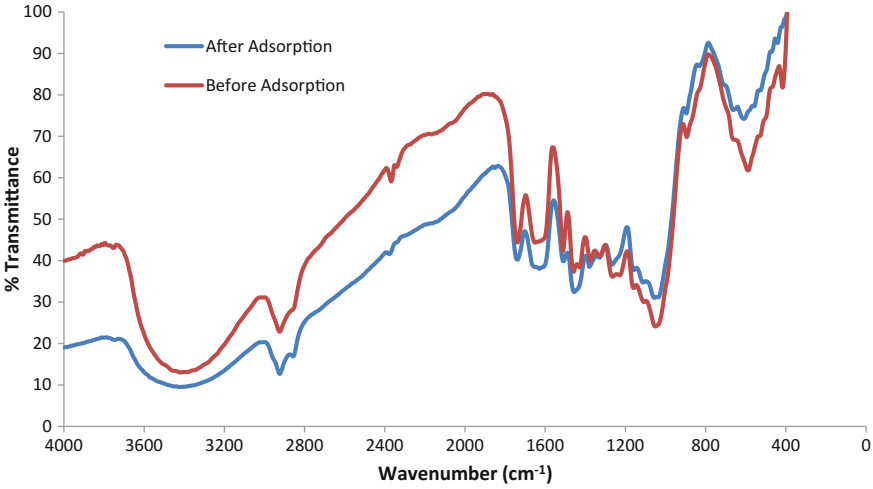

Fig. 1 FTIR spectra of pretreated adsorbent and Ni(II) loaded adsorbent

Table 1 FTIR spectral characteristics of gooseberry seed powder before and after adsorption

IR peak	Before adsorption (cm^{-1})	After adsorption (cm^{-1})	Difference	Assignment
1	3417.86	3425.58	−7.72	O–H stretch, H– bonded
2	1635.64	1627.92	7.72	C=C stretch
3	1450.47	1458.18	−7.71	C–H bend
4	1373.32	1381.03	−7.71	C–H bend (variable)
5	1049.28	1056.99	−7.71	C–N stretch
6	586.36	609.51	−23.15	C–C Stretch

C–Br, C–N, C=O, C=C, and C–C bonds were responsible for the adsorption of Ni (II) (Table 1). Similar results were obtained by Aravind et al. (2013b) when they studied the adsorption of Ni (II) on pigeon pea pod. It was found that C=O, O–H and C–H bonds were involved in the efficient removal of nickel. The involvement of C–C bond in the adsorption process was explained by Malkoc and Nuhoglu (2005) for the adsorption of Cr (VI) on waste acorn.

3.2 Scanning Electron Microscope (SEM) Analysis

SEM analysis was carried out to compare the intraparticle pore size of the sorbent material before and after adsorption. There was significant difference in pore size of the particles which were observed after comparing the SEM micrograph results (Fig. 2). The pore sizes were not distinct in shape and there was irregularities and decrease in pore sizes after adsorption of Ni(II) uniformity since symbol is not used

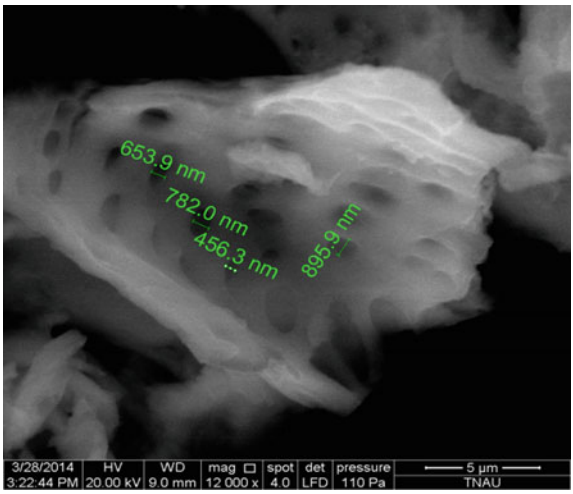

Fig. 2 SEM micrograph of gooseberry seed powder before adsorption

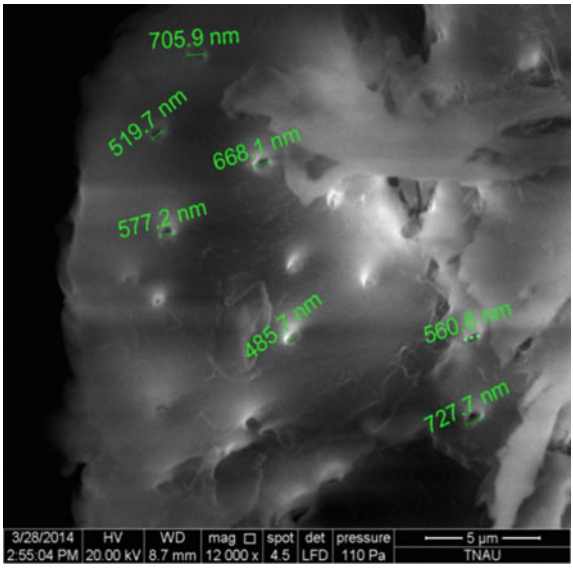

Fig. 3 SEM micrograph of gooseberry seed powder after adsorption

before (II) (Fig. 3). The results obtained were comparable to the SEM results obtained by Rao and Ikram (2011) where the surface of sorbent after and before adsorption were compared for heavy metal Cu (II). This result is comparative to our SEM analysis study were we observed a decrease in pore size after adsorption. Thus it may be concluded that the surface size may have reduced over a period of time leading to the decrease in pore size.

3.3 Batch Adsorption Studies

3.3.1 Effect of pH

pH of the metal solution has been considered as one of the most important parameters in biosorption of metals. Researchers say that with the variation in pH, the availability and characteristics of metal ions also changes. To study the effect of pH on Ni (II) adsorption using gooseberry seed powder as the biosorbent, experiments were conducted by modifying the pH from 2 to 9. During these experiments all the other parameters such as contact time (30 min), adsorbent dosage (0.1 g) and metal concentration (100 mg L^{-1}) were kept constant. At pH 3, maximum adsorption of 59% was obtained. As the pH is increased, further till 9 the adsorption capacity of the biosorbent decreases (Fig. 4). A similar result was shown (Ho et al. 1995) where Sphagnum moss peat was used to remove Ni (II) metal from aqueous solutions where the sharpest increase in removal efficiency was observed between pH 2 and 4.

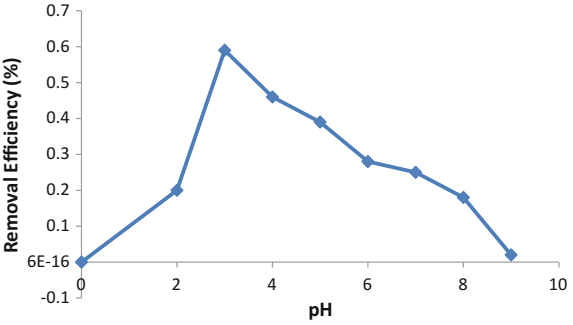

Fig. 4 Effect of pH on adsorption of Ni (II) on gooseberry seed powder (Adsorbent dosage = 0.1 g, contact time = 30 min, metal concentration = 100 mg L^{-1})

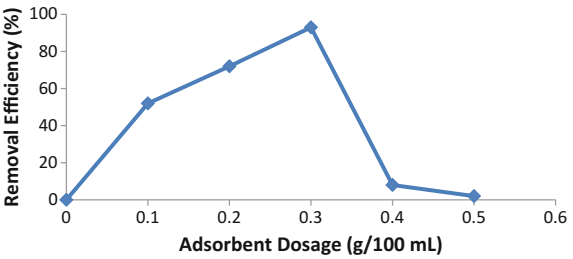

Fig. 5 Effect of adsorbent dosage on adsorption of Ni (II) on gooseberry seed powder (pH = 3, contact time = 30 min, metal concentration = 100 mg L^{-1})

3.3.2 Effect of Adsorbent Dosage

The amount of adsorbent used plays an important role in the adsorption process. As the adsorbent concentration increases the adsorption increases. The pH was maintained at the optimum value 3. The other parameters were also maintained as a constant. The adsorbent dosage was varied from 0.1 g/100 mL^{-1} to 0.5 g/100 mL^{-1}. In this case, maximum adsorption was found at 0.3 g/100 mL^{-1}. The adsorption was found to decrease when the adsorbent dosage was increased (Fig. 5).

3.3.3 Effect of Contact Time

The adsorption experiments were carried out at different time intervals to arrive at the equilibrium time for maximum adsorption. Experiments were conducted by varying the contact time from 15 to 120 min at a pH of 3 and adsorbent dosage concentration of 0.3 gL^{-1}, 100 mL^{-1} and a metal concentration of 100 mg L^{-1}. The adsorption was found to increase with the increase in time. After 90 min, there appeared to be no significant change in adsorption (Fig. 6).

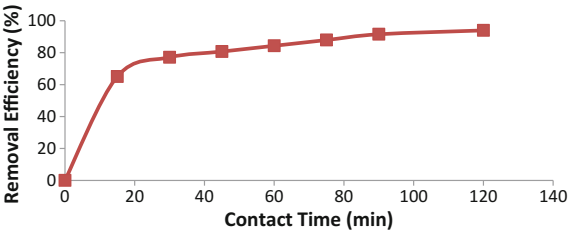

Fig. 6 Effect of contact time on adsorption of Ni (II) on gooseberry seed powder (pH = 3, adsorbent concentration = 0.3 g, metal concentration = 100 mg L^{-1})

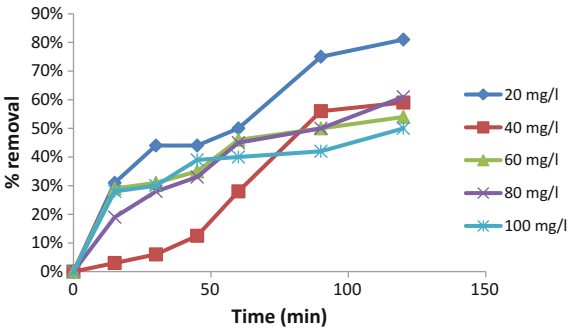

Fig. 7 Effect of initial metal ion concentration on adsorption of Ni (II) on gooseberry seed powder (pH = 3, adsorbent dosage = 0.3 g, metal concentration = 20–100 mg L^{-1})

3.3.4 Effect of Initial Metal Ion Concentration

The effect of initial Ni (II) concentration on the adsorption system involving gooseberry seed powder as biosorbent was studied in the range of 20–100 mg L^{-1}. It was observed that the increase in initial Ni (II) concentration decreased the removal efficiency of the adsorbent (Fig. 7). This decrease in the percentage removal of the metal may be attributed to the saturation of metal binding sites in the adsorbent. Similar observation was made for the adsorption of Ni (II) on pigeon pea pod (Aravind et al. 2013a, b).

3.4 Equilibrium Adsorption Isotherm Studies

The adsorption isotherms are important to design the adsorption system. Each model is checked to see which model best fits our adsorption studies (Malkoc and Nuhoglu 2005). The successful representation of the dynamic adsorptive separation of solute from solution onto an adsorbent depends upon a good description of the equilibrium separation between the two phases. The data obtained from studies were tested for their applicability to the isotherm models namely Langmuir, Freundlich, Temkin and Dubinin–Radushkevich (D–R), H–J and Frumkin isotherm.

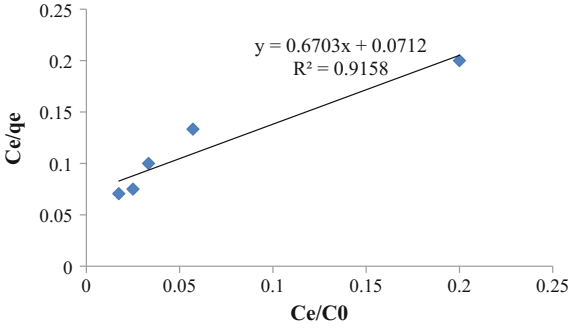

Fig. 8 Langmuir isotherm model for Ni (II) adsorption on gooseberry seed powder

3.5 Langmuir Isotherm Model

Langmuir isotherm model is represented by the equation

$$\frac{C_e}{q_e} = \frac{C_e}{(Q_0 * b) * C_0} + \frac{C_e}{Q0} \tag{2}$$

where, *C*e represents the equilibrium concentration (mg L^{-1}), *q*e is the amount absorbed at equilibrium (mg g^{-1}), Q_0 and *b* are the Langmuir constants related to adsorption capacity and energy of adsorption (Fig. 8). The Langmuir isotherm represents the equilibrium distribution of metal ions between the solid and liquid phases (Dada et al. 2012).

3.6 Freundlich Isotherm Model

This model is commonly used to describe the adsorption characteristics for the heterogeneous surface (Fig. 9). The following equation represents Freundlich isotherm model.

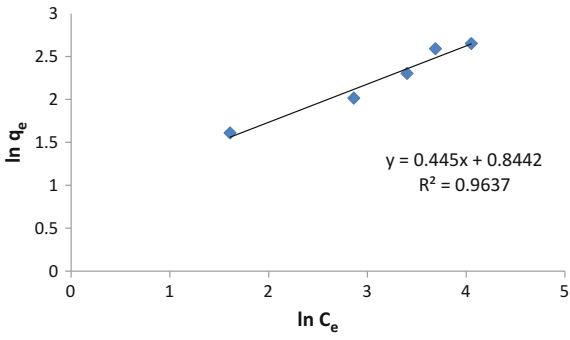

Fig. 9 Freundlich isotherm model for Ni (II) adsorption on gooseberry seed powder

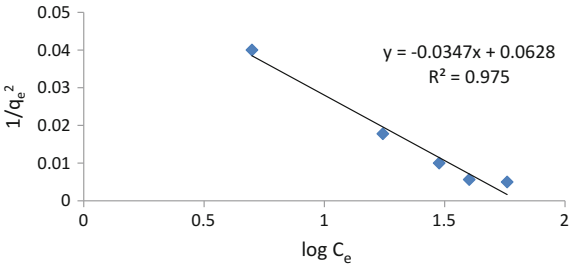

Fig. 10 H–J isotherm model for Ni (II) adsorption on gooseberry seed powder

$$lnq_e = ln\,K + (1/n)ln\,C_e \tag{3}$$

where, q_e is the amount absorbed at equilibrium (mg g^{-1}), C_e is the equilibrium concentration (mg L^{-1}), and K is the Freundlich model constant.

3.7 Harkins–Jura (H–J) Isotherm Model

H–J isotherm equation is given by,

$$\frac{1}{q_e^2}\frac{B}{A} = -\left(\frac{1}{A} * \log C_e\right) \tag{4}$$

where, C_e is the metal concentration at equilibrium (%v/v), q_e is the amount of metal absorbed per unit mass of adsorbent (mg g^{-1}), A and B are H–J isotherm constants (Fig. 10).

3.8 Temkin Isotherm Model

$$q_e = B * \ln A + B * \ln C_e \tag{5}$$

where, C_e is the metal concentration at equilibrium (%v/v), q_e is the amount of metal adsorbed per unit mass of adsorbent (mg g^{-1}), A and B are Temkin constants (Fig. 11). B can be represented as

$$B = RT/b \tag{6}$$

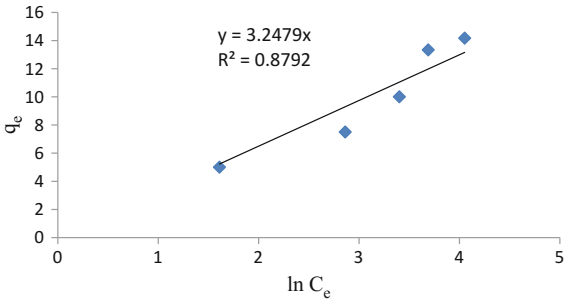

Fig. 11 Temkin isotherm model for Ni (II) adsorption on gooseberry seed powder

3.9 Dublin–Radushkevich (D–R) Isotherm Model

The D–R equation is given by:

$$\ln q_e = \ln q_m - K\,\varepsilon^2 \tag{7}$$

where, q_e is the amount of metal adsorbed per unit mass of adsorbent(mg g^{-1}), q_m is the theoretical monolayer saturation (mg g^{-1}), K is the constant related to adsorption energy and the Polanyi potential ε is given by (Fig. 12),

$$\varepsilon = RT * \ln\left(1 + \frac{1}{C_e}\right) \tag{8}$$

3.10 Frumkin Isotherm Model

The Frumkin isotherm equation is given by:

$$\ln\left[\left(\frac{\theta}{1-\theta}\right) * \frac{1}{C_e}\right] = \ln K + 2a\theta \tag{9}$$

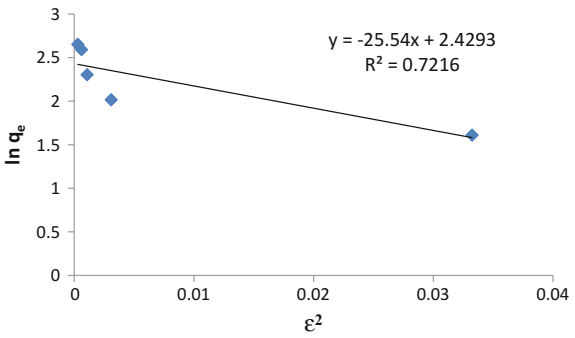

Fig. 12 D–R isotherm model for Ni (II) adsorption on gooseberry seed powder

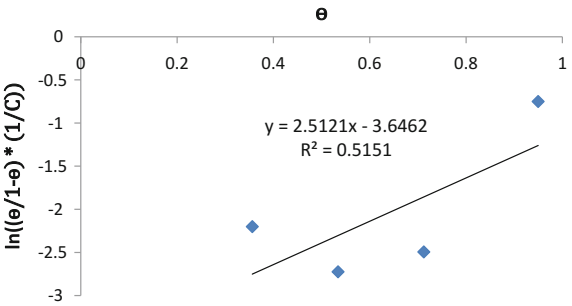

Fig. 13 Frumkin isotherm model for Ni (II) adsorption on gooseberry seed powder

where C is the metal concentration at equilibrium (%v/v), ɵ is the fractional occupation and is given by $\frac{q_e}{q_m}$ (q_e) is the equilibrium adsorption capacity (mg g^{-1}), q_m is the theoretical monolayer saturation capacity (mg g^{-1}), K is the constant related to adsorption equilibrium and C_e is the equilibrium concentration (mg l^{-1}) (Fig. 13).

3.11 Separation Factor

The separation factor R_L is represented as,

$$R_L = \frac{1}{1 + (b * C_0)} \tag{10}$$

C_0 is the initial Ni (II) concentration (mg L^{-1}), b is the Langmuir equilibrium constant (l g^{-1}). The conditions for separation factor are:

R_L values for this adsorption process are 0.653595, 0.350262697, 0.23923445, 0.190839695 and 0.140944327 while the initial concentrations of Ni (II) are 20, 40, 60, 80 and 100 mg L^{-1} respectively. The R_L values lie between 0 and 1 which indicates that the adsorption of Ni (II) on gooseberry seed powder is favorable (Table 2).

Table 2 Separation factor

R_L value	Type of isotherm
$R_L > 1$	Unfavorable
$R_L = 1$	Linear
$0 < R_L > 1$	Favorable
$R_L = 0$	Irreversible

3.12 Equilibrium Adsorption Kinetics Studies

The adsorption kinetics is an important factor to be tested in order to see which order of kinetics the adsorption system follows. The kinetic studies of Ni (II) adsorption on gooseberry seed was studied using both pseudo-first order and pseudo-second order kinetic models on the experimental data obtained from the effect of initial nickel concentrations.

3.13 Pseudo First Order Kinetics

The pseudo-first order kinetics model is given by:

$$\log(q_e - q_t) = \log q_e - \frac{k_1}{2.303} t \tag{11}$$

where, q_t(mg g^{-1}) represents the amount of adsorbed Ni (II) on the adsorbent at the time t (min). Similarly, k_1 (min^{-1}) represents the constant of first-order kinetic model.

From Fig. 14 it is shown that the pseudo-first order model does not fit the present adsorption system. Hence, the adsorption system was studied to check if the model fits the system (Fig. 15).

3.14 Pseudo-Second Order Kinetics

The following equation,

$$\frac{t}{q_t} = \frac{1}{k_2 * q_e^2} + \frac{1}{q_e} t \tag{12}$$

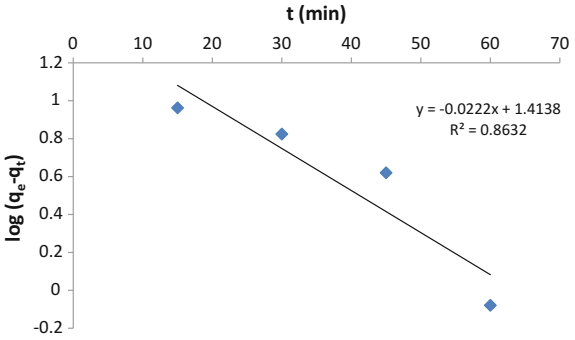

Fig. 14 Pseudo-first order kinetics for adsorption of Ni (II) on gooseberry seed powder

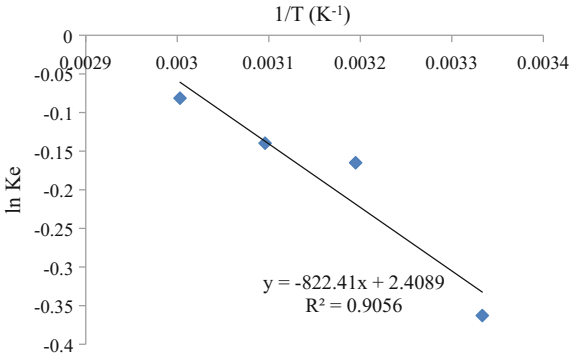

Fig. 15 Adsorption thermodynamics for Ni (II) on gooseberry seed powder

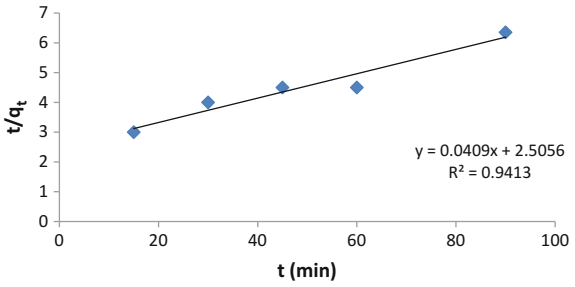

Fig. 16 Pseudo-second order kinetics for adsorption of Ni (II) on gooseberry seed powder

represents the pseudo-second order kinetics model. In the equation, k_2 (gmg^{-1} min^{-1}) is the rate constant of second order adsorbent, q_t(mg g^{-1}) is the amount of adsorbed nickel at time t (min), and q_e and k_2 are constants of pseudo-second order kinetic model (Fig. 16). This model suits the present adsorption system of Ni (II) on gooseberry seed powder. The results were similar to the results obtained by (Aravind et al. 2013a, b) (Table 3).

Based on the R^2 values, it is observed that the H–J isotherm model suits the present adsorption studies. The isotherm accounts for multilayer adsorption and can be explained with the existence of a heterogeneous pore distribution.

3.15 Thermodynamic Studies

3.15.1 Effect of Temperature

The effect of temperature on the adsorption of Ni (II) on the gooseberry seed powder was determined by conducting the experiments at 300, 313, 323, 333 K.

Table 3 Constants evaluated from various isotherms and kinetics

S. No.	Adsorption	Name	Parameters	R^2
1.	Isotherms	Langmuir	Q_0 = 14.04 b = 0.106	0.9158
2.		Freundlich	K = 2.326 n = 2.247	0.9637
3.		H–J	A = 28.81 B = 1.809	0.975
4.		Temkin	A = 0 B = 3.247	0.8792
5.		D–R	q_m = 11.347 K = 25.54	0.7216
6.		Frumkin	K = 0.0260 a = 1.256	0.5151
7.	Kinetics	Pseudo 2nd order	q_e = 24.44 $K_2 = 6.67 \times 10^{-4}$	0.9413

Table 4 Thermodynamic parameters

Temperature (K)	ΔG (J mol^{-1})	ΔH (kJ mol^{-1} K^{-1})	ΔS (k mol^{-1} K^{-1})
300	−81.4643	6837.5	20.02
313	−54.89892		
323	−58.09904		
333	−40.65199		

The adsorption is endothermic as the degree of adsorption increases with the increase in temperature.

Van't- Hoff equation is given by:

$$\ln K_e = \frac{\Delta S}{R} - \frac{\Delta H}{R}\left(\frac{1}{T}\right) \tag{13}$$

where, $K_e = \frac{C_{Ae}}{C_e}$ (C_{Ae} is the amount of Ni (II) ions adsorbed at equilibrium (mg L^{-1}) and C_e is the equilibrium concentration of nickel (mg L^{-1})), R is the gas constant (8.314 J mol^{-1} K^{-1}), T is the temperature (K), ΔH and ΔS are constants. The positive values of ΔH and ΔS suggest that the adsorption process is of endothermic nature and the increased randomness at the solid/solution interface (Table 4). The negative values of ΔG at 300, 313, 323 and 333 K indicate the spontaneous nature of the adsorption process (Fig. 15). The results are comparable to the results obtained by Aravind et al. (2013a, b).

4 Conclusion

The main objective behind this study was to find out the sorption capacity of Indian gooseberry seeds on a heavy metal Ni (II). Nickel is a consistent part of the pollution and affects human health in many ways. This is one main reason for selection of Ni (II) for our study. The sorption nature of gooseberry seeds was characterized using several parameters like effect of pH effect of contact time and adsorbent concentration. The experimental data revealed that the optimum pH was at 3.0, the optimum adsorbent dosage concentration was 0.3 g and the equilibrium time was at 90 min. Under the above conditions, the biosorbent demonstrated 81% removal of Ni (II). To study the best possible fit of isotherm models to the equilibrium data Langmuir, Freundlich, Harkins–Jura, Temkin, Dublin–Radushkevich and Frumkin isotherm models were studied. Based on the R^2 values, it was found that H–J model best suits the adsorption mechanism. This isotherm accounts for multilayer adsorption and can be explained by the existence of a heterogeneous pore distribution. Changes in the vibrational frequencies of functional groups in the biosorbent were noted by comparing FTIR spectroscopy which revealed that O–H and C=O, C–H and C–C bonds were involved in sorption of Ni (II). Surface morphology and pore difference between native and Ni (II) loaded samples were studied using SEM. Observed data shows decrease in porosity of sample after adsorption. Additional research is required to render the practical utility of gooseberry seeds.

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Bioremediation of Congo-Red Dye by Using Silver Nanoparticles Synthesized from *Bacillus sps*

Lakshmi Priya Thyagarajan, Swathi Sudhakar and Thirumoorthy Meenambal

Abstract Bioremediation using a variety of microbes for the xenobiotics degradation seems a green solution to the problem of environmental pollution. Different bacteria have the potentials to degrade complex organic compounds into simpler fragments and sometimes achieve complete mineralization. The present study, deliberate the synthesis of silver nanoparticles (AgNPs) using different bacillus species like *B. megaterium* (SIV01), *B. subtilis* (SIV02), *B. megaterium* (MNS1), *B. subtilis* (001), *B. licheniformis* (P-2) and their ability to decolorize the dye Congo red. The synthesized AgNPs were characterized using UV visible spectrophotometer, FTIR and SEM. The UV Vis Spectrum results showed that the *Bacillus megaterium* (SIV01) to be the most potential organism for the synthesis of AgNPs among the different strains used which is confirmed by high absorbance value at 450 nm. The adsorption and decolourization capacity was found to be high for all the strains which was exihibited by high Langmuir and Freundlich isotherm constants. The synthesized silver nanoparticles showed significant antibacterial activity against *E. coli*. Interestingly, the synthesized silver nanoparticles showed antibacterial activity against its own source microorganisms, this peculiar behaviour exhibited by the bacillus silver nanoparticles, is yet to be explored.

Keywords Bacillus species · Anti-microbial activity · Silver nanoparticles · FTIR · SEM

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1 Introduction

Industrial revolution for fulfilling the demands of increasing population during production, results in pollution of water, air and soil. The discharge of pollutants from various industries poses threat to the surrounding environment. Different types of dyes were used in paper, leather, textile and cosmetics industries. Among all dyes, Congo red dyes are largest and most versatile class of dyes. Congo red is the sodium salt of 3, 3′-([1, 1′-biphenyl]-4,′-diyl) bis (4-aminonaphthalene-1-sulfonic acid) Nithya (2012), Rajeev Wahi (2005). A very small amount of dye in water is highly visible and can be toxic to life and harmful to human beings. Hence, the removal of color from a process or waste effluents becomes of fundamental importance to the environment Bekir (2011), Teng (2011), Barragan (2007).

Several physico-chemical methods such as adsorption, chemical treatment and ion pair extractions have been adopted and proven to be costly while producing large amounts of sludge and thus they also lead to other associated pollution. Virender (2009), Strong (2011). To overcome such problems research has been moving toward biological methods, as these methods are eco-friendly and cost effective. Many microorganisms like bacteria, fungi have been reported to solve the problems regarding environmental remediation Popescu (2010), Fortin (2009), Nelly (2011), Ramanathan (2009), Bansal (2004), Ahmad (2009) but as the load of pollutants in the environment is very high, bioremediation alone is not sufficient. So, enhanced technology like nano-bioremediation can be exploited for better results. Nano-bioremediation technology includes the application of nanoparticles or nanotechnology to augment the process of bioremediation.

The biodegradation of dyes by different species of Bacillus offers an advantage over other processes because of their ability to completely mineralize various dyes Senapati (2005), Nagajyothi (2011), Antarikshsaxena (2012), Mohan (2007), Emil (2007), Moores (2011), Timothy (2011). Nanotechnology enables the development of nano-scale particles of metals with novel and distinctive physico-chemical properties, and a broad range of scientific and technological applications Kadir (2011), Percival (2007a), Wright (2005), Blaser (2006), Buzea (2007), Chompuchan (2009), Elechiguerra (2005), Sivakumar (2007), Jesica (2010), Sahayaraj (2007). In view of these, this work focuses on the comparative study in the microbial synthesis of silver nanoparticles from *B. megaterium, B. licheniformis, B. subtilis* and evaluation of their decolorization potential on Congo-red dye followed by assessing the antimicrobial activity of different pathogenic microbes and tested for self-antimicrobial activity.

2 Methods and Materials

2.1 Microorganisms

B. subtilis strain 001 (MTCC no 7193), *B. megaterium* strain MNS1 (MTCC no 7163) and *B. licheniformis* strain P-2 (MTCC no 7445) were obtained from MTCC, IMTECH, Chandigarh, India, whereas the strains like *B. subtilis* SIV02 (NCBI accession no GU 306176) and *B. megaterium* SIV01 (NCBI accession no GU306175) were registered cultures of NCBI.

2.2 Chemicals

Silver nitrate ($AgNO_3$) was obtained from Sigma Aldrich.

2.3 Synthesis of Silver Nanoparticles

Bacilli cultures were inoculated into a fresh nutrient broth and incubated for 24 h, then the pellets were collected by centrifugation at 10,000 rpm for 10 min Kim (2007), Yang (2007), Linga (2011), Manoj (2011), Guzmán (2008). Around 0.001 g of $AgNO_3$ was added to the pellets to make an overall concentration of 1 mM solution. Samples were incubated in a shaker incubator at 37 °C and 100 rpm for 11 days.

2.4 Studies on Effect of $AgNO_3$ Concentration, pH and Time

Bacilli cultures were inoculated in 150 ml nutrient broth media without NaCl and incubated in dark on orbital shaker (100 rpm) for 24 h at 37 °C. About 50 ml of varying concentrations of $AgNO_3$ (0.5, 1 and 1.5 mM) solutions were added to 50 ml of *Bacilli* culture Mohamed (2007, 2011), and effect of silver concentration on AgNPs synthesis were studied. The same procedure was followed but pH was varied by keeping concentration of $AgNO_3$ constant(1 mM). The reduction of Ag^+ ions were monitored by sampling aliquots (2 ml) of the solution at different time intervals and absorbance was measured Saifuddin (2009), Nalenthiran (2009), Percival (2007b), Favero (1991), Gabriel (1994).

2.5 Characterization Studies of AgNPs

UV-Visible spectrum of the reaction medium was taken after diluting a small aliquot of the sample with distilled water. The absorption maxima of synthesized AgNPs were scanned at the wavelength of 300–900 nm in HACH UV-Visible spectrophotometer Namasivayam (1995). The FTIR spectrum was recorded in the range of 4000–800 cm^{-1} using a Thermo Nicolet Nexus 200 spectrometer in the diffuse reflectance mode operating at resolution of 4 cm^{-1} Bai (2009). Detailed analysis of the morphology, size and distribution of the nanoparticles was documented by Scanning Electron Microscopy (Hitachi S-4500).

2.6 Decolourization Studies

The decolourization studies were carried out by adding synthesized silver nanoparticle solutions to equal volumes of different concentrations (0.1, 0.25, 0.5 mM) of Congo red solution and incubated at 37 °C for 9 days in a shaker. The decolorization was monitored at different time intervals using UV-Visible spectrometer.

The adsorption isotherm was the initial experimental step to determine the feasibility of adsorption treatment. Adsorption data for a wide range of adsorbate concentrations are most conveniently described by various adsorption isotherms, namely Langmuir or Freundlich isotherm.

The Langmuir model Bishnoi (2007) can be described as

$$Q_e = Q_0\, b\, C_e / 1 + b\, C_e \tag{2.6.1}$$

where,

Q_e is the uptake of metal ions per unit weight of the adsorbent.

Q_0 is the moles of solute sorbed per unit weight of adsorbent.

b is the constant relates the affinity between the biosorbents and biosorbate.

C_e equilibrium concentration of ions.

The constants, Q_0 and b are evaluated from the linear plot of the logarithmic equation. The Langmuir model is based on the assumption that maximum adsorption occurs, when a saturated monolayer of solute molecule is present on the adsorption surface.

The Freundlich isotherm is of the form:

$$Q_e = k\, Ce\, 1/n \tag{2.6.2}$$

where,

Q_e is the uptake of metal ions per unit weight of biosorption.

C_e is the equilibrium concentration of metal ions in solution.

k is the Freundlich constants denoting adsorption capacity.

n is the empirical constants, is a measure of adsorption intensity.

The value of k and 1/n were found by plotting the graph between log Q_e and log C_e. A high value of k and n indicates high adsorption throughout the concentration range and vice versa.

2.7 Antibacterial Studies

The antibacterial activity of AgNPs was tested on non-pathogenic bacteria viz., *B. subtilis, B. megaterium, B. licheniformis* and *E. coli* by standard agar well diffusion method. To each well, 20 µL of synthesized AgNPs were loaded and incubated at 37 °C for 48 h. The zones of inhibition with control were recorded and compared. Rashmi (2013).

3 Results and Discussion

3.1 Studies on Effect of $AgNO_3$ Concentration, PH and Time

The effect of concentration, pH and time on synthesis of AgNPS was carried out. It was found that 1 mM was the optimum concentration of $AgNO_3$ and in the case of the pH, pH 5 was the optimum for *B. subtilis* (SIV02) and pH 7 for the *B. megaterium* (SIV01), *B. megaterium* (MNS1), *B. subtilis* (001) and *B. licheniformis* (P-2) species Ibrahim (2015). It was concluded that on the 5th day, maximum synthesis of silver nanoparticles was observed and absorbance values were recorded using UV-Vis spectroscopy at 450 nm (Gajbhiye 2009).

3.2 Characterization of Silver Nanoparticles

3.2.1 UV-Visible Spectra Analysis

The absorbance values were measured at the maximum wavelength 450 nm corresponding to the silver nanoparticle. The analysis showed maximum absorbance to *B. megaterium* (SIV01) strain followed by *B. subtilis* (SIV02) strain. Thus *B. megaterium* (SIV01) showed maximum synthesis of silver nanopaticles (Fig. 1).

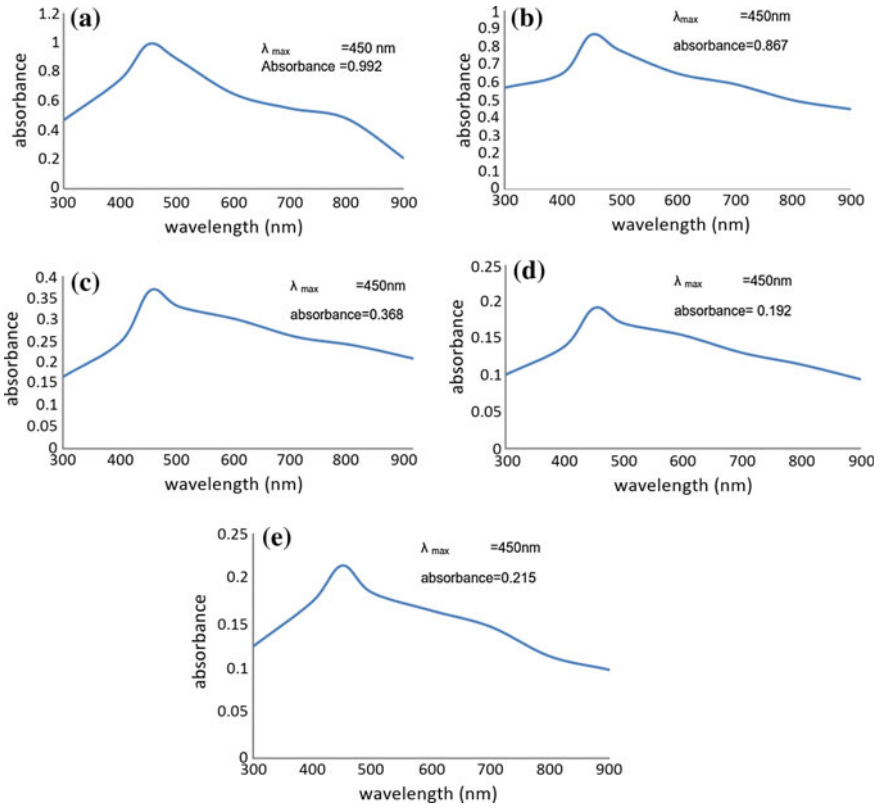

Fig. 1 UV–Vis absorption spectra of silver nanoparticles synthesized by **a** *B. megaterium* (SIV01) **b** *B. subtilis* (SIV02) **c** *B. megaterium* (MNS1) **d** *B. subtilis* (001)

3.2.2 FTIR Spectra Analysis

The FTIR (Fourier Transform Infrared) spectrum indicates various functional groups present at different positions. FTIR spectra of AgNPs from *B. megaterium* (SIV01), *B. subtilis* (SIV02), *B. megaterium* (MNS1), *B. subtilis* (001) and *B. licheniformis* (P-2) are shown in the Fig. 2. The characteristic band at 1627.92 cm^{-1} is attributed to alkyl C=C stretching. Bands at 1203.58, 1145.72 and 1111.00 cm^{-1} indicate aromatic C–H in plane bend. Bands at 1878.67, 1851.66, 1816.94 and 1724.36 cm^{-1} indicate aromatic combination bands. Bands at 3375.43, 3352.28 and 3267.4 cm^{-1} represents hydroxyl group, H-bonded OH stretch. Band at 3086.11 cm^{-1} points aromatic C–H stretching Tolaymat (2010), Rashmi (2013). The characteristic band at 3352.28 cm^{-1} represents aliphatic secondary amine NH stretching.

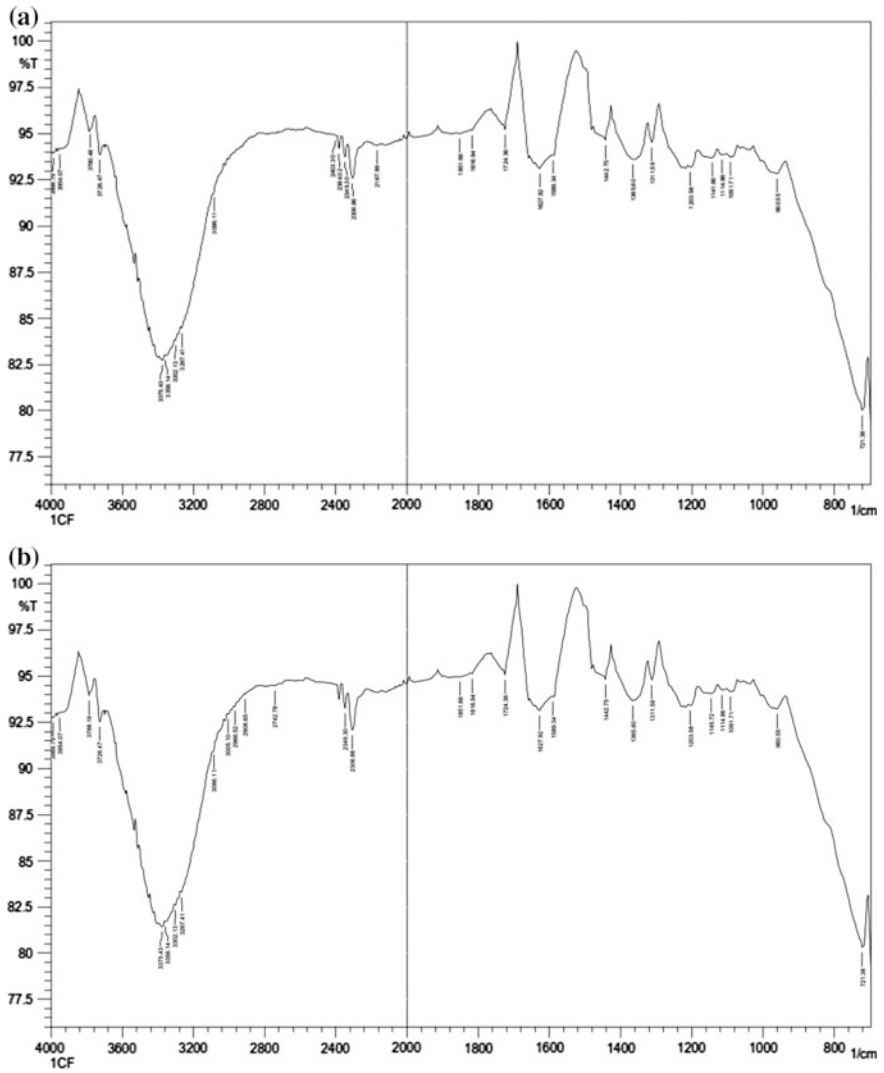

Fig. 2 FTIR spectrum of silver nanoparticles synthesized by **a** *B. megaterium* (SIV01) **b** *B. subtilis* (SIV02) **c** *B. megaterium* (MNS1) **d** *B. subtilis* (001) **e** *B. licheniformis* (P-2)

3.2.3 SEM Analysis

Scanning electron microscopy (SEM) image shows the morphological character of the synthesized silver nanoparticles to be spherical. The SEM analysis showed the particle size between 50 and 100 nm which was shown in the Fig. 3.

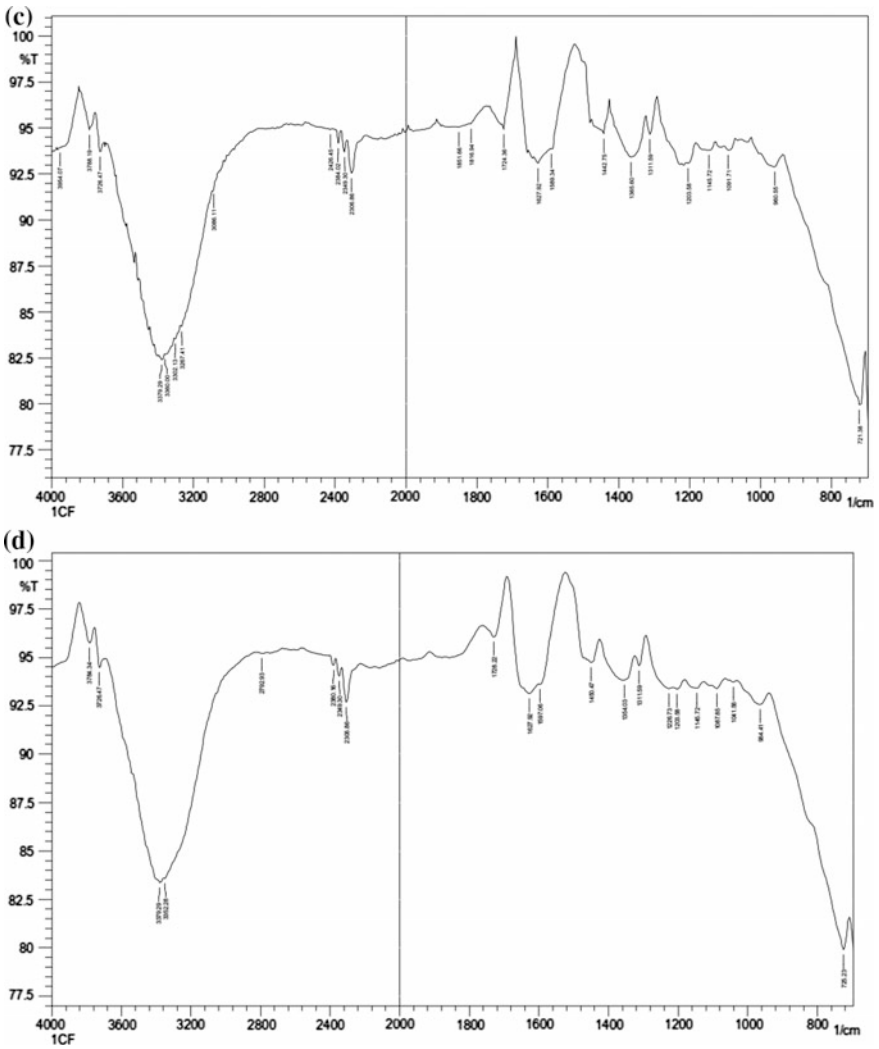

Fig. 2 (continued)

3.3 Decolourization Studies

A significant decolourization rate was observed for the Congo red dye using the synthesized silver nanoparticle solution which was shown in the Fig. 4. The values of Langmuir and Freundlich constants are calculated. The adsorption capacity was found to be high for 5 mL of AgNPs synthesized using *B.megaterium* (SIV01) and *B.subtilis* (SIV02), 2.5 mL of AgNPs synthesized using *B.subtilis* (001) and *B.megaterium* (MNS1) and 7.5 mL of AgNPs synthesized using *B.licheniformis*

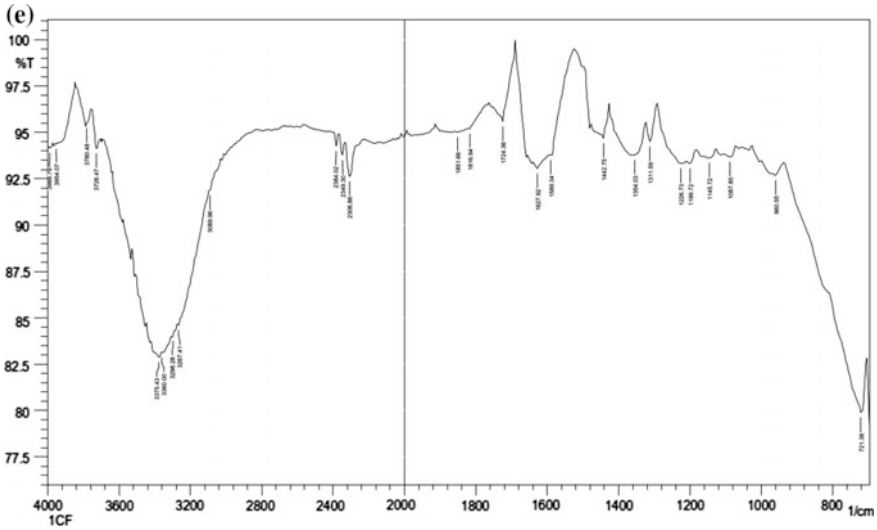

Fig. 2 (continued)

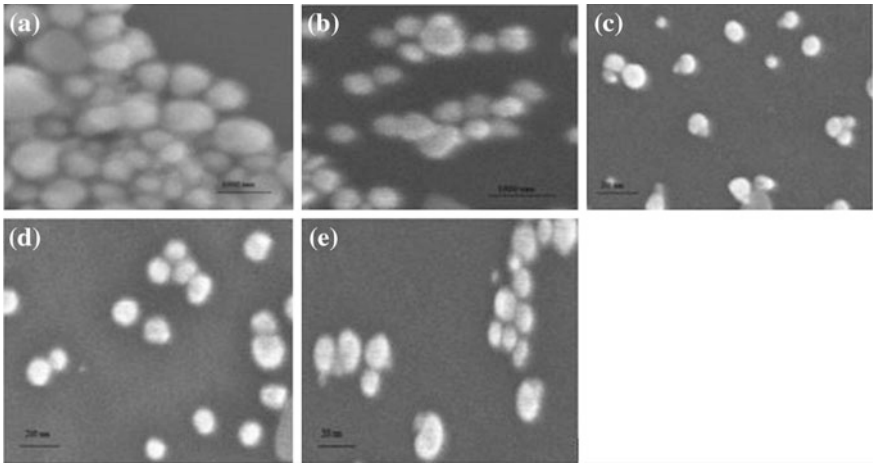

Fig. 3 SEM image of AgNPs synthesized using **a** *B. megaterium* (S1V01) **b** *B. subtilis* (S1V02) **c** *B. megaterium* (MNS1) **d** *B. subtilis* (001) **e** *B. licheniformis* (P-2)

(P-2) as it showed high Langmuir and Freundlich isotherm (Rajeshkannan et al, 2011) constants. High values of Langmuir (Q_0 and b) and Freundlich (K and n) constants indicate high adsorption capacity. Langmuir and Freundlich isotherm constants for dye decolourization was shown in the Table 1.

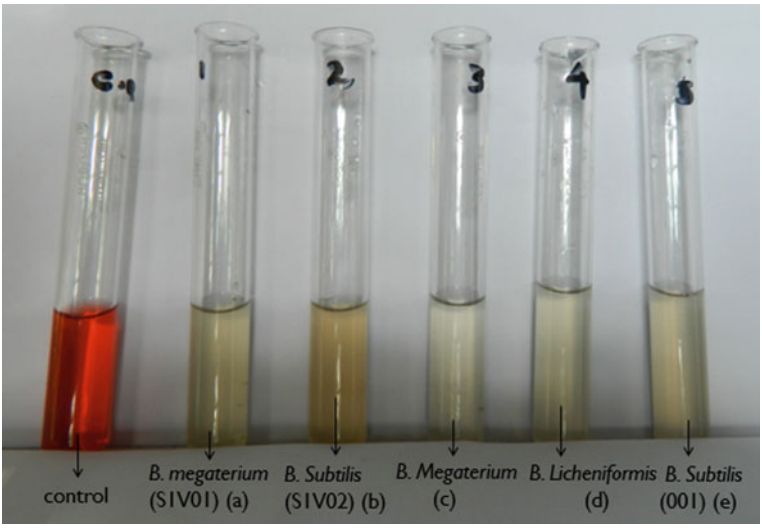

Fig. 4 Decolourization efficiency of different AgNPS synthesized by **a** *B. megaterium* (S1V01) **b** B. subtilis (S1V02) **c** B. megaterium (MNS1) **d** B. licheniformis (P-2) **e** B. subtilis (001)

Table 1 Langmuir isotherm and Freundlich isotherm constants

Silver nanoparticles (Source microorganisms)	Volume of AgNPs in ml	Langmuir constant		Freundlich constant	
		Q_0	B	K	N
B. megaterium (SIV01)	2.5	2	1.14	0.96	1.87
	5	3.33	5.25	0.96	3.33
	7.5	1.42	2.34	0.96	2
B. subtilis (SIV02)	2.5	1.25	1	0.82	2.67
	5	2	4	0.95	5
	7.5	1.67	2.39	0.92	3.4
B. megaterium (MNS1)	2.5	1.25	15	0.98	6
	5	1.11	3.91	0.98	5
	7.5	1	8	0.98	5.75
B. subtilis (001)	2.5	3.33	4.8	0.98	7.25
	5	1	0.5	0.96	2.2
	7.5	1.25	3.52	0.72	2
B. licheniformis (P-2)	2.5	2	0.27	0.7	1.11
	5	1.25	0.08	0.67	0.875
	7.5	5	0.41	0.763	1.28

3.4 Antibacterial Activity

Silver Nanoparticles have shown significant antibacterial activity against *B. megaterium, B. subtilis, B. licheniformis* and *E. coli*. The diameter of inhibition zones around each well with AgNPs was found to be 20, 42, 33, 28, 26, 34, 18, 21 mm, respectively for *B. subtilis*, *B. megaterium*, *B. licheniformis* and *E.coli* which was represented in the Fig. 5. It is interesting to note that the microorganisms involved in the synthesis of silver nanoparticles were also affected by its antibacterial activity Ibrahim (2015), Lakshmipriya (2013, 2015).

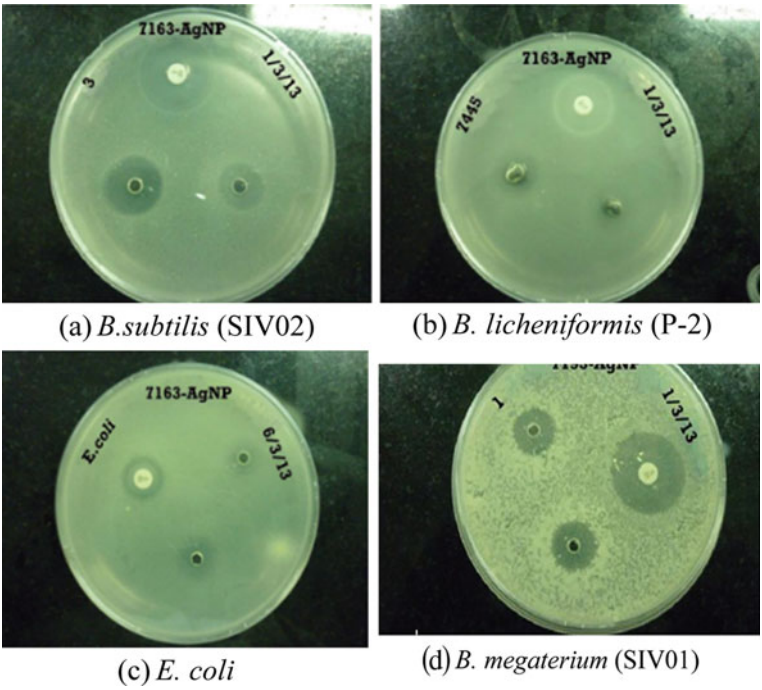

Fig. 5 Anti bacterial activity of silver nanoparticles synthesized using **a** *B. subtilis* (SIV02) **b** *B. licheniformis* (P-2) **c** *E. coli* and **d** *B.megaterium*

4 Conclusion

A green route for synthesizing silver nanoparticles has been employed. Biological methods are used to synthesize silver nanoparticles without the use of any harsh, toxic and expensive chemical substances. In the present study, experiments were conducted for the synthesis of stable silver nanoparticles by the reduction of aqueous $AgNO_3$ solution using cultures of *B. megaterium* (SIV01), *B. subtilis* (SIV02), *B. megaterium* (MNS1), *B. subtilis* (001) and *B. licheniformis* (P-2). From UV-Vis Spectroscopy results, *Bacillus megaterium* (SIV01) was found as the most potential organism for the synthesis of AgNPs among the different strains used. Decolourization efficiency of silver nanoparticles was evaluated for Congo red dye. The adsorption capacity was found to be high for *B. megaterium* (SIV01) and *B. subtilis* (SIV02) followed by *B. megaterium* (MNS1). Synthesized silver nanoparticles showed significant antibacterial activity against *E. coli*. Interestingly, synthesized silver nanoparticles showed antibacterial activity against source organism, where the hidden mystery still had to be unravelled.

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Long Term Impact of Irrigation with Textile Waste Water and an Ecofriendly Approach for Heavy Metal Degradation

M.N. Abubacker and B. Kirthiga

Abstract The major environmental pollution encountered by the textile effluent is the presence of color, dissolved solids and refractory materials such as detergents, organic pollutants, heavy metal ions which might arise from dyes. Discharge of the textile effluent to the nearby water body alters the physical, chemical and biological nature of the water bodies, seep into the aquifer, decreases the oxygen level in water leading to death of the aquatic biota and biomagnification. The present study was developed to comprehend the pollution caused by the local textile mill to the environment and their remediation. The physicochemical analyses of discharged effluent, adjacent soil and the cultivated crop near the discharge point at Tirupur, Tamil Nadu, India were analyzed and found to be altered. The organic matter and total available nitrogen content in the soil was found to be reduced, the high concentration of heavy metal in the seeds of *Pennisetum typhoides* characterized with SEM–EDX indicates soil lost its fertility. The 16S rRNA sequence of the predominant bacterial isolates exhibiting maximum resistant level towards heavy metal was deposited in NCBI Genebank and designated as *Bacillus cereus*, *Pseudomonas putida*. Further, the efficacy of the immobilized strains with *Luffa aegyptiaca* to accumulate/uptake heavy metals (Cr and Zn) under optimized conditions and their subsequent toxicity assessment in response to waste water irrigation were analyzed.

Keywords Textile effluent · Ecofriendly approach · Heavy metal · Degradation

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1 Introduction

The rapid economic growth achieved after globalization has adversely affected the quality of the environment and has become a major threat to sustainable development (World commission on environment and development 1987). The pollution load discharged into the environment has exceeded the assimilative capacity and affects sectors like agriculture, domestic water supply, fisheries, public health and biodiversity. Among the entire, industrial processing units textile processing units use huge quantities of water, the effluents discharged by these units are generally hot alkaline, strong smelling, coloured with large amount of toxic substances and they accumulated into the soil. Most of the small scale industries practice traditional processing technology is not treating their effluents properly (Nelliyat 2007). The use of metals and chemicals in dyeing industries generates large quantities of liquid effluent loaded with high levels of heavy metals, often as bioavailable forms (Calderon et al. 2003). For these reasons, interest has arisen recently in the investigation of some alternative methods involving only ecofriendly materials, that leads to metal recovery with minimal impact on the environment and are cost effective. Potentially bioremediation is cheaper than the chemical and physical methods and can deal with even lower concentration of contaminants more effectively although the process may take longer. Microbial populations in metal polluted environments adapt to toxic concentrations and become metal resistant. Different species of *Aspergillus*, *Pseudomonas*, *Bacillus* have been reported as efficient metal reducers and their detoxifying ability can be manipulated (Rajbanshi 2008; World commission on environment and development 1987). This biological process for removal of metal ions from solution can be divided into biosorption and bioaccumulation. Bioaccumulation is the uptake of metal by species means of metabolism dependent processes which may involve both transport into cell and partitioning into intracellular components.

2 Materials and Methods

In 2011, water, soil and plant (*Pennisetum typhoides*) samples from an irrigation field receiving discharge from small scale textile industries of Tirupur, located on the banks of the Noyyal River were collected (Fig. 1). A portion of the effluent and soil samples were acidified to pH $\leq$ 2 with Conc. HNO_3, refrigerated to prevent the volume change due to evaporation and the concentration of various heavy metal present in all the samples. Crops were washed, air dried, crushed and ground to a powder using mortar and pestle for metal analysis. Soil samples from the rhizosphere region to recover the *Arbuscular mycorrizal* spores. The samples were processed in 24 h for isolation, enumeration, identification and characterization of microbial flora.

Fig. 1 Sample collection area

2.1 Physico-chemical Characterization of Effluent, Soil and Crop

The physico-chemical parameters like color, odour, pH, temperature, TS, TSS, TDS, Chlorides, BOD and COD of the effluent, soil texture (Bouyoucos 1962),

water holding capacity (Rayment and Higginson 1992), electrical conductivity, cation exchange capacity, organic carbon, free calcium carbonate, organic matter, nitrogen content, bioavailable metal ions (Atomic Absorption Spectrophotometer), sulphide (acidified ammonium acetate extract) whereas bio available potassium, nitrate and phosphorus (Spectronic 20 flame photometer) were analyzed according to standard methods (APHA 2005). Rhizosphere was analyzed for the number of AM spores per 50 g by wet sieving and decanting technique using a series of sieves (50, 75, 100 µm mesh size). Spores were counted on filter paper under a dissection microscope and grouped according to their morphological characteristics (Abubacker et al. 2014).

Detection of metal concentration: The effluent (Babyshakila 2009), soil (Banytan et al. 2000) and the crop (Patel et al. 2004)were digested and analyzed using hollow cathode lamps for chromium (Cr), cadmium (Cd), lead (Pb), zinc (Zn) and nickel (Ni) at 357.9, 228.8, 217.0, 213.9 and 232.0 nm respectively at Sophisticated Analytical Instrument Facility (SAIF), in Indian Institute of Technology Madras (IITM), Chennai using Inductively Coupled Plasma Atomic Emission Spectroscopy.

2.2 *Characterization of* Pennisetum typhoides *Seeds Elemental Analysis*

Elemental analysis and Scanning Electron Microscopic images coupled with energy dispersive Xray consisting 3.5 and 2.5 nm resolution for tungsten filament and LaB6 and EDX detector resolution 133 eV of *Pennisetum typhoides* seeds was assessed (Make: SEM—TESCON—Czechoslovakia). The seeds were dried in hot air oven at 50 °C for 1 h and then powdered using mortar and pestle. The powdered material was placed in steel stab with carbon tape and sputter coated with gold particle for 50 s in high vacuum conditions (Vinod and Sasidhar 2010).

2.3 *Enumeration, Isolation and Identification of Bacteria*

The soil and effluent samples were serially diluted and plates yielding 30–300 colonies were selected for enumeration. Further, isolation and identification was carried by following standard procedures (Cappuccino and Sherman 2002) and numbered as TEAK 01–TEAK 13 and identified at generic level using the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Holt et al. 1994). Upon the percentage of distribution, the predominant isolates from soil and effluent sample were chosen and characterized. CTAB method was used to extract large quantities of heavy molecular weight DNA from bacteria. The pellet obtained

was washed with 70% ethanol, dried and dissolved in 50 µl TE buffer with pH 7 or water (Prabha et al. 2013).

For sequencing of 16S rRNA gene region of the selected bacterial isolates, standard PCR amplification was carried out using Ready-To-Go PCR Beads (Sigma Aldrich) containing 5 µl of 10X MTP Taqbuffer, 1 µl of 10 Mm dNTP mix, 0.5 µM of each primer (5′ AACGGCTCACCAAGGCGACG 3′and 5′ GTACCGTCAAGGTGCCGCCC 3′), 0.05 µl of MTP Taq DNA polymerase and 10 ng of template DNA in a 50 µl of reaction mixture in a thermal cycler. The cycling profile included an initial denaturation of 94 °C for 1 min followed by 30 cycles of denaturation (94 °C for 1 min), annealing (5 °C for 60 s) and Extension (72 °C for 65 s) and cooled at 4 °C. 10 µl of the amplified products were subjected to electrophoresis, visualized under UV transilluminator. The amplified PCR products were purified using QIA quick PCR purification kit (Qiagen GmbH, Hilden, Germany) as recommended by the manufacturer. The DNA sequences were run on an ABI 310 automated sequencer using the chain-termination method with big-dye terminators (Applied Biosystems, Foster, CA, USA). Automated base calls were checked by manual inspection of the electrophorogram of both forward and reverse sequences. The base call conflicts were resolved by alignment and comparison of both strands using the Seq Scape® software v 2.5 (Applied Biosystems, Foster, CA, USA) and blasted in the GenBank database (www.ncbi.nlm.nih.gov) for similarity search.

The Phylogenetic tree was constructed using the BLAST tree tool to demonstrate the evolutionary relationship between species, a group of genes related through a process of divergent evolution from a common ancestor or the result of convergent evolution was demonstrated.

2.4 *Maximum Resistance Levels (MRL) of Bacterial Isolates Against Cr and Zn*

MRL of the purified and characterized bacterial isolates against the HM was determined by preparing nutrient agar medium amended with varying concentrations (mg/L) of the heavy metals Zn (100–3600) and Cr (25–3000). The surface of the heavy metal incorporated agar plates were streaked with pure isolates and incubated at 37 °C for 48–72 h. The highest concentration of heavy metal that allows growth after 48 h of incubation was designated as MRL (Rajbanshi 2008). Textile effluent *Bacillus* sp. MTCC 5342 and *Pseudomonas* sp. MTCC 2847 were used as positive control.

2.5 Bioaccumulation Potential of Bacterial Isolates

A stock metal solution of (4000 mg L^{-1}) Zn and Cr was prepared by dissolving AR grade salts of $ZnSO_4$. $7H_2O$ and $K_2Cr_2O_7$ in double distilled water. The working concentration (100, 500, 1000 mg L^{-1}) were prepared from the stock metal solution.

2.5.1 Optimization of the Accumulation Process in Bacteria

Investigation of process dependency on pH: 2% inoculum concentration of bacterial isolates (2 days old cultures) were inoculated separately into 5 sets of 250 ml Ehrlimayar's flask contained 100 ml of minimal salt medium containing 100 mg L^{-1} of chromium or 100 mg L^{-1} of zinc. The pH varied from 6.5 to 8.5 (6.5, 7.0, 7.5, 8.0 and 8.5). The pH of the medium was adjusted using dilute HCL or NaOH and incubated on a rotary shaker in 150 rpm speed at 37 °C with control containing cells inoculated medium without heavy metal. Every 6 h interval, the residual metal concentration was analyzed. Cells were harvested by centrifugation at the end of 48 h incubation. The biomass was weighed, digested and analyzed for accumulation of chromium and zinc ions (Congeevaram et al. 2007).

Dependency of the metal concentration for nourishment: Minimal salt medium (pH 7.5) containing varying concentrations of metal ions ranging from 100 to 3000 mg L^{-1} was incubated with 2% inoculum at 37 °C. Every 6 h interval, a flask of varying metal concentration was analyzed for the residual metal concentration. At the end of 48 h, the cells were harvested and the biomasses were weighed and the concentrations of heavy metals were analyzed (Congeevaram et al. 2007).

After centrifugation, the filtrate and the pellet were digested with conc. HNO_3 in slow boiling, evident by brownish fume (Babyshakila 2009). The concentration of chromium was determined using diphenyl carbazide assay method and concentration of zinc was analyzed using inductively Coupled Plasma Atomic Emission Spectroscopy as described earlier.

Compatibility analysis of the Screened, adapted Bacterial strains: The Compatibility among the isolates, i.e. the antagonistic effect of bacterial isolate on the other bacterial strain was studied by standard well-cut method. Nutrient agar was prepared and poured into sterilized petri dishes and allowed to solidify. Two wells of 6 mm diameter were punctured at equidistant positions adjacent to one another in 9 cm petri dish containing the culture media using sterile cork borers. Hundred µl of each bacterial suspension was added to the wells and the plates were incubated at 37 °C. The diameter of the zone was measured in mm (Chin et al. 2001).

2.6 Bioremoval of Cr (VI) and Zn (II) Ions from Waste Water Through Accumulation Process

Immobilization: An inexpensive matrix *Luffa aegyptiaca*, commonly known as bath sponge was used for immobilization of bacteria. The dry matrix was sliced into pieces, washed with distilled water and dried in oven at 105 °C. The dried matrices weighing approximately 0.1 g were inoculated with 1 ml of bacterial suspension in petri plates containing 20 ml of sterilized nutrient broth and incubated. The Immobilized material was removed after 48 h and tried for remediation studies (Iqbal and Edyvean 2005).

Bioaccumulation of Cr (VI) and Zn (II) from textile dye effluent waste water: 100 ml of filtered Textile effluent aliquots were taken in six 250 ml Erlenmeyer flasks and their initial chromium and zinc ion concentrations were assayed. To this effluent, growing cells of bacterial isolate TEAK 03 and TEAK 06, bacterial consortium immobilized on *Luffa aegyptiaca* were added separately, enriched with 0.01% glucose and incubated under the optimized condition respectively. After incubation, the immobilized bacterial cells were harvested and the aliquots were digested using 10 ml of triple acid solution (HNO_3, H_2SO_4 and $HClO_4$ in 9:2:1 proportion respectively) till the effluent becomes colorless (Ameer Basha and Rajaganesh 2014). Filtered and unfiltered effluent sample without inoculum were used as control. Later, the digested sample was analyzed for the residual metal ions Cr and Zn as per the standard methods.

2.7 Toxicity Assessment of Treated Waste Water—Bioaccumulation Process

The Physicochemical parameters like pH, EC, total suspended solids, total solids, total dissolved solids, chlorides, BOD, COD, metal concentration of the treated effluent was analyzed as per standard procedures (APHA 2005). Seeds of *Pennisetum typhoides* were surface sterilized with 95% ethanol and then treated with 0.2% mercuric chloride and washed three times with sterile distilled water and blot dried under aseptic conditions. Seeds for the study were obtained from the Institute of Agricultural Research, Tamil Nadu. The seeds were soaked in 40 ml of treated wastewater for 30 min and dried under aseptic condition for 4 h to assess the toxic effect of treated waste water where as the seeds soaked in distilled water and untreated wastewater was used as a control. A total of six seeds were placed in each petri plate containing a moist filter paper and the plates were maintained in a growth chamber for 7 days, the germination percentage was calculated (Prashanth and Mathivanan 2009). The concentration of chromium and zinc was analyzed by digesting the germinated seeds and the filtrate samples were analyzed for Cr and Zn concentration using Inductively Coupled Plasma Atomic Emission Spectroscopy (Patel et al. 2004).

3 Results and Discussion

3.1 *Physico-chemical Characterization of Effluent, Soil and Crop*

Various parameters *viz* colour, smell, pH, temperature, electrical conductivity, total dissolved solids, metal concentration of the collected effluent samples, soil samples and VAM spores were analyzed (Tables 1, 2, 3, 4, 5, 6, 7 and 8). The color of the effluents at the sites 1–6 are blackish green, greenish black, slight greenish black, slight greenish, slight greenish, slight greenish and colourless respectively. The offensive odour was found at site 1, pungent odour at site 2, 3 and effluent at site 4–6 were odourless. Because of the effluent pollution, the colour of the soils were dark brown at site 1, reddish brown at site 2–3, brown at site 4, light yellow and site 5 and site 6 remains colourless. There was pungent odour in site 1, fishy in site 2 and rests were muddy in odour. The site 1 is clay loam in texture, whereas in remaining sites were sandy.

The study clearly demonstrates that soil adjacent to the effluent discharge point experiences a change in physicochemical parameters from site to site, well above the CPCB limits (Asfaw 2014; Furaha et al. 2015; Mohabansi et al. 2011; Rohilla et al. 2012). The elevated metal concentrations may be due to high content of metal ions present in the dye and mordants (HRB 38, Nickle-phthalocyanine complex, HRV5 copper containing azo dye, zinc yellow pigment and chrome yellow), and discharge of unbounded metal ions into the waste water and thus direct and continuous exposure alters the physicochemical properties and accumulation of metal ions in the soil (Jayashree et al. 2011). Thus, waste water being used for crops cultivation results in built up toxic substances in the soils (Joshi and Santani 2012; Joshi et al. 2011; Mahawar and Akhtar 2015) and affects the fertility. Reduced agricultural production due to accumulation of heavy metals is concern of regional global scale (Muhammad et al. 2015). Long term irrigation with heavy metal contaminated soil, accumulation of heavy metal in the root and shoot system of the plant (Jayashree et al. 2011).

Significant variation in AMF spore density was observed in all the sites (Table 9). Over all 22 different AMF species were isolated from the rhizosphere of *Pennisetum typhoides,* commonly cultivated near the contaminated sites. *Acaulospora elegans*, *Acaulospora morrowiae*, *Sclerocystis rubiformis* were not present in the contaminated soil (Fig. 2).

Thus, the results conclude plants grown well with heavy metal contaminated soil on long term irrigation reduces the AM spore population and changes its diversity. The symbiotic association formed by the mycorrhiza fungi in the roots of plant responsible for nutrient transfer is affected in the polluted site (Abubacker et al. 2014).

Table 1 Physico-chemical characterization of effluent

S. No.	Parameters	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Reference	CPCB limit (APHA 2005)
1	pH	11.3 ± 0.2	10.4 ± 0.1	9.7 ± 0.2	8.3 ± 0.1	7.9 ± 0.2	7.6 ± 0.2	6.8 ± 0.1	6.0–9.0
2	Temperature (°C)	32 ± 1.0	32 ± 1.0	30 ± 1.0	29 ± 1.0	30 ± 1.0	30 ± 1.0	20 ± 1.0	28–30
3	Total solids (mg/L)	4432 ± 15.0	4168.6 ± 13.4	3868.8 ± 16.9	3347.6 ± 14.1	2744.8 ± 22.9	2238.4 ± 23.5	1986.3 ± 18.0	NA
4	Total suspended solids (mg/L)	283 ± 5.0	211.6 ± 3.4	179.8 ± 6.9	158.6 ± 4.1	127.8 ± 2.9	100.4 ± 3.4	92.3 ± 2.8	100
5	Total dissolved solids (mg/L)	4194 ± 0.0	3957 ± 10.0	3689.0 ± 10.0	3189 ± 10.0	2617 ± 20.1	2138 ± 20.1	1894 ± 10.0	500–2000
6	Chlorides (mg/L)	1134.2 ± 8.0	1094.1 ± 7.0	1003 ± 6.0	989.6 ± 7.2	956.3 ± 5.6	934.8 ± 4.9	928.5 ± 5.6	250–1000
7	BOD (mg/L)	108 ± 10.1	98 ± 20.0	86 ± 20.0	72 ± 10.0	64 ± 20.0	41 ± 10.0	20 ± 10.0	30
8	COD (mg/L)	997 ± 30.1	864 ± 10.1	791 ± 20.1	698.4 ± 10.1	564.3 ± 20.1	498.6 ± 30.1	184 ± 10.1	250

*NA—Not Applicable

Table 2 Concentration of metal ions present in the effluent

S. No.	Metal (mg/L)	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Reference	WHO std (mg/L)	Permissible limit (mg/L)
1	Cr	24.5 ± 0.37	19.6 ± 0.42	14.8 ± 0.11	11.2 ± 0.48	9.41 ± 0.89	7.32 ± 0.03	0.03 ± 0.01	0.05 ± 0.01	0.05
2	Cu	17.7 ± 0.91	13.2 ± 0.16	9.75 ± 0.54	6.49 ± 0.21	4.63 ± 0.21	2.47 ± 0.94	0.72 ± 0.02	1 ± 0.02	0.5
3	Zn	36.1 ± 1.27	31.2 ± 1.01	26.3 ± 2.1	19.8 ± 2.35	15.6 ± 2.36	11.2 ± 0.62	3.97 ± 0.97	5 ± 1.2	0.2
4	Ni	0.78 ± 0.54	0.61 ± 0.01	0.53 ± 0.02	0.41 ± 0.01	0.29 ± 0.02	0.17 ± 0.01	0.06 ± 0.01	0.01 ± 0.00	–
5	Cd	7.28 ± 0.42	6.08 ± 0.02	4.94 ± 0.01	3.96 ± 0.03	2.21 ± 0.01	1.08 ± 0.03	0.02 ± 0.00	0.05 ± 0.23	0.01
6	Pb	1.79 ± 0.13	0.99 ± 0.85	0.29 ± 0.01	0.07 ± 0.01	ND	ND	0.74 ± 0.01	0.07 ± 0.01	0.065

*ND—Not Detected

Table 3 Exchangeable form of metal ions in effluent sample

S. No.	Metal	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
1	Cr	19.32 ± 0.41	16.36 ± 0.21	11.29 ± 0.42	09.49 ± 0.36	8.94 ± 0.81	7.58 ± 0.01
2	Cu	14.84 ± 0.87	11.24 ± 0.20	8.54 ± 0.19	5.68 ± 0.21	4.12 ± 0.21	1.34 ± 0.26
3	Zn	30.42 ± 1.42	28.62 ± 1.31	23.45 ± 2.2	18.81 ± 2.41	13.00 ± 2.01	9.88 ± 0.81
4	Ni	0.57 ± 0.54	0.41 ± 0.02	0.37 ± 0.01	0.28 ± 0.02	0.09 ± 0.01	0.04 ± 0.01
5	Cd	6.13 ± 0.62	5.13 ± 0.03	3.27 ± 0.02	2.54 ± 0.01	1.98 ± 0.02	0.97 ± 0.02
6	Pb	0.99 ± 0.13	0.69 ± 0.12	0.54 ± 0.02	0.32 ± 0.02	ND	ND

*ND—Not Detected

Table 4 Physico-chemical characterization of effluent discharged soil

S. No.	Parameters soil	Site 1	Site 2	Site3	Site4	Site 5	Site 6	Reference site	Permissible limit (CPCB)
1	pH	11.24 ± 0.05	10.31 ± 0.01	9.87 ± 0.02	9.68 ± 0.07	8.9 ± 0.03	8.4 ± 0.04	7.61 ± 0.02	7–8.5
2	EC (μS/cm)	63.8 ± 3.0	61.7 ± 2.1	59.36 ± 2.9	57.7 ± 3.0	54.28 ± 1.0	51.68 ± 2.0	46.7 ± 2.0	0–1.5
3	Holding capacity (%)	42.94 ± 2.0	51.26 ± 2.0	59.26 ± 2.0	64.98 ± 1.0	70.25 ± 1.0	74.26 ± 2.0	80.21 ± 1.0	NA
4	Organic carbon (%)	1.71 ± 0.1	1.6 ± 0.2	1.2 ± 0.1	0.91 ± 0.2	0.93 ± 0.1	0.89 ± 0.2	0.71 ± 0.2	0.5–0.75
5	Free $CaCO_3$ (%)	8.700 ± 0.12	8.13 ± 0.22	7.56 ± 0.24	6.87 ± 0.14	6.26 ± 0.11	5.12 ± 0.23	2.89 ± 0.31	NA
6	Organic matter (%)	0.41 ± 0.01	0.49 ± 0.01	0.54 ± 0.01	0.63 ± 0.02	0.69 ± 0.01	0.74 ± 0.03	0.91 ± 0.02	0.8–1.30
7	Nitrogen content (%)	0.19 ± 0.009	0.11 ± 0.001	0.09 ± 0.001	0.09 ± 0.001	0.08 ± 0.001	0.07 ± 0.001	0.097 ± 0.006	0.043–0.065
8	Potassium (mg/Kg)	589.3 ± 1.3	574.03 ± 1.0	556.09 ± 2.0	543.02 ± 3.0	517 ± 3.0	503 ± 1.0	494.06 ± 1.0	NA
9	Nitrate (mg/Kg)	0.91 ± 0.03	0.83 ± 0.01	0.76 ± 0.02	0.67 ± 0.01	0.54 ± 0.01	0.42 ± 0.02	0.30 ± 0.02	NA
10	Phosphorus (mg/Kg)	19.34 ± 0.24	17.26 ± 0.34	15.41 ± 0.01	11.28 ± 0.12	10.28 ± 0.04	9.24 ± 0.17	7.26 ± 0.07	NA
11	Sulphate (mg/Kg)	29.7 ± 3.0	26.1 ± 2.0	21. 4 ± 2.0	19.5 ± 2.1	17.3 ± 3.0	15.1 ± 2.0	12.18 ± 1.0	200
12	Calcium (mg/Kg)	309 ± 30.0	301 ± 10.0	294 ± 9.0	281 ± 08.0	269 ± 10.0	257 ± 10.0	149 ± 11.0	200
13	Magnesium (mg/Kg)	0.97 ± 0.02	0.86 ± 0.03	0.079 ± 0.01	0.071 ± 0.02	0.065 ± 0.04	0.061 ± 0.01	0.56 ± 0.03	NA
14	Sodium (mg/Kg)	41.38 ± 1.0	57.02 ± 3.0	61.08 ± 2.0	67.04 ± 3.0	71.14 ± 1.0	84.03 ± 2.0	118.07 ± 2.0	200

*NA—Not Applicable

Table 5 Concentration of metal ions present in the soil

S. No.	Metal (mg Kg^{-1})	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Reference site	WHO standard (mg Kg^{-1})
1	Cr	29.89 ± 1.23	21.86 ± 0.77	18.61 ± 0.94	13.47 ± 0.03	11.21 ± 0.55	9.45 ± 0.43	0.03 ± 0.03	0.05
2	Cu	21.55 ± 0.57	19.27 ± 0.27	12.43 ± 0.31	9.51 ± 0.27	7.61 ± 0.21	4.97 ± 0.25	0.98 ± 0.12	1
3	Zn	47.83 ± 3.14	41.32 ± 1.98	33.28 ± 2.40	24.78 ± 1.69	20.03 ± 1.87	14.98 ± 2.11	4.52 ± 1.01	5
4	Ni	1.27 ± 0.97	0.99 ± 0.03	0.78 ± 0.01	0.61 ± 0.02	0.43 ± 0.01	0.31 ± 0.02	0.24 ± 0.01	–
5	Cd	9.97 ± 0.69	8.77 ± 0.21	6.28 ± 0.31	5.89 ± 0.71	4.37 ± 0.42	2.17 ± 0.22	0.91 ± 0.02	0.05
6	Pb	2.31 ± 0.72	1.87 ± 0.51	0.97 ± 0.24	0.77 ± 0.14	0.58 ± 0.05	0.27 ± 0.04	0.84 ± 0.03	0.1

Table 6 Exchangeable form of metal ions in soil sample

S. No.	Metal (mg Kg^{-1})	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
1	Cr	26.23 ± 1.54	19.87 ± 0.81	15.28 ± 0.23	10.68 ± 0.01	8.68 ± 0.21	7.42 ± 0.49
2	Cu	19.23 ± 0.57	16.73 ± 0.15	11.84 ± 0.26	7.88 ± 0.32	5.96 ± 0.33	3.01 ± 0.07
3	Zn	40.08 ± 2.98	36.41 ± 0.21	29.62 ± 0.31	21.02 ± 1.02	16.47 ± 1.42	09.27 ± 3.84
4	Ni	0.94 ± 0.02	0.68 ± 0.01	0.54 ± 0.02	0.31 ± 0.02	0.15 ± 0.02	0.09 ± 0.03
5	Cd	8.41 ± 0.21	6.95 ± 0.34	5.87 ± 0.42	3.94 ± 0.81	2.88 ± 0.62	1.42 ± 0.31
6	Pb	1.23 ± 0.11	0.85 ± 0.62	0.43 ± 0.26	0.26 ± 0.21	0.09 ± 0.01	0.07 ± 0.01

Table 7 Concentration of metal ions present in the plant

Crop cultivated	Plant part	Irrigated near to effluent discharge point		Irrigated 1 km awy from discharge point		Reference	
		Cr	Zn	Cr	Zn	Cr	Zn
Pennisetum typhoides	Root	0.73 ± 0.02	6.97 ± 0.83	0.59 ± 0.08	5.61 ± 0.51	0.001 ± 0.00	1.03 ± 0.01
	Seed	0.49 ± 0.04	4.87 ± 0.06	0.37 ± 0.02	3.46 ± 0.05	ND	0.04 ± 0.01

*ND—Not Detected

Table 8 AMF Spore density associated with plantation near the effluent discharge point (Total No. of spores/100 g of soil)

S. No.	AMF Spores	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Reference
1	*Acaulospora elegans*	ND	ND	ND	ND	02	09	18
2	*Acaulospora morrowiae*	ND	ND	ND	ND	ND	11	29
3	*Glomus amphisporum*	96	101	117	139	146	154	194
4	*Glomus albidium*	29	37	49	56	63	74	91
5	*G. citricolon*	19	25	34	48	53	61	73
6	*G. claroideum*	ND	ND	ND	ND	ND	ND	19
7	*G. deserticola*	ND	ND	ND	ND	ND	ND	61
8	*G. dimorphicum*	ND	ND	ND	ND	ND	ND	29
9	*G. etunicatum*	ND	ND	ND	ND	ND	ND	34
10	*G. geosporum*	17	29	35	47	61	73	79
11	*G. macrocarpum*	ND	ND	ND	ND	ND	ND	52
12	*G. melanosporum*	29	42	51	68	91	86	113
13	*G. microcarpum*	18	31	48	67	74	83	96
14	*G. mossae*	62	87	93	114	128	153	263
15	*G. multisubstensum*	ND	ND	ND	ND	ND	ND	22
16	*G. occulatum*	ND	ND	ND	ND	ND	ND	39
17	*G. pulvinatum*	ND	ND	ND	ND	ND	ND	28
18	*G. radiatum*	ND	ND	ND	ND	ND	ND	35
19	*G. tortuosum*	ND	ND	ND	ND	ND	ND	33
20	*G. fulvum*	ND	ND	ND	ND	ND	ND	47
21	*Sclerocystis rubiformis*	ND	ND	ND	ND	ND	07	26

*ND—Not Detected

3.2 *Scanning Electron Microscopy—Energy Dispersive X-Ray (SEM–EDX) of* Pennisetum typhoides *Seeds Elemental Analysis*

SEM–EDX elemental analysis confirms the presence of Cr and Zn in the seeds of *Pennisetum typhoides* irrigated near the effluent discharge point and the concentration was found to be 0.22 and 1.30% and the seeds cultivated at 1 km distance from the discharge point showed decreased concentration (0.08 and 0.44%) whereas the control seeds does not show the occurrence of metals (Figs. 3, 4 and 5).

Table 9 Biochemical characterization of isolated bacterial stains from effluents

Test	ETEAK1	ETEAK2	ETEAK3	ETEAK4	ETEAK5	ETEAK6	ETEAK7	ETEAK8	ETEAK9	ETEAK10	ETEAK11	ETEK12	ETEAK13
Gram stain	+	+	−	−	+	+	−	+	+	+	−	+	+
Motility	+	+	+	−	+	+	+	+	−	−	−	+	−
Spore	+	−	−	−	−	+	−	−	+	−	−	−	+
Indole	−	−	−	−	−	−	−	−	−	−	+	−	−
MR	+	+	+	+	−	−	−	+	−	+	+	+	+
VP	+	+	+	+	−	+	+	+	−	+	+	+	+
Citrate	+	+	+	+	+	+	−	+	−	−	+	+	+
Catalase	+	+	+	+	+	+	+	−	+	+	−	−	+
Oxidase	+	+	−	−	−	+	+	−	+	+	−	+	+
TSI	∓	∓	+	∓	+	−	∓	∓	±	±	∓	−	+
H2S	−	−	−	+	−	−	−	−	−	−	−	−	−
Urease	−	−	−	+	−	−	−	−	+	−	+	−	−
Starch	+	−	+	−	−	−	−	−	+	−	+	−	+
Gelatin	−	−	+	−	−	+	+	−	−	+	±	−	−
Nitrate	+	−	+	+	−	+	+	+	−	+	+	−	+

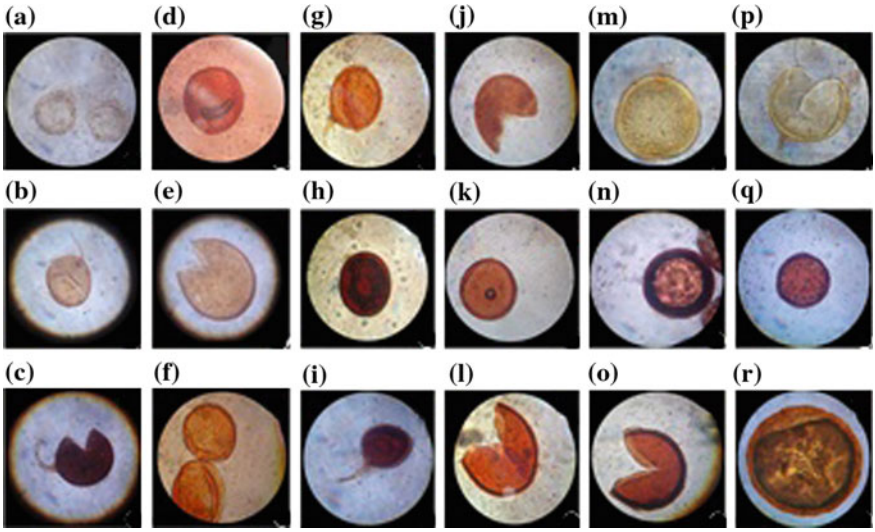

Fig. 2 AM spore (400X) identified in contaminated site. **a** *Sclerocystis rubiformis*; **b** *Acaulospora elegans*; **c** *Acaulospora morrowiae*; **d** *Glomus albidium*; **e** *G. citricolon*; **f** *G. claroideum*; **g** *G. deserticola*; **h** *G. dimorphicum*; **i** *G. geosporum*; **j** *G. occulatum;* **k** *G. melanosporum*; **l** *G. etunicatum*; **m** *G. mossae*; **n** *G. fulvum*; **o** *G. radiatum*; **p** *G. pulvinatum*; **q** *G. microcarpum* and **r** *G. macrocarpum*

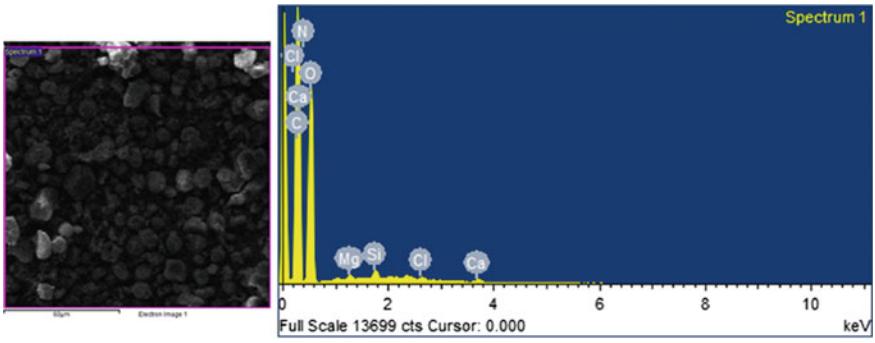

Fig. 3 SEM–EDX: Seeds of *Pennisetum typhoides* irrigated with borewell water (control)

3.3 Enumeration, Isolation and Identification of Bacteria

Bacterial load was calculated in all the sites (Soil and effluent). The soil sample contained more cfu/mg in site 1 to site 6 ($3.70 \pm 0.01 \times 10^6$ to $2.99 \pm 0.02 \times 10^6$) than the reference sample ($2.40 \pm 0.02 \times 10^5$). Similarly the effluent sample has more cfu/ml varied from $5.81 \pm 0.03 \times 10^3$ to $4.21 \pm 0.01 \times 10^3$ and the reference site showed $3.29 \pm 0.01 \times 10^3$. The colony of the bacterial isolates from effluent ETEAK 1–13 were round/irregular, undulate margin; circular; small

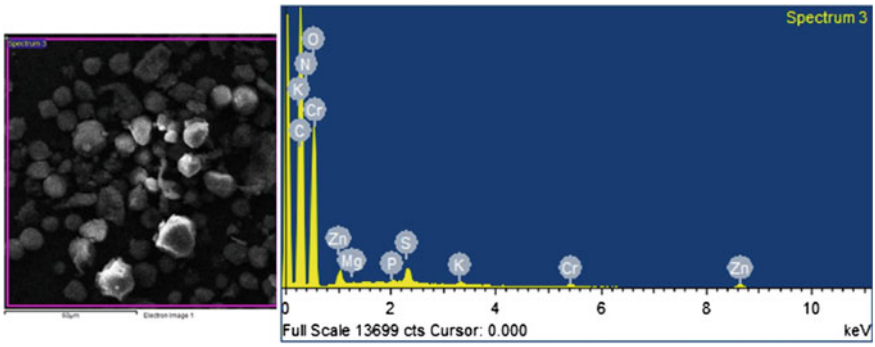

Fig. 4 SEM–EDX: Seeds of *Pennisetum typhoides* irrigated with effluent near discharge point

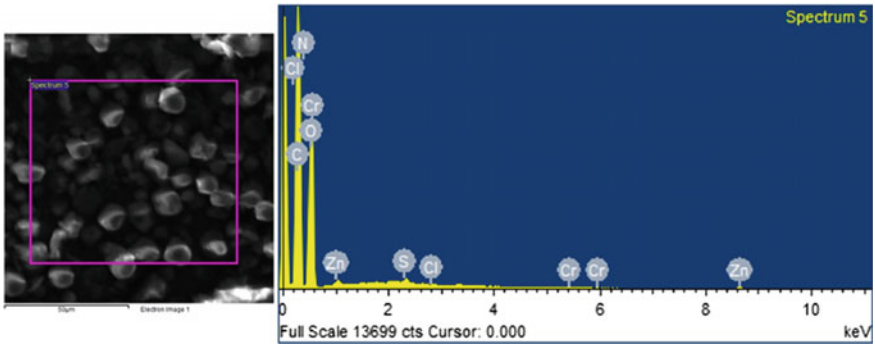

Fig. 5 SEM–EDX: Seeds of *Pennisetum typhoides* irrigation field 1 km away from discharge point

mucoid, entire umbonate; small circular; small circular flat, entire, powdery; large, irregular flat; circular convex, smooth entire; small, pinpoint convex; irregular round, medusa head; circular, pinhead, convex, entire; mucoid, umbonate elevation; circular, pinpoint, entire raised depressed center and circular, flat, undulated margin respectively. The ETEAK 1–13 were yellow; bright yellow non-diffusible pigment; red pigment; No pigment; light yellow green with black centre; smooth waxy whitish to cream mousy smell; tan to buff-color; grey color; golden-brown color; diffusible green pigment, fruity odor; white and cream colour, whereas the shapes were rods, diplo cocci, rods thin, diplorods, diplo rods, rods in chain, short rods, coccirods, diplo coccic, rods, diplo cocci and rods respectively. Based on the morphological and biochemical characteristics the isolates were identified as *Lactobacillus* sp., *Agrobacterium* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Alcaligenes* sp., *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., *Enterococcus* sp., *Aerococcus* sp. (Adesemoye et al. 2006; Malekzadeh et al. 2010; Das et al. 2010) (Table 9).

The colony morphology of soil bacterial isolates STEAK 1–13 isolated from soil contaminated by textile effluent were formed the colony and seemed as small, round, convex, entire; small, round, convex, entire; small, circular, convex, entire; small, circular elevated entire; small round, biconvex spreading edge; small circular, flat undulate margins; small irregular round, medusa head; small irregular, wrinkled, opaque; pinhead circular, convex, entire; pinhead circular, convex, entire; small, mucoid, umbonate elevation, entire; small, pinpoint convex entire and small, circular, convex, entire respectively. The colour and pigment of them were as follows off-white to cream, shiny; watery white cream no pigment; shiny wet, diffusible green fluorescent pigment; pale yellow white; greyish pigment; translucent, colorless, fruity smell no pigment; Diffusible cream no pigment; Smooth grayish pigment; slightly yellow colour, no pigment; bright yellow, non-diffusible pigment; Whiteno pigment; diffusible green pigment, fruity odor; Grey, no pigment and semi-transparent, white grey, surrounded by narrow zone of green color. The shape of the colony were rods, except for STEAK 5, 10, 12 (Diplococci), STEAK 9, 13 (Cocci). From the morphological and biochemical characteristics (Table 10), the genus semi-transparent, white grey, surrounded by narrow zone of green color were identified as *Lactobacillus* sp., *Agrobacterium* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Alcaligenes* sp., *Bacillus subtilis*, *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Enterococcus* sp. and *Aerococcus* sp. respectively.

Long term exposure to heavy metals microbial community adapts to the polluted environment. In the present investigation, bacterial isolates *Pseudomonas putida* STEAK 03, *Bacillus cereus* ETEAK 06 exhibited their survival and tolerance towards heavy metal polluted environment. Results imply that heavy metals promote the selection of resistant species and decrease the other microbial diversity (Ezzouhri et al. 2009; Hemamalini and Sneha 2014; Singh et al. 2013).

The soil isolates STEAK 03 (29%) and effluent isolate ETEAK 06 (21%) has the highest frequency of occurrence and they belong to *Pseudomonas* and *Bacillus* genus. The evolutionary history of *Pseudomoas putida* STEAK 03 *and Bacillus cereus* ETEAK 06 was inferred using the Neighbor-Joining method. The optimal tree is drawn to scale with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The 16s rRNA sequence (*Pseudomonas putida* STEAK 03 and *Bacillus cereus* ETEAK 06) determined in this study was deposited in NCBI Genbank under accession number KP731884 and KP731883 (Figs. 6 and 7).

3.4 Maximum Resistance Levels (MRL) of Bacterial Isolates Against Cr and Zn

Genotypically characterized *Pseudomonas putida* STEAK 03 and *Bacillus cereus* ETEAK 06 were assessed for their maximum tolerance level towards chromium

Table 10 Biochemical characterization of isolated bacterial stains from soil contaminated with textile effluent

Test	STEAK1	STEAK2	STEAK3	STEAK4	STEAK5	STEAK6	STEAK7	STEAK8	STEAK9	STEAK10	STEK11	STEAK12	STEAK13
Gram stain	+	−	−	−	−	+	+	+	+	+	−	+	+
Motility	−	−	+	−	−	−	−	−	−	−	−	+	−
Spore	−	−	−	−	−	−	+	+	−	−	−	−	−
Indole	−	−	−	−	−	−	−	−	−	−	+	+	−
MR	−	+	−	+	−	−	+	−	+	+	+	−	+
VP	−	+	−	+	−	+	+	+	+	+	+	+	+
Citrate	+	−	−	+	−	−	−	−	−	−	+	−	+
Catalase	+	+	+	+	+	+	+	+	+	+	−	−	−
Oxidase	+	+	+	−	+	+	−	+	+	+	−	+	−
TSI slant	∓	±	+	−	+	+	∓	∓	+	±	∓	+	±
H2S	−	−	−	−	−	−	−	−	±	−	−	−	−
Urease	−	+	−	−	−	−	−	−	+	−	+	−	−
Starch	−	−	−	−	−	+	+	−	+	−	+	−	−
Gelatin	−	−	−	−	−	−	−	+	+	+	±	+	−
Nitrate	+	+	−	−	−	−	+	−	+	+	+	+	−

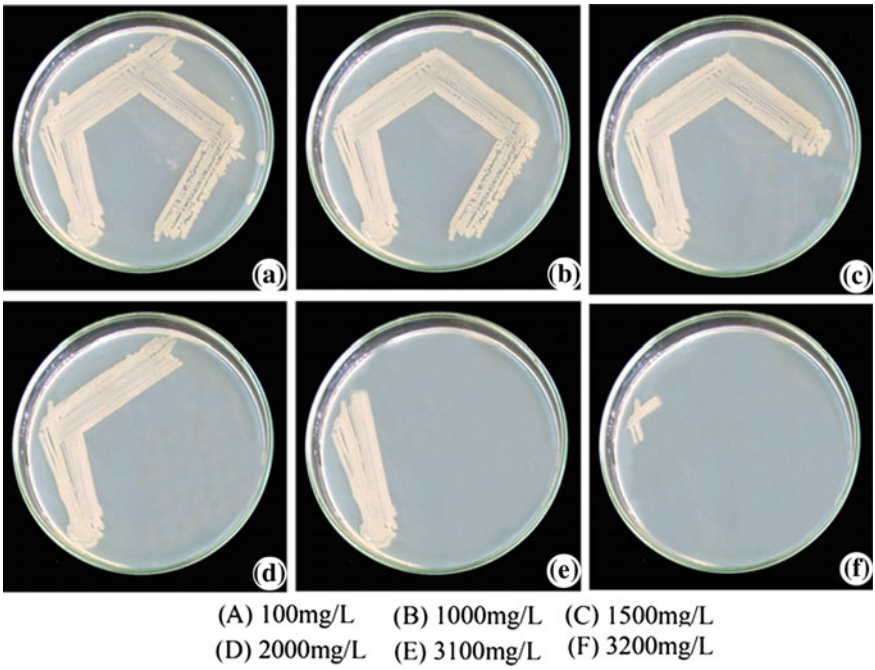

Fig. 6 MRL of *Becillus cereus* ETEAK 06 against zinc

and zinc. *Pseudomonas putida* STEAK 03 exhibited a greater tolerance level of 2,400 mg L^{-1} of Cr (VI) (Figs. 6 and 7). *Bacillus cereus* ETEAK 06 showed moderate growth in chromium concentration as high as 2100 mg L^{-1} with good growth at 1900 mg L^{-1}. For Zn, both the strains showed the highest degree of tolerance, 3200 and 3100 mg L^{-1}.

The time-course data for heavy metal removal and cellular growth were observed for each isolate under its optimal pH. The bacterial isolates removed Cr and Zn at pH 7.5. In the present study, the accumulation of bacterial isolates peaked at 2000 and 3000 mg L^{-1} of Cr and Zn and no growth at 3000 mg L^{-1} of Cr and lowered at 3000 mg L^{-1} of Zn. Thus, the viable cells used in shake flasks for removal of metal ions from aqueous solution offered several advantages including self replenishment, continuous metabolic uptake of metals after physical adsorption (Qazilbash 2004; Leung et al. 2000; Tam et al. 2001).

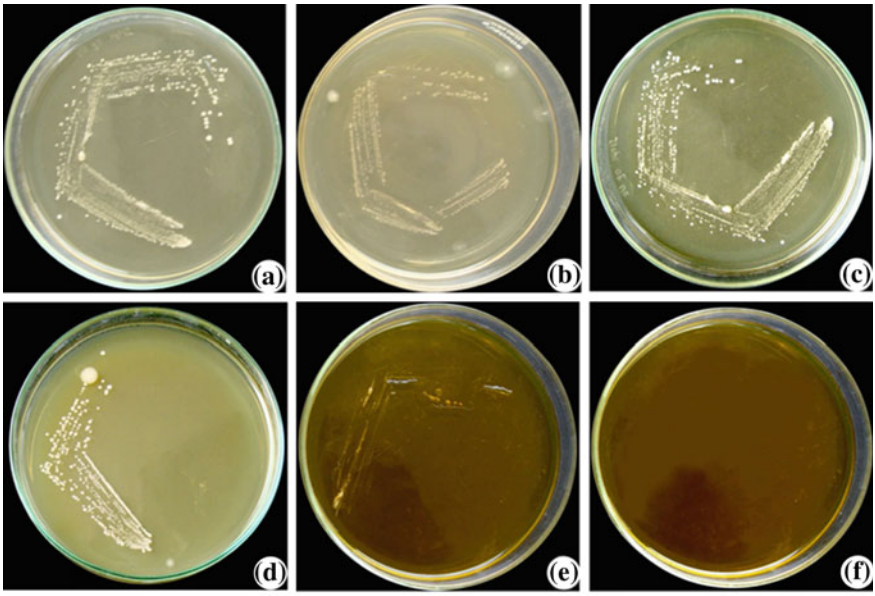

Fig. 7 MRL of *Pseudomonas putida* STEAK 03 against chromium

3.5 Bioaccumulation Potential of Bacterial Isolates

3.5.1 Optimization of the Accumulation Process in Bacteria

A series of experiments was designed to determine the tolerance/resistance levels of the *Pseudomonas putida* STEAK 03 and *Bacillus cereus* ETEAK 06 and indeed these strains remove Cr and Zn ions from the minimal salt media. Both the strains were cultured on minimal salt media with varying pH and varying metal concentration and observed for 2 days at the interval of 6 h (Figs. 8 and 9) represents the optimal pH and metal concentration.

The maximum uptake, of Cr and Zn by *Pseudomonas putida* STEAK 03 and *Bacillus cereus* ETEAK 06 was observed at pH 7.5 and complete accumulation was absorbed at 1000 mg L^{-1} with the maximum biomass ranges between (0.36–0.42 g) Cr and (0.49–0.51 g) Zn by *Pseudomonas puitda* STEAK 03 and *Bacillus cereus* ETEAK 06 respectively.

To build a consortium both bacterial isolates should be compatible, *Pseudomonas putida* STEAK 03 and *Bacillus cereus* ETEAK 06 was analyzed for their compatibility in the nutrient agar medium and the isolates were found to be compatible.

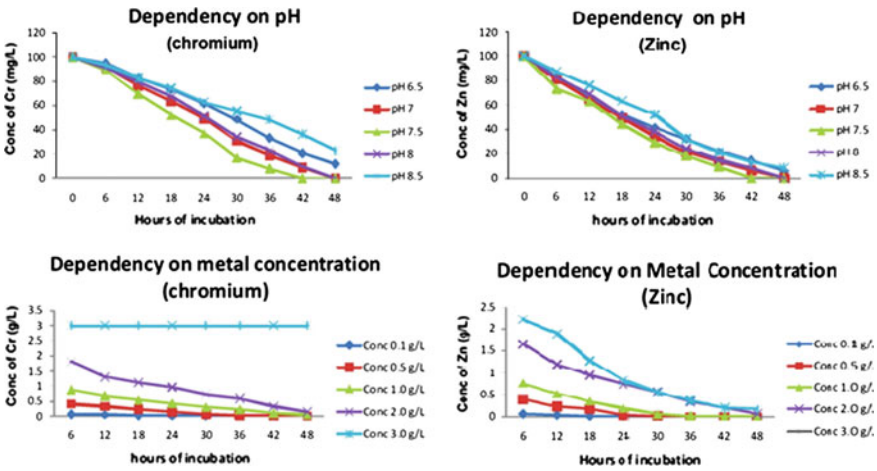

Fig. 8 Optimization of resistance level of *P. putida* STEAK 03 against chromium and zinc

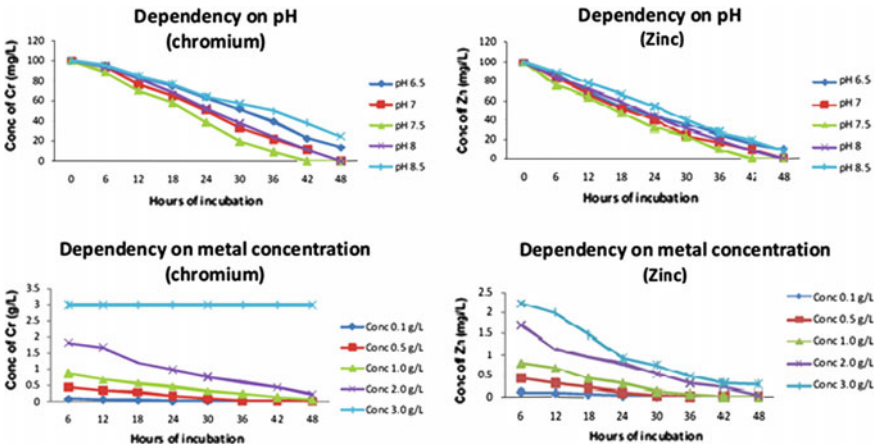

Fig. 9 Optimization of resistance level of *B. cereus* ETEAK 06 against chromium and zinc

3.6 Bioremoval of Cr (VI) and Zn (II) Ions from Waste Water Through Accumulation Process

Immobilization: Improvement in the removal of heavy metal using live cells depends on contact between the effluent and the isolates. Thus, immobilized growth was preferred to increase the contact time. Matrix with better holding capacity like *Luffa aegyptiaca* (bath sponge) was chosen and found to be most efficient. *Pseudomonas putida* STEAK 03 and *Bacillus cereus* ETEAK 06 and consortium of bacterial isolates were immobilized on *Luffa aegyptiaca* separately (Fig. 10a, b and

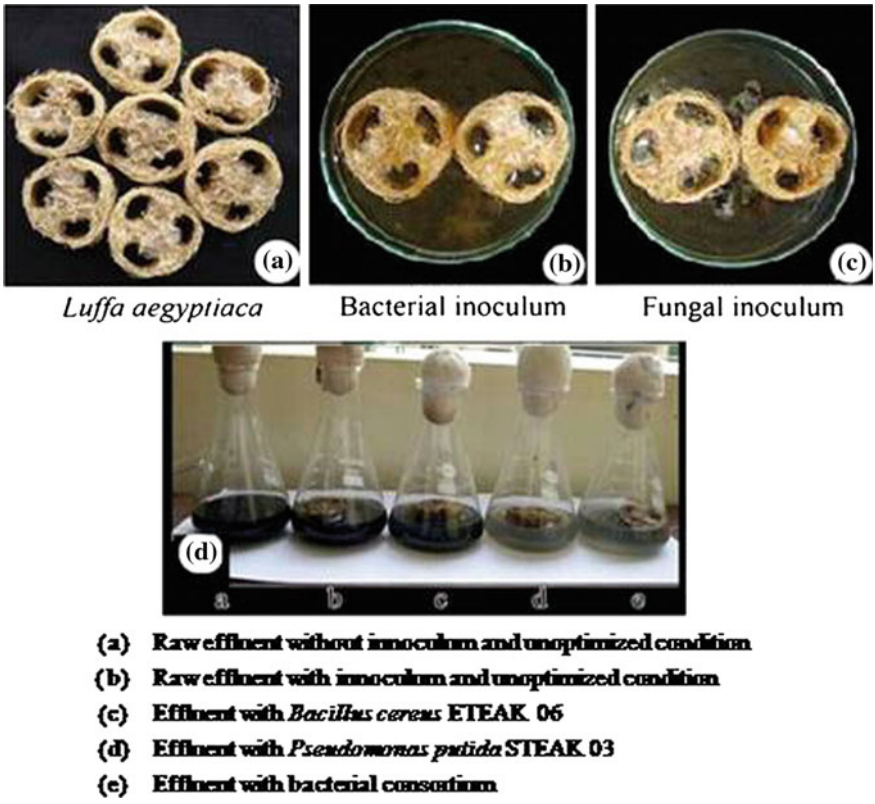

Fig. 10 Bioremoval of Cr (VI) and Zn (II) through bioaccumulation

c). Obtained results highlighted the potential use of immobilization with *Luffa aegyptiaca* for the removal of heavy metals from textile effluents.

Bioaccumulation of Cr (VI) and Zn (II) from textile dye effluent waste water: Over the stipulated period of incubation time, the results of the accumulation studies with immobilized consortium under optimized conditions showed noticeable changes in reduction of heavy metals and cellular growth than the individual bacterial isolates (Fig. 10d). 29.7% of Cr and 44.5% of Zn was adsorbed with *Bacillus cereus* ETEAK 06 and *Pseudomonas putida* STEAK 03 removed 32.2% of Cr and 48.5% of Zn, whereas Bacterial consortium removed 38.5 and 51.2% of Cr and Zn (Table 11). The results prove the accumulation by adsorption over the cell wall of microorganisms. However, it is to be noted that, more interestingly, the unfiltered effluent (raw effluent) which contained a mixture of microorganisms worked more effectively and removed (37.23% of Zn and 23.6% of Cr). This translated to the fact, bioremediation occurs naturally in the polluted environment, results also indicate consortium of microorganisms were doing equally good on the removal of heavy metal from the contaminated sites.

Table 11 Physiochemical parameters of the bacterial consortium treated effluent

S. No.	Parameters	Raw effluent	Bacterial consortium treated effluent	Reference	CPCB limit
1	Colour	Blackish green	Light yellow	Colourless	Absent
2	Smell	Offensive	Odourless	Absent	Absent
3	Ph	11.3 ± 0.2	8.1 ± 0.2	6.8 ± 0.1	6.0–9.0
4	Temperature (°C)	32 ± 1.0	30	20 ± 1.0	28–30
5	Total solids	4432 ± 15	1779.5 ± 20.0	1986.3 ± 18.0	Not applicable
6	Total Suspended solids (mg/L)	283 ± 5.0	101.5 ± 5.0	92.3 ± 2.8	100
7	Total dissolved solids (mg/L)	4194 ± 10	1678 ± 15.0	1894.0 ± 10.0	500–2000
8	Chlorides (mg/L)	1134.2 ± 8	896 ± 4.0	928.5 ± 5.6	250–1000
9	BOD	108.0 ± 0.1	30.0 ± 2.0	20.0 ± 10.0	30
10	COD	997.0 ± 30.1	213.9 ± 15.0	184.0 ± 10.1	250
Metal concentration					
S. No.	Metals	Raw effluent	Bacterial biosorbent treated effluent	WHO standards	Permissible limit for irrigation (mg/L)
11	Cr	24.51 ± 0.37	10.59 ± 0.02	0.05 ± 0.01	0.05
12	Zn	36.13 ± 1.27	7.23 ± 0.05	5.0 ± 1.2	0.2

3.7 *Toxicity Assessment of Treated Waste Water—Bioaccumulation Process*

The Physicochemical parameters like pH, EC, total suspended solids, total solids, total dissolved solids, chlorides, BOD, COD, metal concentration of the treated effluents was analyzed. The results reveal all the physiochemical parameters are within the permissible limit of central pollution control board. Observed average concentration (56.7 and 79% of Cr and Zn ions) was removed from the effluent sample.

Seed germination test: Phytotoxic studies on *Pennisetum typhoides* and *Vigna radiata* was carried out with consortium treated effluent and untreated effluent from industry. In seed germination study, there was no inhibition of germination by the bacterial consortium treated effluent and with the seeds soaked in bore-well water devoid of effluent where as 100% inhibition was observed with untreated effluent on 7 days of incubation and the germination was observed after 14 days of incubation and the metal concentration in the crop was also less and within the permissible limits for irrigation (Fig. 11).

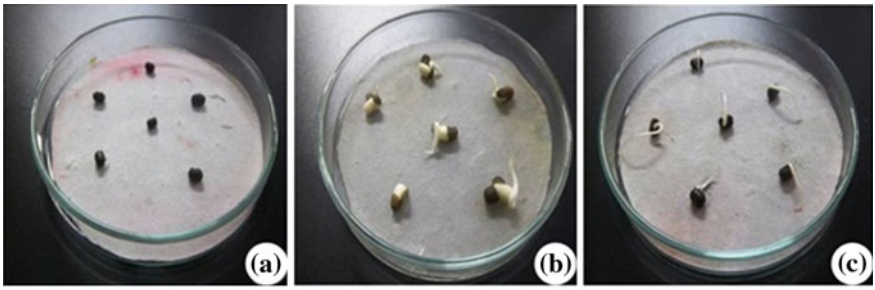

Fig. 11 Seed germination test of treated water on *Vigna radiate*

The reduction in germination percentage with untreated textile effluent might have been due to presence of high concentration of metals and other toxic organic compounds that cause a range of cellular toxicities (Kadar and Kastori 2003) and the presence of high amounts of salts and organic compound in untreated textile effluent reduces the availability of water thereby resulting in reduced germination (Ashraf 2004).

4 Conclusion

Thus, *Pseudomonas putida* STEAK 03 and *Bacillus cereus* ETEAK 06 strains sufficiently supported the process of bioremediation. This technology may indeed be labeled as the panacea for the removal of toxic substances from the environment. Bioremediation is without doubt an innovative technique that needs to be adapted to help cleanse the environment of such toxic pollutants such as Cr and Zn, save the biodiversity of AM Spores, conserve them. Based on the above results, it is concluded the biological treatment of textile dye effluent with *Luffa aegyptiaca* immobilized bacterial isolates for the recovery of heavy metals are proven to be the best and the effluent treated is safe for irrigation.

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World commission on environment and development 1987

Preparation of *Ficus benghalensis* Bark Activated Carbon and its Use as an Adsorbent for the Removal of Endocrine Disruptor Bisphenol-A

M. Kamaraj, K. Satheesh and S. Rajeshwari

Abstract Bisphenol A (BPA), is one of the phenolic endocrine-disrupting compound (EDC) which has estrogenic activity and are acutely toxic to aquatic organisms. Due to an increase in products containing epoxy resins and polycarbonate plastics, human exposure to BPA has sequentially increased. Therefore it becomes mandatory to remove the available BPA from aqueous phase. In this study, a novel adsorbent, *Ficus benghalensis* bark activated carbon (FBBAC) was prepared and proposed for BPA removal from aqueous phase. The adsorption characteristics of BPA on FBBAC were studied by batch experiments. A maximum value of 97.27% BPA removal was obtained using an adsorbent dosage of 1000 mg/50 mL at pH range 6–8. BPA removal equilibrium was reached within 24 h. The results indicated that obtained experimental data has high relativity with selective isotherm kinetic models.

Keywords *Ficus benghalensis* · Bisphenol-A · Adsorption · Removal · Pollutant

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1 Introduction

Currently majority of endocrine-disrupting compounds (EDC's) are released to the environment (Vandenberg et al. 2009). These EDCs are naturally occurring compounds or chemicals of man-made actions, which act like hormones in the endocrine system and disrupt the physiological functions of endogenous hormones (Gong et al. 2009). Bisphenol A (BPA) common name for 2,2-bis(4-hydroxylphenyl)-propane, is known as one of the EDC and widely used as a monomer in plastics industry for production of epoxy resins and polycarbonates. It can be released into the aquatic environment through various routes; can contaminate surface water and raw water supply of drinking water treatment plants (Loos et al. 2010). Hence it poses a risk to the aquatic animals and human reproductive health and fecundity (MOE 2005; Nakamura et al. 2010). Therefore significant effort must be made to remove it from the aqueous phase (Sui et al. 2011) and it's important to remove of BPA from wastewater for protection of human health and ecological environment. Various methods such as chemical oxidation (Li et al. 2008), membrane filtration (Dong et al. 2008), photocatalytic degradation (Kamaraj et al. 2014a, b), sorption (Liu et al. 2009), phytoremediation (Nakajima et al. 2004) and biodegradation (Kamaraj et al. 2014a, b) have been employed to eliminate BPA from wastewater.

Adsorption by activated carbon is generally considered to be one of the most efficient methods among various BPA removal process due to its simplicity, cut-price reusability of non-toxic adsorbent and will not produce more toxic intermediates than BPA itself (Mohanty et al. 2006). However, wood or coal is mainly used for commercially available activated carbon production and therefore it's considered expensive (Molina-Sabio and Rodriguez-Reinoso 2004). So there is a growing need for finding a renewable and locally available low cost material for economically large scale production of activated carbon for BPA removal purposes. Agricultural wastes are an apt option, as it makes a significant contribution for the sustainability of a healthy environment by using activated carbon produced, in the purification of contaminants from liquid and gas streams that evolves from industrial and domestic sources (Moyo et al. 2012). A wide variety of low cost materials, have been exploited for the removal of pollutants from aqueous solutions, including lemon peel, jute fiber, oil palm, orange peel, coir pith, cotton seed shell, beech sawdust, kernel shell, ground nut shell etc., (Olusegun and Ugba 2013). On contrary, Banyan (*Ficus bengalensis*) tree received much less attention as a precursor for the preparation of activated carbon. To the best of our knowledge, the adsorption behavior of BPA over FBBAC has not yet reported.

Therefore, the present investigation was designed with the following objectives: (1) To prepare activated carbon from *Ficus bengalensis* tree bark by physical and chemical activation (2) To study the adsorption capacities of BPA onto these activated carbon by batch experiments by adopting different kinetic models to analyze the experimental data and (3) To examine the effects of contact time, adsorbent dose, BPA concentration and solution pH on the adsorption of BPA onto FBBAC.

2 Methodology

2.1 Preparation and Characterization of FBBAC

Ficus benghalansis bark (FBB) used in this study was obtained from Coimbatore District, Tamil Nadu. FBB was cut into small pieces of 2–3 cm and then it was dried in sunlight until all the moisture evaporated and crushed into desired mesh size (1–2 mm). After grinding to fine powder using steel blender, the obtained mass was oven dried at 110 °C for 2 h. Dried mass was impregnated with $ZnCl_2$ in a ratio of 1:1 (g $ZnCl_2$/g FBB) and allowed to carbonize in a tube furnace (SRJK-2-13, Beijing) at 450 °C for 1 h. The carbon so obtained was characterized using Standard procedures (Rajeshwari et al. 2001).

Scanning electron microscopy (SEM) and pH of point of zero charge (pHPZC) using pH drift method were performed. Changes in the surface structures of activated carbon, prior to and after adsorption, were examined by ABB BOMEM FTIR Spectrometer Series 102 (Quebec, Canada) operating in the range of 400–4000 cm^{-1}, with a resolution of 4 cm^{-1}. XRD was performed at D8 Advanced XRD, Bruker and spectra were analyzed using Origin 6.0 software.

2.2 Adsorption Study

BPA (GC grade > 99%) was purchased from Sigma-Aldrich, India. A stock solution of 1.0 g/L was prepared by dissolving appropriate amount of BPA in 100 mL and made up to 1000 mL with distilled water. All chemicals used throughout this study were of analytical-grade reagents. Solution and reagents were prepared using double-distilled water. Factors like Agitation time, Dosage and pH which influence rate and extent of BPA uptake by adsorbents are studied at λ_{max} of 276 nm, using UV-Vis spectrophotometer (UV2450—Shimadzu, Japan) by batch mode experiments.

2.2.1 Contact Time and Initial BPA Concentration Study

Studies were carried out by shaking (model L-orbital) the adsorbents with 50 mL aqueous solution of BPA at different concentrations, at their neutral pH and at room temperature in 250 mL conical flasks at 120 rpm. The preliminary kinetic studies had led to select the necessary data for fixing the concentration of BPA and dose of the adsorbent used for adsorption experiments. The BPA concentration varied as 25, 50, 75, 100, 250, 500, 750 and 1000 ppm.

The amount of adsorbed dye per gram FBBAC at equilibrium, q_e (mg/g), and its dye removal efficiency in percentage were calculated by the following equations:

$$q_e = (C_0 - C_e)V/W \quad (1)$$

$$\% \text{ removal} = C_0 - C_e/C_0 \times 100 \quad (2)$$

where, C_0 and C_e are the initial and equilibrium concentrations of dye, respectively (mg/l). V is the volume of dye solution and W is the weight of adsorbent used (g).

2.2.2 Adsorption Dose Studies

Effect of the adsorbent dose was studied by agitating 50 mL of different concentration of BPA with different doses of adsorbent (200, 400, 600, 800 and 1000 mg) for a time greater than their equilibrium time at their natural pH. Experimental conditions were selected to assess the effect of adsorbent dose.

2.2.3 Effect of pH Study

The effect of pH on the removal of BPA was studied by using 50 mL of BPA (25 ppm) solution of desired concentration adjusted to a desired initial pH value mixed with known concentration of carbon and agitated for greater than equilibrium time interval. pH of the BPA solution was adjusted using HCl (0.1N) or NaOH (0.1N). After agitation, the adsorbent was separated by centrifugation and followed by supernatant was taken for spectrophotometry estimation at 276 nm.

2.3 Adsorption Isotherm Kinetics Studies

Kinetics of BPA adsorption onto the adsorbent were analyzed using Langmuir and Freundlich (Weber 1972), pseudo-first-order (Langergren and Svenska 1998), pseudo-second-order (Ho and McKay 1999), Elovich (Chien and Clayton 1980), Intraparticle diffusion (Weber and Morris 1963) and fractional power models (Venckatesh et al. 2010). The conformity between experimental data and model-predicted values were expressed by correlation coefficients (R^2, values close or equal to 1). The relatively higher value is more applicable model in kinetics of adsorption of BPA onto the adsorbent.

2.4 Batch Desorption Study

After adsorption experiments with desired concentration of BPA solution and known amount of adsorbent, the BPA-laden adsorbent was separated out by centrifugation

and the supernatant was discarded. The adsorbent was then gentle washed by using double time distilled water to remove any unabsorbed BPA molecules. Desorption studies of treated carbon was carried out by agitating it with 50 mL of distilled water with various concentrations of known desorption media (0.5 and 1.0N NaOH, HCl, H_2SO_4, and Acetic acid) for a time greater than the equilibrium time and the desorbed BPA was estimated Spectrophotometrically at 276 nm.

3 Results and Discussion

3.1 Characterization of Prepared Activated Carbon

Prepared activated carbon surface area was found to be 256 m^2/g, with total pore volume of 70% and average pore diameter was found to be 2.5 nm (Table 1). High surface area and total pore volume of prepared activated carbon was due to activation process used which involved both chemical and physical activating agents of $ZnCl_2$ and CO_2. Pore development in the activated carbon during pyrolysis is also important as this would enhance BET surface area and pore volume of activated carbon by promoting diffusion of $ZnCl_2$ and CO_2 molecules into the pores and thereby increasing $ZnCl_2$-carbon and CO_2-carbon reactions, which would then create more pores in activated carbon, creation of a large specific surface area and high pore volume.

Figure 1 shows SEM image of derived activated carbon. Many large pores were clearly found on the surface of activated carbon. The well-developed pores had led to large surface area and porous structure of activated carbon (Jinggeng et al. 2009).

Table 1 Characterization of FBBAC

S. No	Characterization of activated carbon	
1	pH	5.7
2	Conductivity	0.27 mhos
3	Bulk density	1.39 mg/ml
4	Porosity	70%
5	Specific gravity	4.77 m^2/g
6	Ion exchange capacity	2 g/ml
7	Decolorizing power	1.65 g/ml
8	Silica	Nil
9	Moisture content	0.1 g/ml
10	Surface area	256 m^2/g
11	Phenol adsorption capacity	3.19 mg/g
12	Matter soluble in water	3.46
13	Total surface charge (pHzpc)	2.5
14	Surface functional groups	0.516 mmol/g
15	Yield	50%

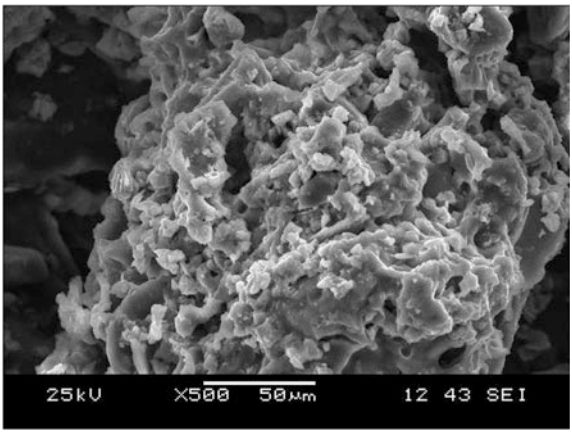

Fig. 1 SEM analysis of *Ficus benghalansis* bark activated carbon

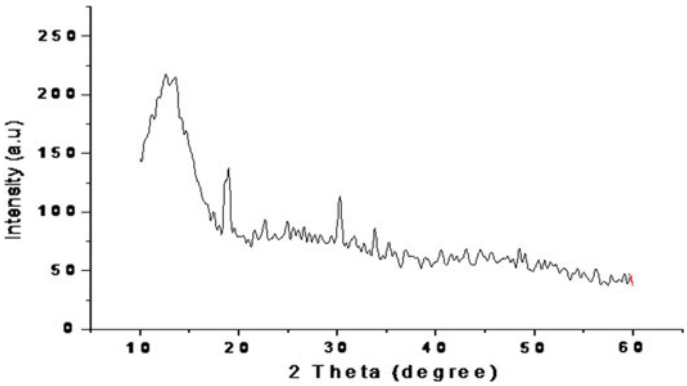

Fig. 2 XRD analysis of FBBAC

Graphitization process of activated carbon under high temperature (200 °C) can be reflected by X-ray diffraction patterns, as shown in Fig. 2. This spectrum indicated a shape of typical amorphous carbon and it's showed broad asymmetric peaks corresponding to 2Θ 13.03, 2Θ − 18.78 and 2Θ − 30.25. According to peak intensity evolution, such disordered graphitic carbon was termed as 'turbostratic structure' by Biscoe and Warren (1942). In this experiment, the graphitization temperature of activated carbon is lower than the previous results (Jinggeng et al. 2009).

Figure 3 displays FTIR spectra of desired activated carbon. This type of analysis used for identification of organic functional groups presented on the surface (Namasivayam and Kavitha 2006). FT-IR analysis of FBBAC displayed the following bands: 3541, 1610, 3421 and 3611 cm^{-1}: O–H stretching of carboxyl

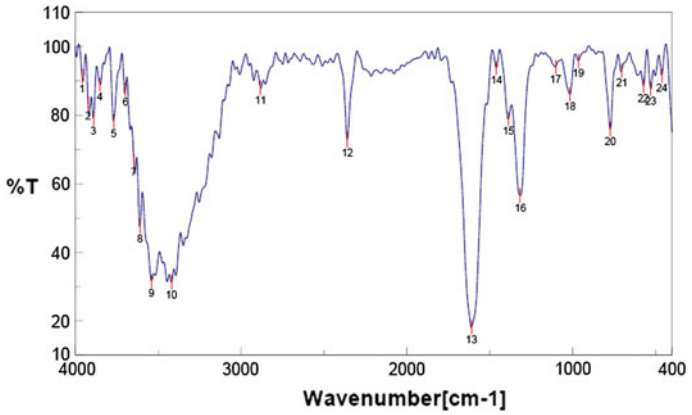

Fig. 3 FTIR analysis of FBBAC

groups, 1315 and 2359 cm^{-1}, symmetric stretching vibrations. Main surface functional groups present in derived activated carbon were phenolic groups. The OH stretching vibrations found were probably due to incorporation of hetero atoms at the edge of aromatic sheet or within carbon matrix (Guo and Lua 2002). Association of several bands with presence of groups containing oxygen is also agreed by Bohem titration determinations, given that the functional groups at surface of adsorbent, characterized by Boehm method, exhibited high acidity (0.32 mmol/g sorbent). High acidity is attributed to high value of activation temperature, given that acidic groups disappeared for temperatures over 500 °C (Chun et al. 2004). After adsorption, significant reduction of small peaks was observed within the range of 400–800 cm^{-1}, associated to C–H vibrations (Crini et al. 2007). This is reflected in decrease of basic groups according to the Bohem titration after adsorption (1.09 mmol/g sorbent). Results for pHPZC determination indicated a pHPZC value of approximately 2.5, and therefore pH values should be maintained greater than this value to ensure a predominant negatively charged surface and favor adsorption by electrostatic attraction (Shawabkeh and Tutunji 2003).

3.2 *Effect of Contact Time, Initial BPA Concentration and Carbon Dose*

Effect of contact time on adsorption of BPA on Banyan Bark (*Ficus benghalansis* L) indicated that rate of BPA removal progressively increased as agitation time increased. Rate of removal increased with agitation time and may be attributed to decrease in diffusion layer thickness surrounding of adsorbent particles. The percent removal of BPA increased with increase in adsorbent concentration and attained a plateau after a particular adsorbent concentration for BPA studied (Table 2).

Table 2 Effect of adsorbent dose and BPA dose in adsorption of BPA by FBBAC

BPA concentration (ppm)	BPA removal (%)					
	Control	Activated carbon load				
		200 mg	400 mg	600 mg	800 mg	1000 mg
25	0	34.24 ± 2	56.0 ± 2	88.40 ± 2	96.74 ± 2	97.27 ± 1
50	0	27.13 ± 2	42.01 ± 2	67.37 ± 2	92.59 ± 2	93.75 ± 1
75	0	24.72 ± 2	38.46 ± 2	59.40 ± 2	85.83 ± 1.5	91.29 ± 1
100	0	16.58 ± 2	26.71 ± 2	39.16 ± 2	76.49 ± 1.5	89.49 ± 1
250	0	16.69 ± 2	16.69 ± 2	27.18 ± 2	27.23 ± 1.5	27.23 ± 1
500	0	0	0	0	16.21 ± 1.5	21.22 ± 1
750	0	0	0	0	0	0

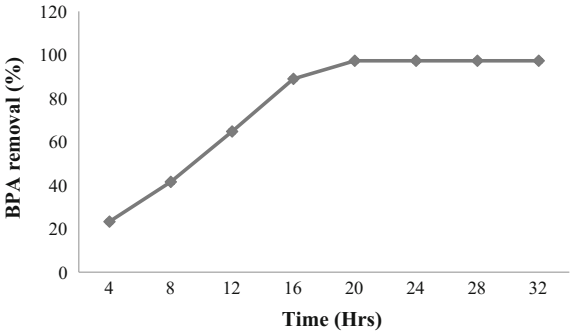

Fig. 4 Effect of contact time on removal of BPA by FBBAC

Maximum quantitative removal 97.27% was obtained at an adsorbent dose 1000 mg/50 mL. Further it revealed that with increase in dose of absorbent, the percentage of BPA removal has been increased. This was attributed to availability of more adsorption sites and increased carbon surface area (Minguang 1997). Equilibrium time increased with BPA concentration and depends on initial BPA concentration for the range of concentrations used for study. The maximum equilibrium time recorded was 20 h in this study (Fig. 4).

The removal curves were single, smooth and continuous. Further, it revealed that with increase in BPA concentration, percentage removal of BPA decreased whereas amount of BPA adsorbed/unit weight of adsorbent (mg/g) increased in the range of concentration tested suggesting that BPA removal using concentration dependent adsorption techniques. Similar results have been reported by several authors for adsorption of dyes using low cost materials (Rajasekhar and Haribabu 2009).

3.3 Effect of pH on BPA Adsorption

BPA solution pH was adjusted along with adsorbent and BPA solutions were agitated at their respective equilibrium time. Solutions pH varied between 3.0 and 11.0 with no

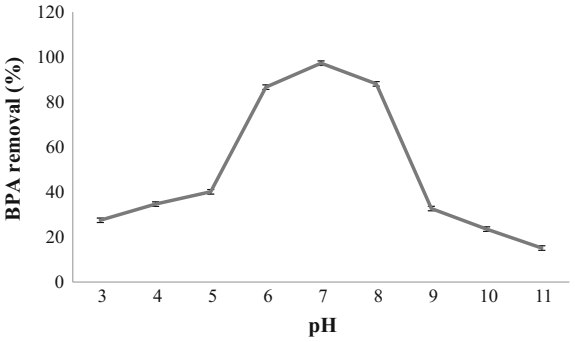

Fig. 5 Effect of pH on removal of BPA from aqueous solution by FBBAC

measurable variations due to solute concentration variations during adsorption process. Obviously, equilibrium adsorption amount of BPA decreased with increase of solution pH and minimum q_e occurred at pH 11.0 (Fig. 5). Adsorption rate reduced in the order of 7.0 > 8.0 > 6.0 > 9.0 > 5.0 > 10.0 > 3.0 > 11.0 as 97, 88, 86, 40, 34, 32, 27, 23 and 15% respectively. Results represented high adsorption amount at neutral and mildly basic pH values. Adsorptive behavior of BPA is pH dependent because pH values affect the grade of ionization/dissociation of BPA molecule, and charge on adsorbent surface as well. At high pH, surface groups become anionic (negative charge) and they are not favorable for adsorption of negatively charged dye molecules. Therefore, slight decrease in adsorption capacity at basic pH may be attributed to beginning of repulsion between anionic dye molecules and negatively charged adsorbent surface (Cotoruelo et al. 2010). Nevertheless, high adsorption capacity observed at neutral or weakly acid media could be explained taking into account the contribution of ion exchange mechanisms such as hydrogen bonds and forces as Vander-Waals type (Kannan and Murugavel 2008).

These results were due to surface charge density of carbons and charge of BPA species both of which depend on the solution pH. When solution pH was similar to pH_{PZC} value of carbons, the net surface charge density was near to zero, thus enhancing the π–π dispersion interaction. Consequently, when solution pH was above 8.0, surface charge density of carbon was negative and BPA deprotonated. As a result, repulsive electrostatic interaction was intensified and this was the main mechanism that contributed to worst equilibrium adsorption amount of BPA onto carbon at pH 11.0.

3.4 Adsorption Isotherm Analysis

Adsorption isotherm constants and correlation coefficient (R^2) values of selected kinetic models were calculated using standard formula (Table 3). Langmuir isotherm assumes monolayer adsorption onto a surface containing a finite number of adsorption sites of uniform strategies of adsorption with no transmigration of

Table 3 Selective adsorption isotherm constants for BPA adsorption by FBBAC

Langmuir constant	R^2	0.98
	Q_m	2.99
	b	0.33
	R_L	0.005
Freundlich constant	R^2	0.97
	K_f	0.19
	1/n	3.33
Lagergren constant	R^2	0.85
	K_{ad}	0.250
Pseudo second order constant	R^2	0.96
	K_2	511.5
	h	7.67
Elovich constant	R^2	0.94
	α	0.23
	β	0.74
Fractional power model constant	R^2	0.95
	a	0.352
	b	0.817
Intra particle diffusion constant	R^2	0.86
	K_P	1.42

R^2 = Correlation coefficient; Q_m = 1/Slope; b = slope; R_L = equilibrium parameter; n = how favourable the adsorption process; K_f (mg/g (l/mg)^n) is the adsorption capacity of the adsorbent; K_{ad}.2.303 × slope; k_2 is the equilibrium rate constant; $h = k_2 q_E^2$ (q_E = equilibrium mg/g); α = initial adsorption rate (mg/g min); β = adsorption constant (g/mg); a = intercept; K_P = (mg/g min$^{0.5}$)

adsorbate in plane of surface, while, Freundlich isotherm model assumes heterogeneous surface energies, in which energy term in Langmuir equation varies as a function of surface coverage. The R^2 values of Langmuir and Freundlich isotherms confirmed that Langmuir model yields a somewhat better fit (R^2 = 0.986) than Freundlich model (R^2 = 0.9771) at different concentrations and indicated favorable adsorption on adsorbent (Gupta et al. 2005). Correlation coefficient values of Lagergren at high concentration are higher than 0.80, the experimental q_e values do not agree with calculated ones, obtained from linear plots (Table 3). This showed that, adsorption of BPA onto activated carbon is not a first-order kinetic. The correlation coefficients for pseudo second-order kinetic model obtained were greater than 0.862 for all concentrations.

The calculated q_e values also agree very well with experimental data. These results indicated that adsorption system studied belongs to second order kinetic model. The similar phenomena was also observed in cross-linked chitosan beads adsorption of dye RR189 (Chiou and Li 2002), in adsorption of dye BB69 and DR227 on activated clay and in adsorption of AB9 on mixed sorbents (activated

Table 4 Desorption studies of BPA on FBBAC

Media	BPA (25 ppm)
	Desorption (%)
0.5N HCl	40.69
1NHCl	82.05
0.5 NH_2SO_4	32.18
1N H_2SO_4	74.30
0.5N CH_3COOH	33.88
1N CH_3COOH	30.43
0.5N NaOH	38.83
1N NaOH	56.48
Water	18.85

clay and activated carbon) (Wu et al. 2001; Ho and Chiang 2001) and in adsorption of Chromium hexavalent on used black tea leaves (Hossain et al. 2005). Correlation coefficients for Elovich kinetic model and Fractional power model which obtained at all the studied concentrations were high and suggested that this adsorption system is acceptable. Intra particle diffusion isotherm indicated a boundary layer effect while second linear portion is due to intraparticle or pore diffusion (Vadivelan and Vasanth Kumar 2005).

3.5 Desorption Studies

Desorption studies helps to elucidate the nature of adsorption and recycling of spent adsorbent and BPA (Table 4).

In this study maximum desorption rate was observed in different solutions in the order as follows $HCl > H_2SO_4 > NaOH > CH_3COOH > H_2O$. If adsorbed BPA can be desorbed using neutral pH water, then the attachment of BPA on adsorbent is by weak bonds. If sulphuric acid or alkaline water desorbed the dye then adsorption is by ion exchange. If organic acids, like acetic acid can desorb the dye, then dye had been held by adsorbent though chemisorption. Reversibility of adsorbed BPA in mineral acid or base is in agreement with pH dependent results obtained. Desorption of dye by mineral acids and alkaline medium indicated that dyes were adsorbed onto activated carbon though physic-sorption mechanisms (Arivoli et al. 2007).

4 Conclusion

Activated carbon prepared from *Ficus benghalansis* (Banyan) bark by using $ZnCl_2$as an activating agent was adopted for BPA removal from aqueous solution. Characterization by SEM, XRD and FT-IR demonstrated that prepared FBBAC had

an ordered porous structure with a specific surface area of 256 m^2/g. Furthermore, effects of different operating conditions like contact time, solution pH, adsorbent dose and BPA dose on adsorption of BPA on to FBBAC were also examined. The adsorption experiments indicated that BPA was effectively removed from aqueous phase by prepared FBBAC. The adsorption kinetics of BPA onto FBBAC can be well described by selective isotherm models. Langmuir and Freundlich isotherm models and Lagergen pseudo second order model, Elovich, Fracitonal power model were found to be best fitting isotherm and kinetic models. However, adsorption isotherms revealed the adsorbent can only uptake limited amount in relatively low concentration of BPA in aqueous solutions. Kinetic parameters thus obtained from fittings of model are dependent on adsorbent particle size and initial BPA concentration.

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Cow Dung Bioremediation of Pharmaceuticals, Pesticides and Petrochemicals for Cleaner Environment

S. Prakash, M. Selvaraju, K. Ravikumar and A. Punnagaiarasi

Abstract Cow dung, an excreta of bovine animal is a cheap and easily available bioresource on earth. Many traditional uses of cow dung such as burning as fuel, mosquito repellent and as cleansing agent are already known in India. Cow dung harbours a diverse group of microorganisms that may be beneficial to humans due to their ability to produce a range of metabolites. Nowadays, there is an increasing research interest in developing the applications of cow dung microorganisms for biofuel production and management of environmental pollutants. Use and misuse of pharmaceuticals, pesticides and petrochemicals by man is causing havoc with nature, as they persist as such or as their toxic metabolites. These pollutants bioaccumulate in environment, and they ultimately reach man through various means. They are hazardous because of potential toxicity, mutagenicity, carcinogenicity and genotoxicity. Bioremediation methods use naturally occurring microorganisms to detoxify man-made pollutants so that they change pollutants to innocuous products that make soil fertile in the process. This review discusses about using cow dung as bioremediation for pharmaceuticals, pesticides and petrochemicals for cleaner environment.

Keywords Cow dung · Bioremediation · Pharmaceuticals · Pesticides · Petrochemicals

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1 Introduction

Recent years, pharmaceuticals have received growing attention from environmental and health agencies all over the world and have become one of the emerging pollutants due to their frequent detection in the environment (Benotti et al. 2009). Medicines have an important role in the treatment and prevention of disease in both humans and animals. But it is because of the very nature of medicines that they may also have unintended effects on animals and microorganisms in the environment. Once released into the environment, pharmaceuticals will be transported and distributed to air, water, soil or sediment. A range of factors, such as the physico-chemical properties of the compound and the characteristics of the receiving environment, will affect their distribution.

Cow dung is a cheap and easily available rich source of microflora. According to Ayurveda, Gomeya/cow dung is not a waste product, but it is a purifier of all wastes in the nature. When spread over urban and rural waste in solution form (1:10–1:25 solution), it biodegrades the waste in time. It is a "gold mine" due its wide applications in the field of agriculture, energy resource, environmental protection, and therapeutic applications. Bioremediation is the use of naturally occurring microorganisms or genetically engineered microorganisms (bacteria and fungi) by man, to detoxify man-made pollutants (Ogden and Adams 1989). Microorganisms have a unique ability to interact both chemically and physically with a huge range of both man-made and naturally occurring compounds leading to a structural change to/the complete degradation of the target molecule.

2 Manure Production Per Animal

The amount of manure produced by animals is very variable even within species, partly due to differences in dry matter content of the manure. As water is not a very interesting component, it is deled mainly with the dry matter (DM), the organic matter (OM) and the nutrients N and P. The little data on DM excretion reported in literature is difficult to interpret, partly because the amount of bedding included often varies, while the weight of the animals and the feeding situation is often poorly described. Fleming and Mordenti (1993), Muller (1980) and Romney et al. (1994) gave manure production specifications for different animal species in different regions of the world but the basis for these estimates is unclear. It includes ruminants (30–55), pigs (10–35) and poultry (10–35).

3 Bioremediation of Pharmaceuticals, Pesticides and Petrochemicals

Use and misuse of pharmaceuticals, pesticides, and petrochemicals by man is causing havoc with nature, as they persist as such or as their toxic metabolites. These pollutants bioaccumulate in environment, and they ultimately reach man through various means. They are hazardous because of potential toxicity, mutagenicity, carcinogenicity, and genotoxicity. To rejuvenate nature, remediation methods currently available are usually expensive and might convert one toxic pollutant to another. Bioremediation methods use naturally occurring microorganisms to detoxify man-made pollutants so that they change pollutants to innocuous products that make soil fertile in the process. Taking cue from Ayurveda, Gomeya/cow dung is used as an excellent bioremediation method. Thus, utilizing freely available cow dung as slurry or after composting in rural areas, is a cheap and effective measure to bioremediate the harmful pollutants. Yet, more research in this direction is warranted to bioremediate non biodegradable, potentially toxic pollutants (Fig. 1).

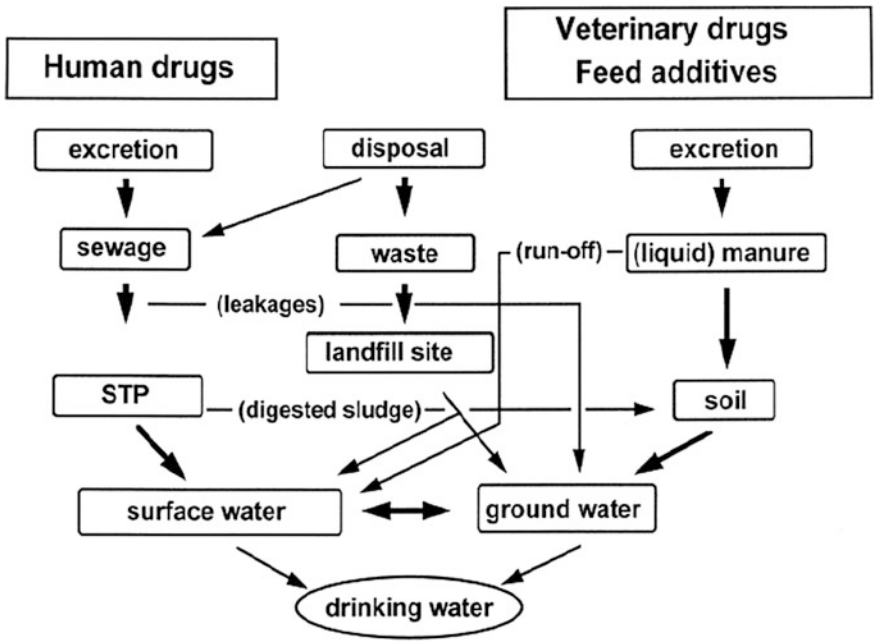

Fig. 1 Fate of pharmaceuticals in the environment (*Source* Ternes 1998)

4 Fate of Pharmaceuticals in the Environment

The fate of veterinary and human drugs, urinal and faecal excretion are quite different to each other. Generally all the municipal sewage and human pharmaceuticals have to pass through a STP prior to entering rivers or steams. Whereas veterinary drugs are more likely to contaminate soil and ground water when liquid manure is used for top soil dressing. Additionally after rainfall incidents surface water can be polluted with human or veterinary drugs by run-off from fields treated with digested sludge or livestock sluries respectively. Industrial waste water may be another possible source for the contamination of surface waters, but are surely not responsible for their ubiquitous occurence. Another possible contamination of soil and ground water may be caused by the application of digested sludge from municipal STP on agricultural areas. Additionally transport of drugs via bank filtration from highly contaminated surface water into ground water is also a possibly as the infiltration of waste water directly from leakage in drains. Also drugs disposed together with domestic waste can reach landfill sites which could lead to ground water contamination by leaching. A unique pathway for the contamination of soil and ground water by medicinal residues, disposal of raw sewage/STP effluents by spray and broad irrigation in agricultural areas.

4.1 Antimicrobial Agents

In medicine, 6% of prescriptions are for antimicrobial agents, while in veterinary medicine, more than 70% of prescriptions contains them (Bruhn 2003). Therefore, non-degradation of antibiotics can theoretically lead to the development of multidrug resistant strains which can indirectly infect the humans, causing increased morbidity and mortality. Manure (e.g., cattle dung) could serve as a relevant model ecosystem to study the fate of drugs, more so because some of the known coprophilous basidiomycetes can degrade enrofloxacin. Two such basidiomycetes were isolated fromaged cattle dung by Wicklow and colleagues two decades ago (Wicklow et al. 1980; Wicklow1992). The bacterial strain *Cyathus stercoreus* has high activity in the degradation of lignocellulose in vitro. *C. stercoreus* is able to degrade enrofloxacin (Freer and Detroy 1982; Wicklow et al. 1980).

4.2 Biomedical Waste Degrader

Proper and cheap method of disposal of biomedical waste is a burning issue in view of the expanding health care system in India. Current method of biomedical waste disposal is the use of incinerators which are not only expensive but also not environment friendly. Incinerators produce toxic gases (polychlorinated

dibenzofurans and polychlorinated dibenzo p-dioxins) in the process. Dioxins are known to cause genetic aberrations, hormonal imbalances, and damage to immune and reproductive systems. *Periconiella* species of fungus isolated from cow dung was found to be an excellent degrader of biomedical waste. Fifty grams of biomedical waste, kept in the form of used bandages and cotton in culture media, were effectively and completely reduced by 50th day. It was found to be cheap, safe, and environment friendly method of biomedical waste disposal (Pandey and Gundevia 2008).

4.3 Pesticides

At present, India is the largest producer of pesticides in Asia. The Indian pesticide industry with 82000 MT of production for the year 2005–2006 is ranked second in Asia (behind China) and ranks twelfth in the world for the use of pesticides with an annual production of 90,000 tons (Boricha and Fulekar 2009). 2–3% of pesticide is actually utilized and the rest persists in soil and water causing environmental pollution leading to toxicity (WHO 1990). Pesticide residues remain in surface soil, leading to toxicity in the soil water environment. A vast majority of Indian population (56.7%) is engaged in agriculture and is therefore exposed to the pesticides used in agriculture. At present, the pesticide waste is being treated by physico-chemical methods which are not efficient and effective. As a result, pesticide residue remains in the soil-water environment causing toxicity to the biota and thereby entering into the food chain. Pesticide residue in environment ultimately affects the health of man and is a cause of morbidity. Immuno-pathological effects of pesticides in animals and man are acquired immune deficiency or immunosuppression, and autoimmunity and hypersensitivity reactions, like eczema, dermatitis, allergic respiratory diseases, and pesticides might be the cause of recurrent infections. Many pesticides are known to cause mutations in chromosomes of man and animals, thereby may lead to carcinoma of liver and lung. They are teratogenic and mutagenic in nature, and can cause neuropathy, nephropathy, hepatotoxicity, and reproductive disorders (Chauhan and Singhal 2006).

In a study, pesticides (chlorpyrifos, cypermethrin, fenvalerate and trichlopyrbutoxyethyl ester) were analyzed for bioremediation with cow dung (specific breed of cow not mentioned) slurry. Fresh cow-dung slurry in the ratio of 1:10 with distilled water was taken as a source of microbial biomass. The cow dung slurry biomass was activated for a period of three days by continuous aeration and by addition of one dose of nutrient-glucose 150 mg/L, potassium dihydrogen phosphate 80 mg/L and ammonium sulphate 80 mg/L. Chlorpyrifos was rapidly hydrolyzed to 3, 5, 6 trichloro-2-pyridinol (TCP) in 25 and 50 mg/kg chlorpyrifos amended soil, while in 100 mg/kg chlorpyrifos amended soil, it was present till the 3rd day of the experiment. More than 75 and 50% Cypermethrin (25 and 50 mg/L, resp.) was hydrolyzed to 3-phenoxy benzaldehyde and 3-phenoxybenzyl alcohol by 7th day. The compounds trichlopyracid and 3, 5, 6 trichloropyridinol were found to

be the principal metabolites of Trichlopyr butoxy ethyl ester biodegradation within 24 h. The higher nutrient availability and larger microbial population of the cow dung slurry and soil-pesticide mix was found to affect bioremediation of pesticides under controlled environmental conditions (Geetha and Fulekar 2008). Research studies showed that adaptability of microorganisms during bioremediation releases enzymes, which metabolizes wide spectrum of anthropogenic chemicals. The remediation of pesticide residue from soil and water is of prime importance to decontaminate the environment.

Pseudomonas plecoglossicida is a novel organism for bioremediation of hazardous compounds like cypermethrin (Boricha and Fulekar 2009) and chlorpyrifos (Organophosphate insecticide) by *Pseudomonas aeruginosa* (Fulekar and Geetha 2008). These microorganisms obtained from cow dung though have the ability for bioremediation in laboratory setups, can also be applied in pesticide contaminated soil and water. Fenvalerate (a synthetic pyrethroid) is used as a pesticide in agriculture. It has the property to adsorb soil particles and causes contamination leading to the toxicity in soil water environment. The activated cow dung slurry was used as a source of microbial consortium for bioremediation often valerate amended soil. Fenvalerate was degraded with the formation of prominent intermediates like 4-chloro alpha benzene acetic acid and 3-phenoxy-benzoic acid over a period of seven days. These intermediates are less toxic than the parent compound and on longer acclimatization in the environment would be mineralized into inorganic biomass and carbon dioxide (Geetha and Fulekar 2010). Alternatively to the use of pesticides in agriculture, organic farming can be adopted to stop the onslaught of pesticides in the environment.

5 Conclusion

Currently, pharmaceutical compounds are being merged into the environment in extremely large quantities regularly and present system of regulations of their release is not able to control the untreated or partially treated pharma effluents. The impacts of drugs are entering into and occurring on ecosystems, biota and humans. The side effects on human, aquatic and animal health need to be investigated through thorough safety and toxicological studies. Sincere efforts are required to reduce the problem along with some adequate regulations to monitor or to control them.

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Treatment of Textile Effluent by Phytoremediation with the Aquatic Plants: *Alternanthera sessilis*

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Abstract India has a large network of textile industries of varying capacity and textile effluent is one of the main contributors of water pollution and it adversely affects fauna and flora. Phytoremediation technology can be effective approach for remediating contaminated sites of such textile dyeing effluents. The objective of this research is to study the efficiency of aquatic macrophytes plant contributed for remediation of textile waste water. In this study, lab scale wetland was constructed and tested with *Alternanthera sessilis*, sand and gravel as filter. Results indicated that, textile effluent degradation and significant reductions in TS (78%), TDS (83%), TSS (38%), COD (65%), BOD_5 (66%), chloride (44%), hardness (76%) and TDS (59%) were observed by the combined use of plants within 24 hrs. The removal efficiency of unplanted were TS (50%), TDS (50%), TSS (29%), COD (27%), Hardness (57%), Chloride (26%), BOD_5 (27%) respectively. When compared the removal efficiency of planted with unplanted, planted shows a maximum removal the results showed that the removal efficiencies of the organics TDS, COD and BOD_5 were improved significantly with the extension of HRT. This objective of study and efficiency of plants and proposed mechanism of aquatic macrophytes plant contributed for remediation of textile waste water.

Keywords Textile effluent · Phytoremediation · *Alternanthera sessilis* · Aquatic macrophytes · Efficiency

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1 Introduction

Industrial revolution, the beginning has accelerated toxic metal in polluted water is a major problem and is leading cause of diseases/death worldwide (Sayyed and Sayadi 2011). Textile industrial production uses different chemicals and dyes according to textile wastewater practices, their dilution after production and wastewater management. Worldwide, textile industries commercially produce around 10,000 different dyes with an annual production more than 7 × 105 metric tons. The textile processing produce wastewater that in different stages of dyeing, fixing, washing and other processing, which contain suspended solids, high amount of dissolved solids, unreacted dyestuffs and other chemicals (Mohan et al. 2005). Textile effluents are dark color, high turbidity of the receiving water body due to usage of dyes and chemical. Improper disposal of textile wastewater causes direct contamination of ground water and surface water (Thilakar et al. 2012).

Treatment process include physical, chemical and biological methods for achieving color removal and reduction of TDS, BOD_5, COD, which have merits and economical limitations (Prajapati et al. 2012). The physical, chemical dye removal methods are expensive and have limited versatility (Zee and Villaverde 2005). Recycling the wastewater from the industries involve various methods, including aerobic and anaerobic microbial degradation, coagulation (Guendy 2010), absorption (Sivakumar 2014; Sivakumar et al. 2014), activated carbon (Syafalni et al. 2012), electrochemical process (Dogan and Haluk 2012), reverse osmosis (Ramesh Kumar et al. 2013), ozonation (Guendy 2007), adsorption (Shankar and Sreenivasa 2012; Sivakumar and Shankar 2012), catalytic oxidation (Hussein and Abass 2010) and membrane processes (Abdulraheem and Abiodun 2012) etc. for color removal from wastewater, these processes have high operational costs and limited applicability.

Phytoremediation is one of the waste water treatment methods by using plant based systems for removing the contaminants from various natural sources. To clean up the contaminated water, selection of an appropriate and efficient plant system is highly essential. Those plant systems should have high uptake of both organic and inorganic pollutants, grow well in polluted water and be easily controlled in quantitatively propagated dispersion (Roongtanakiat et al. 2007). Phytoremediation is easier to manage because it is an autotrophic system of large biomass that requires little nutrient input. Moreover, plants offer protection against water and wind erosion, and prevents contaminants from spreading (Cluis 2004). Phytoremediation treatment is used for treating many classes of contaminants like petroleum hydrocarbons, chlorinated solvents, pesticides, explosives heavy metals and radionuclides and landfill leachates. These technologies involve moderately relatively high capital expenditure and manpower as well as long-term operating costs (Shenbagavalli 2007; Prithabai 2008). In recent years, considerable attention has been focused on absorption process using aquatic plants because of its advantage over conventional treatment methods include low cost, high efficiency, minimization of chemical and biological sludge (Roy et al. 2010). In India,

terrestrial plants like *Helianthus annuus*, *Phragmites karka*, *Datura innoxia*, *Brassica juncea*, *Alternanthera sessilis* and *Zea mays* have been used to treat different metals contaminating effluent soil and sludge from various types of industries (Meagher 2000; Kramer and Chardonnens 2001; Peuke and Rennenberg 2005; Prithabai 2008). The objective of study is to examine the proposed mechanism of aquatic macrophytes plant contribution for remediation of textile waste water.

2 Materials and Methods

2.1 Experimental Setup

The lab-scale vertical-flow container beds with 44, 34 and 14.2 cm (length, width and height) dimension were constructed 2 in number. The beds were white plastic tray and planted with *Alternanthera sessilis* (Fig. 1). Each bed was filled with the media consisting 5 cm of gravel layer and sand on the top as the supporting layer. Approximately 10 L of raw effluent from factories was brought to the laboratory in plastic containers and the experiments were setup in plastic containers. The experiment plants were initially subject to stabilization in tanks containing tap water for one month for acclimatization.

Ten liters of the particularly diluted effluents were prepared and then transferred to plastic tank. For each experimental set, one control was maintained in stock tanks and were collected, cleaned, introduced in the experimental tanks. Approximately 250 g each experimental plant used for the study, each occupying half of tanks. Triplicate of each experimental setup was maintained and 500 ml from each effluent were examined. The wastewater was treated at different hydraulic retention time for 24 h. The textile effluent was brought to the laboratory, diluted with tap water and

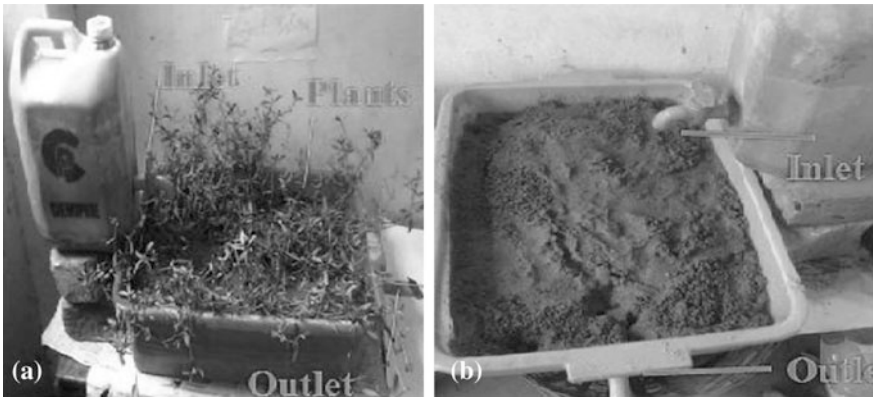

Fig. 1 Schematic diagram of vertical flow constructed wetland (**a**) *A. sessilis* (**b**) control

placed in the storage tank with control flow to feed 0.3 L wastewater to the bed in a one minute interval on a daily basis.

2.2 Analytical Methods

The effluent samples were collected in a sterile 500 ml plastic bottles, stored at 4 °C and physico-chemical parameters like temperature, pH, electrical conductivity (EC), total solids (TS), total dissolved solids, (TDS), total suspended solids, chemical oxygen demand (COD) and biological oxygen demand (BOD_5) were analyzed as described by APHA (1998). The percentage concentration decrease efficiency was evaluated according to Eq. (1) proposed by the International Water Association.

$$E = C_i - C_O/C_O * 100 \quad (1)$$

where, E = percentage concentration decrease efficiency (%), Ci = inlet concentration (mg/l) and Co = outlet concentration (mg/l).

3 Result and Discussions

3.1 Physico-Chemical Characterization of Textile Effluents

The characteristics of textile effluent collected from Tirupur common effluent treatment plant was brought to laboratory and was diluted with tape water. The physico-chemical parameters are showed in Table 1.

Table 1 Characteristics of textile effluent (Raw wastewater 10:1 with diluted tape water)

Parameter	Mean	Minimum	Maximum	Std. deviation
Temperature (°C)	28	28.6	28.8	0.03
pH	8.75	8.68	8.86	0.02
Electrical conductivity (mS/cm)	7.08	7.05	7.12	0.01
TS (mg/l)	6500	6200	6700	61.72
TDS (mg/l)	6000	5800	6400	87.28
TSS (mg/l)	500	200	800	92.58
Total alkalinity (mg/l)	250	230	270	4.87
Total hardness (mg/l)	277	260	310	6.15
Chloride (mg/l)	648	610	680	9.11
DO (mg/l)	2.5	2.2	2.8	0.09
BOD_5 (mg/l)	362	345	385	4.61
COD (mg/l)	1400	1200	1600	141.42

3.2 Effect on Temperature

The temperatures determine the solubility of dissolved oxygen in water, its concentration and its availability to aquatic organisms (Anon 1996). The effect of the textile effluent, on the phytoremediation was studied at temperature values ranging from 24.5 to 25.5 °C (Fig. 2). The effluent from the bed planted with *A. sessilis* was effectively removed (14%) at temperature of 24.5 °C during hydraulic retention time (24 h) when compared to control.

3.3 Effect on PH

The pH of textile effluent was 8.25, the alkaline nature might be due to dyes and other chemicals. The effluent from the planted *A. sessilis* had a mean pH of 7.76 and unplanted bed had a mean pH of 7.8 during hydraulic retention time of 24 h (Fig. 3). This result is consistent with the behaviour of pH in other treatment wetlands (Belmont et al. 2004). The pH reduction in effluents is due to nitrification that produces the hydrogen ion in constructed wetlands (Lin et al. 2005).

3.4 Effect on Electrical Conductivity

The EC of textile effluent was observed (Fig. 4), the *A. sessilis* planted bed had mean value 0.89 µS/cm, whereas the unplanted bed had a mean of 1.32 µS/cm during retention time 24 h. EC was reduced due to evapotranspiration and/or movement of substrate by plant roots (Hench et al. 2003) and earlier reports for pilot-container experiments in papyrus-based treatments showed higher reductions (Kyambadde et al. 2004).

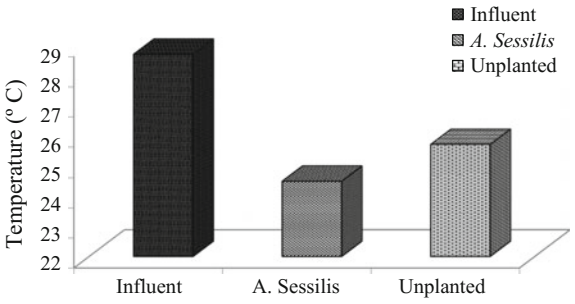

Fig. 2 Effect of *A. sessilis* treatment and control on temperature

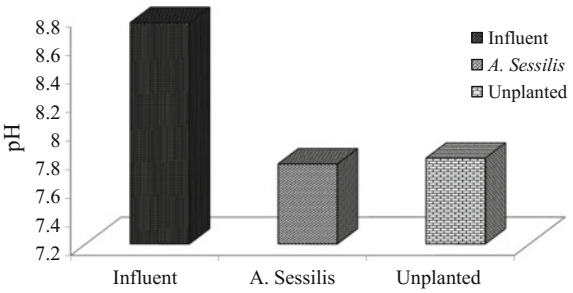

Fig. 3 Effect of *A. sessilis* treatments and control on pH

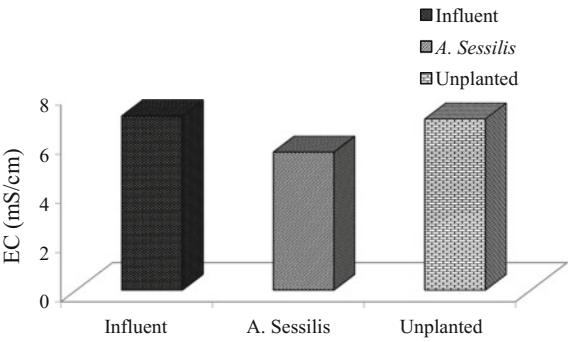

Fig. 4 Effect of *A. sessilis* treatment and control ion electrical conductivity

3.5 *Effect on Total Solids*

Total solids of textile effluent 6814 mg/l, when treated with *A. sessilis*, had a mean of 1500 mg/l whereas unplanted bed had mean of 3500 mg/l during HRT of 24 h. In the present study, plants removed TS in 78% textile effluent whereas in the unplanted it is 49% (Fig. 5).

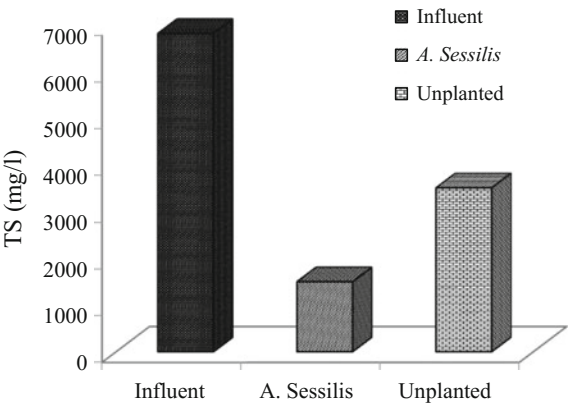

Fig. 5 Effect of *A. sessilis* treatment with planted and control on total solids

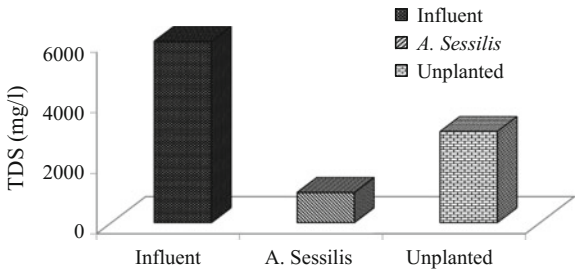

Fig. 6 Effect of *A. sessilis* treatment and control on TDS

3.6 Effect on Total Dissolved Solids

The TDS removal by *A. sessilis* was good in almost all concentrations (Fig. 6) while the textile effluent was observed with 577.48 mg/l TDS. The effluent from the planted had a mean TDS of 73.26 mg/l and unplanted bed had a mean 115.14 mg/l during the HRT of 24 h. There occurred an 83% increase in planted while lowest occured at 50% in unplanted.

3.7 Effect on Total Suspended Solids

The TSS of textile effluent was 814 mg/l, after treated with *A. sessilis* the mean was 300 mg/l, whereas unplanted bed had a mean TSS 714 mg/l during the HRT of 24 h (Fig. 7). TSS removal efficiencies were reported in similar studies (Neralla et al. 2000; Steer et al. 2002). In the present study the performance efficiency was 38% which is higher than the control (12%).

3.8 Effect on Total Alkalinity

The average TA concentration of textile effluent was 250 mg/l, after treatment with *A. sessilis* it reduced to 90 mg/l, during the HRT of 24 h (Fig. 8) which is 64% effective whereas in the unplanted bed TA reduced at 18% rate in 24 h.

3.9 Effect on Total Hardness

The hardness of textile effluent was 272 mg/l and reduced to 90 mg/l by phytoremediation during the HRT of 24 h and 160 mg/l was found in control (Fig. 9).

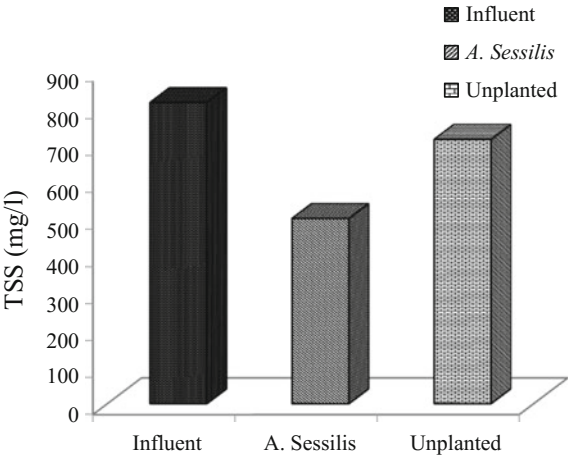

Fig. 7 Effect of *A. sessilis* treatment and control on TSS effect on total alkalinity

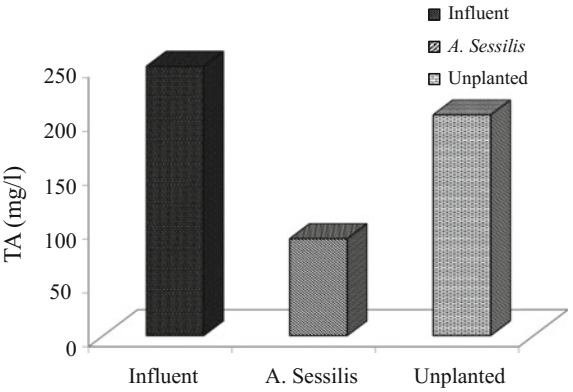

Fig. 8 Effect of *A. sessilis* treatment and control on total alkalinity

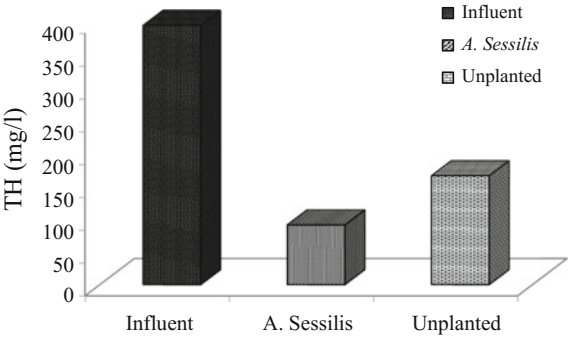

Fig. 9 Effect of *A. sessilis* treatment and control on total hardness

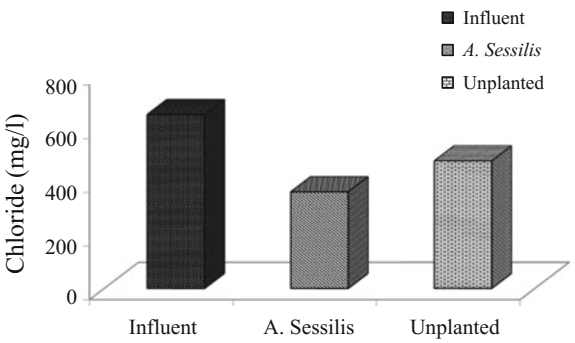

Fig. 10 Effect of *A. sessilis* treatment and control on chloride

3.10 Effect on Chloride

The chloride content in textile effluent was 648 mg/l which are alarmingly high values. The effluent from the treatment had a mean chloride of 425 mg/l and unplanted bed had a mean chloride of 551 mg/l during the HRT of 24 h (Fig. 10). In the present study it has been found that *A. sessilis* removed 44% of chloride whereas in unplanted it reduced to 26%.

3.11 Effect on Dissolved Oxygen

The dissolved oxygen was increased (5.85 mg/l) with in 24 h by *A. sessilis* treatment than the 2.5 mg/l of textile effluent (Fig. 11). According to Reddy (1981), it might be the reason that the presence of plants in wastewater depletes dissolved CO_2 during the period of photosynthetic activity and an increase in DO of water, thus creating aerobic conditions in wastewater.

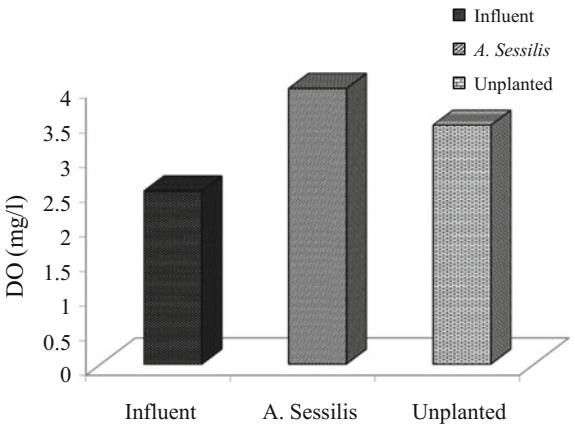

Fig. 11 Effect of *A. sessilis* treatment and control on dissolved oxygen

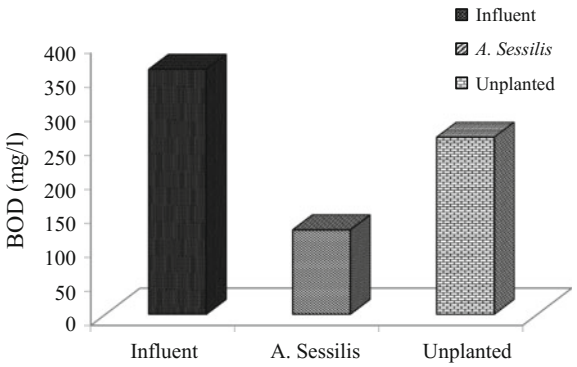

Fig. 12 Effect of *A. sessilis* treatment with planted and control on BOD_5

3.12 Effect on Biological Oxygen Demand

The BOD_5 of textile effluent was 162 mg/l and increased by *A. sessilis* treatment to 110 mg/l which is efficient than unplanted bed which had a mean BOD_5 of 130 mg/l during the HRT of 24 h (Fig. 12). BOD_5 removal between planted and unplanted wetlands may be due to microbial degradation of organics coupled with root zone oxygen input (Klomjek and Nitisoravut 2005). The BOD reduction study by Steer et al. (2002) reported that the sedimentation, adsorption and microbial metabolism are considered to be the primary mechanisms for BOD removal, it is likely that the plant roots and falling residues provide a more effective settling medium than gravel alone, while at the same time increasing surface area for attachment and food sources for the microbial populations. In the present study the plant removed BOD_5 at maximum of 66% and minimum of 27% in textile effluent.

3.13 Effect on Chemical Oxygen Demand

The COD was reduced by *A. sessilis* to 180 mg/l (65%) from the observed 460 (20%) mg/l in the textile effluent (Fig. 13). In control the COD was reduced to 280 mg/l during the HRT 24 h, this data agrees with the finding of Mashauri et al. (2000) who found that longer retention time would reduce COD. The removal of COD is attributed to microbial degradation of substrate by the plants roots (Greenway and Woolley 1999; Vymazal 2002; Steer et al. 2002). This is in agreement with the observations made by Lim et al. (2001) but also in contrast to the results reported by Tanner et al. (2005). However, daytime photosynthes is done by plants could actually generate oxygen to balance the oxygen used for organic mineralization and nitrification (Lin et al. 2005).

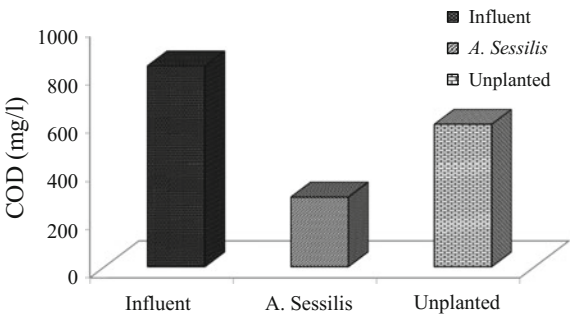

Fig. 13 Effect of *A. sessilis* treatment and control on COD

4 Conclusion

There are various methods employed for effluent treatment in textile industry. But, all the methods are not significantly used in textile industry because of various difficulties such as time, cost, raw material unavailability, etc. In this study, the investigation was carried out in the textile dye effluent by natural adsorbents namely phytoremdiation. The results indicated that the removal of organic pollutant was enhanced by the presence of plants. It is possible that the presence of plants roots with rhizobacteria had an advantageous effect on improving the organic pollutant degradation. It is observed that these adsorbents are active in removal of dye and harmful pathogenic bacteria. From this study, the promising attributes of *Alternanthera sessilis* includes its tolerance to dye and dye absorption along with good root development, low maintenance and ready availability in contaminated regions. These characteristics helps to prove that the suitability of the plant in dyeing industry effluent treatment ponds. Hence, it is concluded that *A. sessilis* has the potential in removal of dye and harmful pathogenic bacteria and more useful for waste water treatment applications.

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Part III
Bioenergy Production for Cleaner Environment

Bioconversion of Cellulosic Waste into Bioethanol—A Synergistic Interaction of *Trichoderma Viride* and *Saccharomyces Cerevisiae*

C. Elizabeth Rani Juneius and J. Kavitha

Abstract Bioconversion offers a cheap and safe method of not only disposing the agricultural residues, but also it has the potential to convert lignocellulosic wastes into usable forms such as reducing sugars that could be used for ethanol production. In this present study, a formulated bio-control agent *Trichoderma viride* collected from the farmers of Wayanad, Kerala, India and an indigenous *Trichoderma* spp isolated from the paddy fields of Thiruporur area, Tamil nadu, India were used to evaluate the efficiency of cellulolytic property for the conversion of cellulosic wastes such as vegetable waste and sugarcane bagasse to simple sugars. Further the same was used as substrate for the synthesis of bio-ethanol by *Saccharomyces cerevisiae.* Cellulase production ability of both fungi using substrates carboxy methyl cellulose, vegetable waste and bagasse were estimated and the amount of cellulase produced by *Trichoderma viride* was 396.6, 361.6 and 466.6 U/ml and the amount of cellulase produced by *Trichoderma* spp KT was estimated as 198.3, 233.3 and 245 U/ml from the corresponding substrates. Among the all substrate used, sugarcane bagasse had served as a good substrate when compared to others. Bioconversion of the same saccarified broth was used for bio-ethanol production. The production rate estimated in carboxy methyl cellulose, vegetable waste and sugarcane bagasse was 70 and 100% respectively by *Trichoderma viride* where as it was 54.6, 66.3 and 83.3% in the fermented broths of *Trichoderma* spp KT. In conclusion, the cellulosic waste can be very well used as a resource for bio-ethanol production. The synergistic interactions of *Trichoderma viride and Saccharomyces cerevisiae* can play a significant role in bioconversion of waste into a renewable energy.

Keywords Cellulase · Bioethanol · Synergism · Sugarcane waste

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1 Introduction

Conversion of solid waste into an useful byproducts are in demand in Indian scenario because of the plenty of availability of degradable wastes. Cellulosic waste is nothing but a waste from plant resources are the raw material used for this study. These cellulosic wastes are utilized by fungi such as *Trichoderma viride, Aspergillus niger, Mucor* spp etc. These fungi can produce cellulase enzyme and hence the complex polysaccharide can be converted to simple fermentable sugars. Further these sugars can be utilized by yeasts such as *Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Candida, Hansenula* spp etc., for the production of ethanol. Always there is a demand for bioethanol because of the problems associated with petroleum based fuels. Recently people have started using edible crops to produce ethanol by fermentation reactions but the world is already in food crisis for some underdeveloped countries. Therefore, the use of wastes such as vegetable wastes, paper waste, waste from sugar cane industry are gaining interest to produce bioethanol. The lignocellulosic biomass has the advantage of highly abundant, being economical in nature, reduced greenhouse gases emissions and does not have the socioeconomic issues regarding the use of food crops. All these factors make lignocellulosic biomass, one of the most promising technological approaches available for supplementing the current source of transportation fuel. The biofuel, ethanol production involves some steps such as pretreatment which involves grinding of solid waste, cellulolysis that is nothing but lysis of cellulose into simple sugars by cellulase enzyme and then fermentation by brewers yeasts or bakers yeasts or bacteria which has the ability to produce ethanol (Figs. 1, 2 and 3).

Fig. 1 Sugar cane waste. http://www.thehindu.com

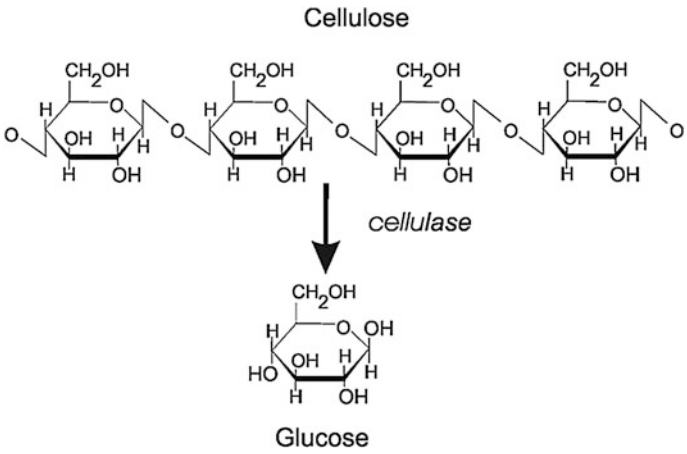


Fig. 2 Steps involved in bioconversion of cellulose to glucose. Jinliang et al. (2013)

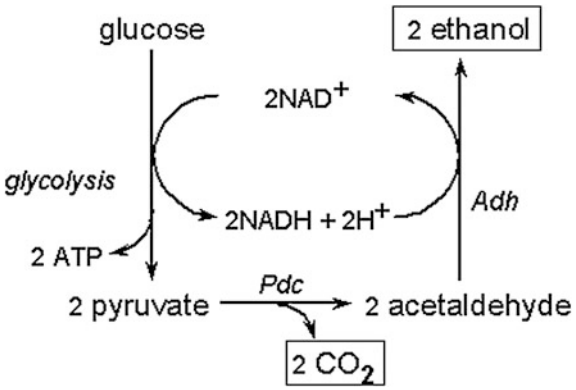


Fig. 3 Steps involved in bioconversion of glucose to ethanol. Paul Held, http://e3f99a4a3891332df177-b511c07cae915b9d1958179eaa4432ea.r82.cf1.rackcdn.com/assets/tech_resources/Beer_Ethanol_Concentration_App_Note.pdf, Determination of Ethanol Concentration in Fermented Beer Samples and Distilled Products

2 Methods

2.1 Production of Bioethanol

A known bio-control agent in the form of formulated powder of *Trichoderma viride* was collected from the farmers of Wayanad, Kerala. Soil sample was collected from paddy fields of Thiruporur area to isolate indigenous *Trichoderma* spp to carry out the comparative study. The unknown fungi were identified by morphological, cultural characteristics and molecular characteristics. Cellulolytic activity of fungi

was examined by using media containing carboxymethyl cellulose. Further cellulase was produced in a broth containing chopped sugarcane bagasse, ground vegetable waste juice as substrates. Amount of cellulase produced by the known and indigenous *Trichoderma viride* were estimated by DNS method. The selected wastes which was already used for cellulolysis was further used for the fermentation by *Saccharomyces cerevisiae*. Production of ethanol was carried out at 28 °C, for 48 h at 250 rpm agitation in a shaking incubator. Amount of ethanol produced was estimated using a known concentration ethanol by spectrophotometric method.

3 Results

3.1 Phenotypic Characterization of Known and Unknown Fungi

The known bio-control agent *Trichoderma viride* collected from the farmers of Wayanad, Kerala was again reconfirmed by phenotypic methods (Fig. 4 and 5). The fungi isolated from the paddy soil sample around Thiruporur, Kanchipuram, Tamil

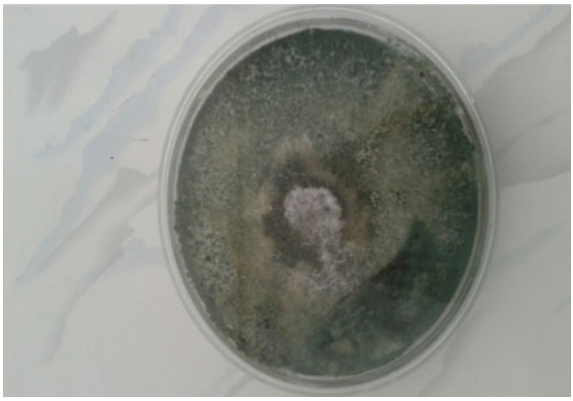


Fig. 4 *Trichoderma viride*—culture on Sabourad's dextrose agar—a strain collected from Wayanad

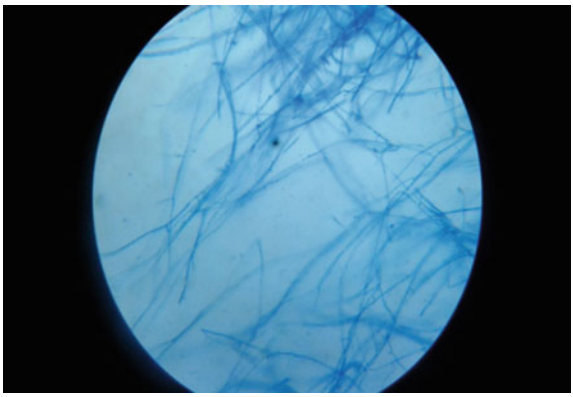


Fig. 5 *Trichoderma viride*—Lacto phenol cotton blue staining—a strain collected from Wayanad

Nadu, was also characterized by phenotypic methods. There were only two morphologically distinct fungi isolated and their morphological and cultural characteristics were analyzed (Figs. 5 and 6). Based on the results, the unknown fungi were identified as *Trichoderma* spp *and Mucor* spp *and Trichoderma* spp.

3.2 *Cultural and Morphological Characteristics of* Trichoderma Viride *Isolated from Paddy Fields of Thiruporur Area*

See Figs. 6 and 7.

3.3 *Identification of Unknown Fungi by Molecular Method Using Gene Specific Primers*

A region of nuclear rDNA, containing the internal transcribed spacer regions 1 and 2 and the 5.8S rDNA gene using a forward primer 5′-TCCGTAGGTGAA

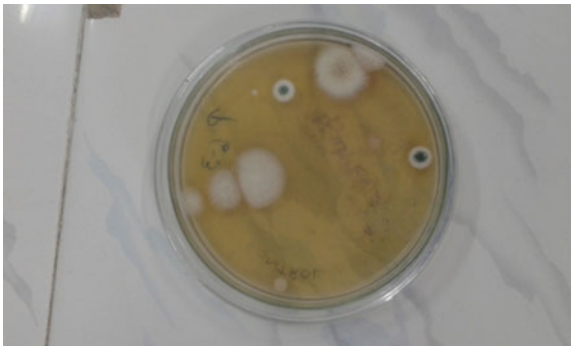


Fig. 6 *Trichoderma viride*—culture on Sabourad's dextrose agar—a strain collected from Thiruorur

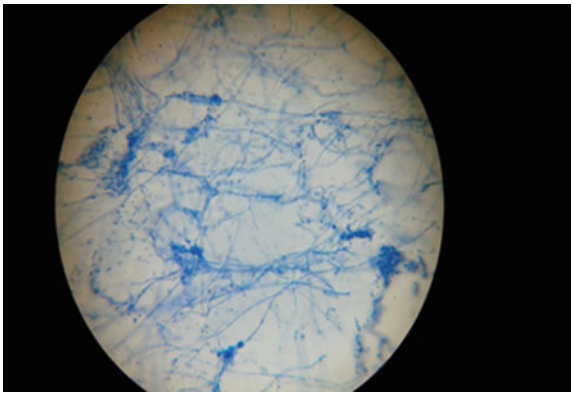


Fig. 7 *Trichoderma viride*—Lacto phenol cotton blue staining—a strain collected from Thiruporur

CCTGCGG-3′ and a reverse primer 5′-TCCTCCGCTTATTGATATGC-3′ and another gene specific for Tef t forward primer 5′-GTGAGCGTGGTATCACCA TCG-3′ and reverse primer 5′-GCCATCCTTGGAGACCAGC-3′ for *Trichoderma* spp were amplified. The phenotypically characterized *Mucor* spp and *Trichoderma* spp were subjected to PCR amplification. *Mucor* spp failed to be amplified whereas *Trichoderma* spp was amplified and the molecular weight of the amplified product for ITS1 was found to be 450 bp and TEF1 was 259 bp.

3.4 Cellulolytic Activity of Standard Trichoderma Viride

The qualitative method of cellulolytic property of the known and indigenous fungi were carried out and the results were positive. (Figs. 8 and 9).

Both the fungi were inoculated on carboxymethyl cellulose containing media. Congo red was flood into the media and discolored area indicates cellulolytic activity of the fungi.

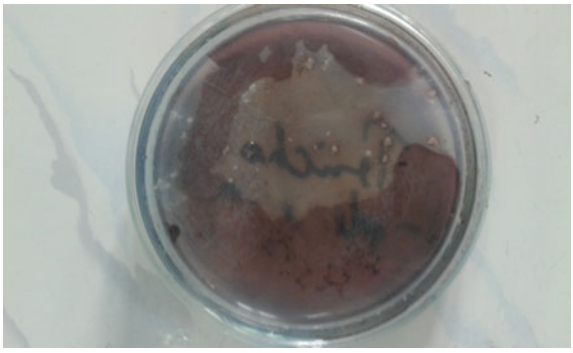


Fig. 8 Cellulolytic activity of standard *Trichoderma viride*

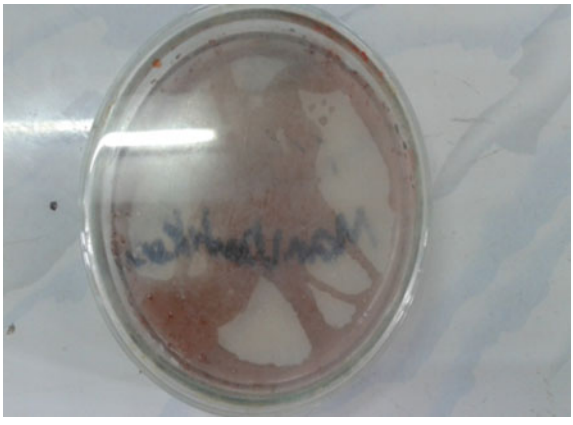


Fig. 9 Cellulolytic activity of *Trichoderma viride* isolated from Thiruporur

Table 1 Cellulase production by species according to substrate

S. no	Substrate	Incubation period in days	*Trichoderma viride*		*Trichoderma* spp KT	
			Glucose in mg	Cellulase U/ml	Glucose in mg	Cellulase U/ml
1.	Carboxymethyl cellulose	3rd day	340	396.6	170	198.3
2.	Vegetable waste	3rd day	310	361.6	200	233.3
3.	Sugarcane bagasse	6th day	400	466.6	210	245

3.5 Cellulase Production and Estimation by DNS Method

Cellulase was produced by the known and indigenous *Trichoderma viride* and the amount of cellulase was estimated by DNS method. Amount of cellulase liberated was calculated by using the following formula and results were tabulated (Table 1).

$$\text{Cellulase activity U/ml} = \frac{(\text{CG} - \text{CRB})\text{XD}}{10\text{XV}}$$

CG Reading from the standard curve for sample µg
CRB Reading from the standard curve for reagent blank
D Dilution factor for the sample
10 incubation time in minutes
V Volume of sample

Maximum amount of cellulase activity was archived on 3rd day for the substrates Carboxymethyl cellulose and Vegetable waste whereas it was 6 days for sugarcane bagasse. The conversion rate is expressed in percentage in Fig. 10.

3.6 Production of Bioethanol

The saccarified broth was used as a substrate for the production of bioethanol by the fungi *Saccharomyces cerevisiae* and the amount of ethanol produced was estimated by spectroscopic method using known standards and the results were depicted in figure number 8. Maximum quantity was gained on the 6th day of incubation period by both candidates. The known fungi showed better result when compared to the unknown in all the three substrates and sugarcane bagasse was found to be a good substrate when compared to be other substrate in terms of product yield but the duration was longer than the other two substrates. (Table 2 and Fig. 11, 12, 13 and 14).

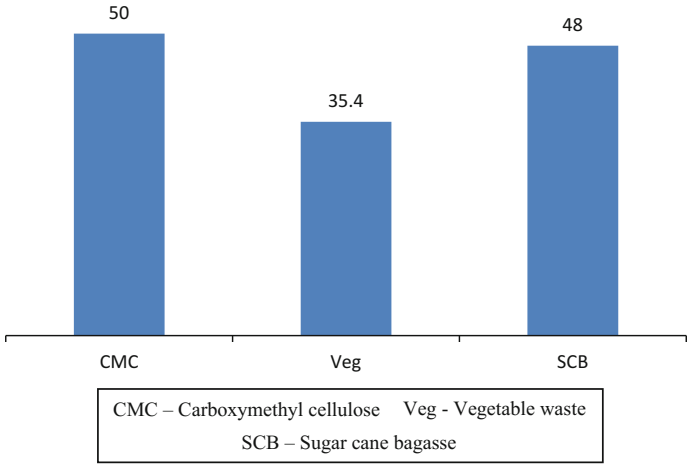
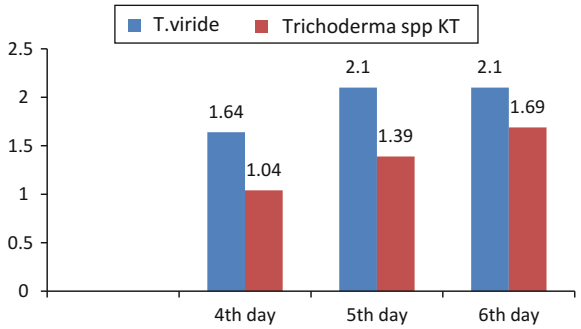


Fig. 10 Cellulase production ability of *Trichoderma viride* in compared to *Trichoderma* spp KT —Rate difference in %

Table 2 Percentage production yield according to substrate

S. no	Substrates	*T. viride* in %	*Trichoderma* spp KT in %
1.	Carboxymethyl cellulose	70	54.6
2.	Vegetable waste	70	63.3
3.	Sugarcane bagasse	100	83.3

Fig. 11 Bioethanol from carboxymethyl cellulose



4 Discussion

Bindu Elizabeth Koshy et al. (2014) analyzed the cellulolytic properties of thermo tolerant *Trichoderma viride and* grown on variously pre-treated sugarcane bagasse. CMCase and FPase enzyme activities were determined as a measure of suitable

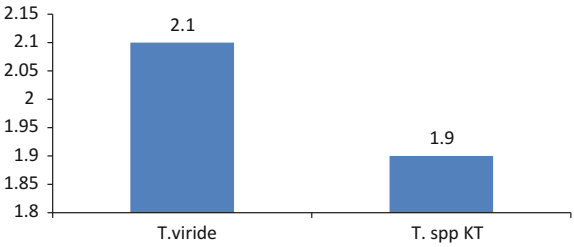


Fig. 12 Bioethanol from vegetable waste in ml/3 ml (6th day)

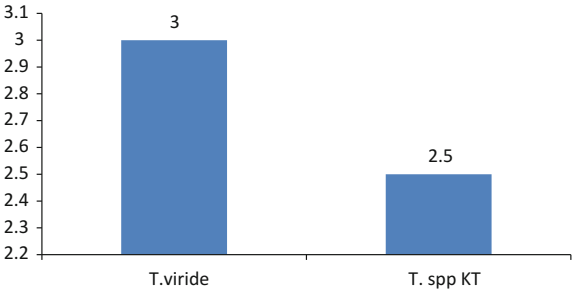


Fig. 13 Bioethanol in sugarcane bagasse in ml/3 ml (6th day)

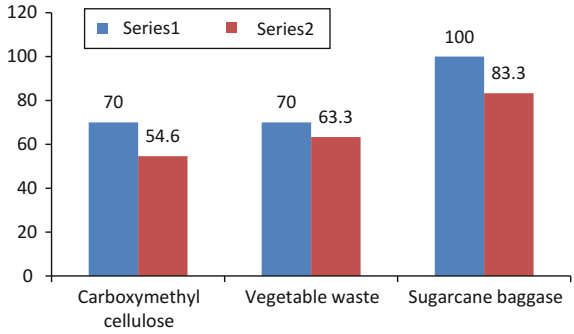


Fig. 14 Percentage conversion of substrates into ethanol

substrate pre-treatment and optimum condition for cellulolytic enzyme production. They have reported that the highest CMCase and FPase activity was found to be 1.217 and 0.109 U/ml respectively under the production conditions of 200 rpm, pH 4.0 and 50 °C using steamed NaOH treated bagasse as substrate. In our study we have found out that the formulated strain *T. viride* could able to produce 396.6 U/ml in carboxymethyl cellulose media, 361.6 U/ml in vegetable waste broth and 466.6 U/ml in sugar cane bagasses. The indigenous *Trichoderma* spp KT could also produce higher amount when compared to the previous study (198.3, 233.3 and 245 U/ml respectively).

Seema et al. (2007) reported that bioconversion offers a cheap and safe method of not only disposing the agricultural residues, but also it has the potential to convert lignocellulosic wastes into usable forms such as reducing sugars that could be used for ethanol production. This paper reports a preliminary study on the microbial pretreatment and fermentation of the agricultural residues like wheat straw, rice straw, rice husk and bagasse. A combination of six different fungi obtained from screening were used for pretreatment and *Saccharomyces cerevisiae* (NCIM 3095) was used for carrying out fermentation. In case of Wheat straw and rice straw, pretreatment with *Aspergillus niger* and *Aspergillus awamori* and fermentation yielded highest amount of ethanol (2.5 gl-1 and 2.2 gl-1 respectively), for rice husk and bagasse it was with *Aspergillus awamori* and *Pleurotus sajor-caju* (8.5 gl-1 and 9.8 gl-1 respectively).

The production of ethanol from different substrate by co-culture of *S. cerevisiae* and *Trichoderma* spp gives the good yield in bioethanol production. The ethanol was produced from the different substrate by using two enzymes namely Amylase from *Aspergillus niger* and Cellulase from *Trichoderma viride* to hydrolyse the starch and cellulose present in the raw materials. The hydrolysed and filtered extracts were fermented using *Saccharomyces cerevisiae.* The used substrate are cellulase base media, vegetable waste and bagasses. All those produces the better amount of cellulase and also it produces the good quantity of bioethanol. Among these bagasse shows the best result in the production of bioethanol *Trichoderma viride* (100%) and indigenious *Trichoderma* (83.3%) while comparing to vegetable waste substrate *Trichoderma viride* (70%) and indigenious *Trichoderma* (63.3%) and carboxy methyl cellulose *Trichoderma viride* (70%) and indigenious *Trichoderma* (54.6%).

5 Conclusion

It can be concluded that ligno-cellulosic waste materials such as vegetable waste from domestic kitchen & sugarcane waste have the capability to undergo acid and enzymatic hydrolysis and fermentation to produce bioethanol. Ligno-cellulosic wastes are rich in cellulose thus can be used to produce cellulosic ethanol. Ligno-cellulosic wastes consist of high amount of glucose which can be converted to bioethanol. Use of ethanol as a fuel can reduce greenhouse gas emission thus reduces air pollution. Fuel ethanol reduces the dependency on the fossil fuels by reducing the use of petroleum for automobile transportation. Apart from the bio-control ability of *Trichoderma viride*, its synergistic interactions with *Saccharomyces cerevisiae* can play a significant role in bioconversion of waste into a renewable energy.

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Efficient Hydrolysis of Lignocellulosic Biomass: Potential Challenges and Future Perspectives for Biorefineries

Gunjan Mukherjee, Gourav Dhiman and Nadeem Akhtar

Abstract Dwindling of petroleum-based fuels together with their frightening environmental effects has enforced the emergence of lignocellulose-based biorefineries. Being most abundant and bio-renewable, lignocellulosic biomass holds enormous potential for production of biofuels, bio-based chemicals and materials for a sustainable energy future. Utilization of lignocellulosic materials in a biorefinery requires a well-designed pre-treatment technology with reasonable processing cost for deconstruction of the lignocellulose complex. Current technologies rely on chemical, physico-chemical and biochemical conversion routes for effective hydrolysis of lignocellulosic materials. Identification of novel enzymes and microbes to counteract the pre-treatment-induced inhibitory products is a prime area of research as chemical detoxification method carries financial constrains. Valorization of biomass is greatly influenced by generation of co-products in biorefining processes and their selective recovery, which may considerably reduce the cost involved in biofuel production. The lab-to-industry transition of a bioprocess technology necessitates a sound techno-economical evaluation for optimum product yield to hit the market place.

Keywords Biofuels · Biorefinery · Hydrolysis · Pre-treatment

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1 Introduction

The growing energy demand and dependence on fossil fuels have raised severe concerns over the depletion of energy resources, increase in fuel prices and effects on climate change. The current energy crisis intensifies the interest for the establishment of biofuel-based biorefinery using lignocellulosic biomass as raw material. Lignocellulose-dependent bioenergy can lessen petroleum-based fuel dependence and green house gas (GHG) emissions (Sanderson 2011; Barakat et al. 2015). To decipher the mystifying effect of global warming, utilization of diverse biomasses need to be globalized at industrial-scale to attain a sustainable economy. An inexpensive, widely available and bio-renewable nature of lignocelluloses allocates a good platform for production of biofuels and other bio-based commodity products. Lack of environmental risk and low capital investment make lignocelluloses more promising over fossil fuels (Akhtar et al. 2016b). Efforts are continuously being made in this direction for production of bioethanol (Koradiya et al. 2016; Zhuang et al. 2016), biobutanol (Kumar et al. 2014), and biodiesel (Ghosh et al. 2016; Liu et al. 2014) using biomass as a raw material through different biochemical conversion routes. Furthermore, delineation of physico-chemical characteristics of leaf litter biomasses has also been studied for their potential use in biorefinery (Akhtar et al. 2016a).

Lignocellulose is composed of cellulose, hemicellulose, lignin along with some proteins, lipids, and several other inorganic materials (Kim 2013). The major component of lignocellulosic biomass is cellulose (30–50%), followed by hemicellulose and lignin, which are usually in the range of 15–35 and 10–20%, respectively. Cellulose rich lignocellulosic material is the preferred candidate for production of biofuel (Isikgor and Becer 2015) and other bio-based polymers due to its inherent property of bio-compatibility and bio-degradability (Ahn et al. 2012). Encapsulation of cellulose in a hemicelluloses-lignin matrix limits the cellulose accessibility for enzymatic actions, which hinders its efficient bio-conversion to monomeric sugars (Meng and Ragauskas 2014).

On the contrary, production of biofuels, bio-based chemicals and materials for a sustainable energy future is a matter of debate. Considering economic feasibility, lignocellulosic biomass can easily be grown and harvested at a much lower cost over food-valued feedstocks such as corn starch and sugar cane (Isikgor and Becer 2015). Furthermore, a huge amount of agricultural and agro-industrial waste is frequently being disposed affecting human and environmental health. Thus, research is on its peak to address these concerns across the globe by using diverse biomasses as suitable substitutes for production of renewable oil and green monomers. Management of inhibitory products is another big challenge to obtain a good quality product with high yields, while minimizing the expenses on raw material, enzymes and microbial inoculants (Jönsson and Martín 2016).

Cellulose-to-biofuel industry could be an alternative to replace petroleum derived products due to their feasibility to convert lignocelluloses to bioenergy and other green chemicals (Adsul et al. 2011). However, development of the

technologies involved in conversion of lignocellulosic biomass to green chemicals and polymers remains a big challenge (Barakat et al. 2015). To manipulate the physico-chemical characteristics of the biomass, the development of a compatible, inexpensive and eco-friendly pre-treatment technology is a prerequisite for an established biorefinery.

2 Pre-treatment Strategies for Lignocellulosic Biomass

The key characteristics of an efficient and reliable pre-treatment technique are to facilitate the biocatalytic reactions on plant polysaccharides with reasonable processing cost and feeble effect on the environment. During the past few years, a large number of pre-treatment techniques have been studied and reviewed for hydrolysis of lignocellulosic biomass (Akhtar et al. 2016b; Alvira et al. 2010; Chaturvedi and Verma 2013; Kim et al. 2016; Li et al. 2016; Sindhu et al. 2016). Crystallinity, degree of polymerization, particle size, accessible surface area, lignin and moisture content of lignocellulosic biomass often limits deconstruction of lignocellulosic complex (Akhtar et al. 2016b).

Physico-chemical characteristics of different leaf litter biomasses were also studied to delineate the chemistries involved in biofuel production (Akhtar et al. 2016c). Addressing these issues, research needs to focus its attention to scout an efficient pre-treatment technique which uses low doses of enzymes and less processing time with minimum inhibitory products to obtain an optimal product yield. Recent development in pre-treatment techniques such as physical, chemical, thermo-physical, thermo-chemical and biological (Fig. 1) have been discussed for their performance and pre-treatment severity. Furthermore, integrative uses of chemical reagents and machinery for pre-treatment have also been highlighted.

2.1 Physical Pre-treatment

2.1.1 Physical Comminution

Physical pre-treatment is focused to increase the accessible surface area for the enzymes to act upon cellulose and hemicelluloses to obtain monomeric sugars. Various physical methods viz. grinding, chipping or milling, microwave irradiations are used for enhancing enzymatic hydrolysis of the biomass (Taherzadeh and Karimi 2008). Mechanical disintegration (grinding, chipping or milling) can significantly reduce the size of lignocellulosic material to different size range (μm–nm), thereby reducing crystallinity and increasing accessible area for the enzymatic actions (Yeh et al. 2010a). There are different means of size reduction of the biomass like hammer milling, ball milling, two-roll milling, colloid milling, wet-disk milling and vibro-disk milling, vibratory ball milling, jet milling and

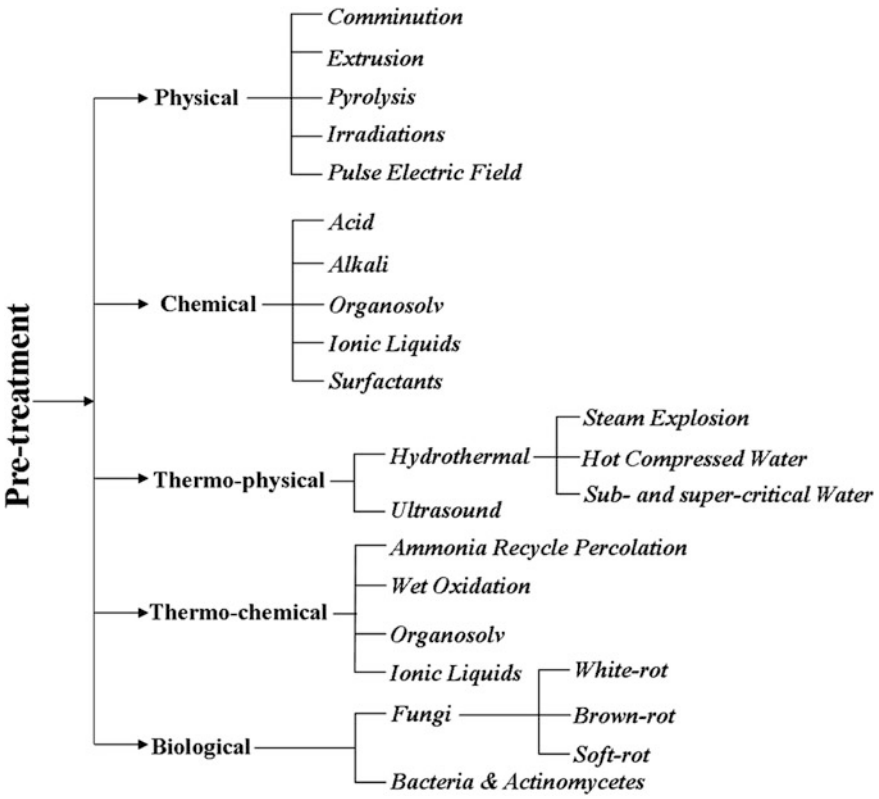

Fig. 1 Commonly used pre-treatment techniques for lignocellulosic biomass

centrifugal milling (Taherzadeh and Karimi 2008). Similarly, reduction of the biomass to nanofibrils increases the surface area to a greater extent and results in a complete enzymatic digestion in reasonable time (Martin-Sampedro et al. 2012). The drawback of these methods lies in the requirement of huge amount of energy, which is economically not viable (Taherzadeh and Karimi 2008). However, comparative study of different milling methods comes with the conclusion that the vibratory ball milling is more efficient than that of the simple ball milling, jet milling and centrifugal milling when applied to sugarcane bagasse biomass (Licari et al. 2016).

2.1.2 Extrusion

Another approach for physical pre-treatment is extrusion, where lignocellulosic biomass is heated, mixed and sheared simultaneously to reduce the size of the cellulose fibrils for efficient enzymatic hydrolysis (Karunanithy and Muthukumarappan 2010). The extrusion pre-treatment of soyabean hull resulted in

95% conversion of cellulose to glucose thereby (Yoo et al. 2011). Pre-treatment of sweet sorghum bagasse using extrusion technique at 100 °C and 200 rpm with 30% moisture showed 70% recovery of fermentable pentoses and hexoses (Heredia-Olea et al. 2015).

2.1.3 Pyrolysis

The decomposition of lignocellulosic biomass at very high temperature (>300 °C) into H_2 and CO along with residual char is the new pyrolysis technique, used as a pre-treatment strategy (Akhtar et al. 2016c). The residual char is rich in glucose and other fermentable sugars, which can be recovered after leaching with water or mild acid treatment and can effectively support the microbial growth for fermentation (Sarkar et al. 2012). Studies have revealed that 80–85% of cellulose converts to fermentable sugars, 50% of which is the glucose recovered by the mild acid leaching of residual char resulted from the lignocellulosic pyrolysis (Bonelli et al. 2001). In a newer combined approach, the formic acid-containing microwave-assisted catalytic pyrolysis was found to be very effective in disruption of the lignocellulosic structure for enhanced enzymatic hydrolysis (Feng et al. 2016).

2.1.4 Irradiation

Pre-treatment using irradiation technology is effective when it is used along with other treatment methods, as it cannot reduce the particle size and is less effective in deconstruction of lignin-hemicellulose matrix. It has been reported that the heat-assisted chemical pre-treatments are less effective than that of microwave-assisted chemical pre-treatment (Moretti et al. 2014). Similarly, γ-ray combined with mechanical crushing or chemical pre-treatment significantly increase the enzymatic hydrolysis (Duarte et al. 2012). However, high cost involved in these processes limits the technique for industrial use (Taherzadeh and Karimi 2008). A pre-treatment study of sugarcane bagasse with γ-radiations followed by mild acid pre-treatment has been shown to increase the enzymatic hydrolysis by 30% (Duarte et al. 2012).

The application of microwave-assisted pre-treatment technologies requires a techno-economical survey to explore its viability for industrial use. Microwave-based irradiation pre-treatment can be employed for heat-assisted chemical pre-treatment for diverse biomasses. The microwave pre-treatment with high boiling solvents, ionic liquids, alkali, salts and acids at atmospheric pressure have been found to have a quite significant result (Li et al. 2016). Liquids of high boiling point are being used in the breaking of lignin bonds and therefore enable the easy degradation of lignocellulosic materials. The combined pre-treatment using microwave and glycerol has resulted in degradation of 5.4% of lignin and 11.3% of the xylan in sugar cane bagasse (Moretti et al. 2014). Similarly, ionic liquid with microwave results in 25% of cellulose dissolution, microwave with

alkali yields almost 100% of cellulose dissolution while the dilute acid microwave-acid pre-treatment yields 48.3% of sugar liberation in hyacinth biomass (Li et al. 2016). Surfactant-assisted microwave pre-treatment of *Eucalyptus globulus* leaf litters also showed 93% increase in reducing sugar as compared to the native biomass (Akhtar et al. 2016a). The combined pre-treatment using different chemical (acid, alkali, surfactant etc.) in presence of microwave is the prime focus of the researchers for lignin removal to achieve high reducing sugar yield. The microwave alkali pre-treatment for 5 min removed 90% of lignin from sugarcane bagasse, however, 0.665 and 0.83 g/g dry weight of reducing sugar was recovered using microwave-alkali and microwave-alkali-acid pre-treatment, respectively (Binod et al. 2012).

2.1.5 Pulse Electric Field

Pulse electric field pre-treatment technique is usually employed for pre-treatment of different biomass for structural alteration (Akhtar et al. 2016b). Application of a potential difference to the lignocellulosic biomass results in increase in porosity, hence allows easy access to the enzymes for hydrolysis (Kumar et al. 2011). The basis of pulse electric field is that it increases the membrane permeability, therefore, other than pre-treatment it has a vital role in the phytochemical extraction and production of various value-added products in a biorefinery (Golberg et al. 2016).

2.2 Chemical Pre-treatment

Chemical pre-treatment of lignocelluosic biomass is done by various means such as acid, alkali, oxidative delignification, organosolv, ionic liquids and surfactants.

2.2.1 Acid

Acid pre-treatment potentially helps in breaking the glycosidic bonds of hemicelluloses and cellulose, thereby providing more exposed carbohydrates for hydrolytic activity. Both concentrated and dilute acids could be used for pre-treatment studies. Since concentrated acids severely damage the cellulosic part and incur more chemical cost, weak acids are preferably used. Different acids like H_3PO_4, H_2SO_4 and HCl have also been used for the pre-treatment studies (Alvira et al. 2010), but H_2SO_4 and HCl in the diluted form are the most widely explored in currently employed cost-effective pre-treatment strategies. Dilute H_2SO_4 (0.5–2.5%) pre-treatment of *Moringa olifera* empty pods at higher temperature (100–200 °C) resulted in 87% of cellulose recovery followed by its 80% of enzymatic hydrolysis in the fermentable sugars (Hernandez et al. 2013). Similarly, 80% cellulose conversion in *Jatropha curcas* fruit shells was observed for enzymatic hydrolysis after

dilute H_2SO_4 (0.5–2.5%) pre-treatment at 110–150 °C (Garcia et al. 2014). Other than mineral acid, various organic acids have also been employed for pre-treatment due to their lignin dissolving properties. Pre-treatment of *Miscanthus x giganteus* with formic acid and acetic acid resulted in 75.3% enzymatic conversion of cellulose with nearly 80% lignin removal (Vanderghem et al. 2012). As the acid pre-treatment is the only commercially exploited pre-treatment technology, its combination with other advance methods is now being potentially utilized in biomass-based biorefinery. For instance sono-assisted acid pre-treatment has lead to effective lignin degradation with minimal inhibitor formation. While the sugar generation was 0.465 g/g on enzymatic hydrolysis and the fermentation efficiency was found to be 71.03% (Sindhu et al. 2016).

2.2.2 Alkali

The alkali pre-treatments mainly focus on the removal of hemicelluloses and lignin. There are various alkaline reagents which are used for this purpose among which NaOH and $Ca(OH)_2$ are the most commonly used. The alkali results into either swelling of the cellulose thereby reducing its crystallanity or by dissolving the lignin and hemicelluose, allowing enzymes to bind effectively with the substrate. The cellulose can swell at low temperature (Cai and Zhang 2005) and therefore reduction in crystalline nature of the cellulose takes place, which allows the enzymatic hydrolysis (Zhao et al. 2008). However, pre-treatment at elevated temperature ranges in the presence of NaOH result into dissolution of lignin and hemicellulose fragments (Haque et al. 2012). Barley straw when exposed to NaOH (0.5–2.0%) for 10 min at 105 °C resulted in nearly 80% lignin removal and 87% sugar liberation after enzymatic treatment. Similarly, $Ca(OH)_2$ pre-treatment of corn strover at 55 °C for 16 weeks resulted in 48% lignin removal, while more effective lignin removal (58–88%) was observed with oxidative lime treatment under similar conditions (Kim and Holtzapple 2005). Green liquids (Na_2CO_3 and Na_2S) are also being used for alkali pre-treatment and showed 45% lignin removal in corn strover and liberated 70% sugar as a result of enzymatic hydrolysis (Gu et al. 2012). One of the most important features of this pre-treatment method is that it selectively degrade the lignin without affecting carbohydrate part. On the other hand, increase in membrane porosity is another advantage for effective enzymatic hydrolysis (Kim et al. 2016).

2.2.3 Organosolv

The lignin dissolving properties of organosolvents are very favorable for effective enzymatic hydrolysis as it increases the pore size for an effective enzymatic attack. There are many organic solvents like ethanol, methanol, acetone etc. which have been employed for the enzymatic conversions. A 99% cellulose conversion in wheat straw was observed by enzymatic hydrolysis after ethanol pre-treatment

while it was 87% for willow wood (Park et al. 2010). Another organosolv pre-treatment called atmospheric glycerol organosolv (AGO) had shown promising results in the pre-treatment procedures, and was able to remove 70% of lignin and hemicelluloses; also the production of the inhibitory compounds was significantly reduced (Sun et al. 2016a). The pre-treatment yields highly amorphous and pure cellulose for the enzymatic hydrolysis, which signifies the effectiveness of the pre-treatment.

2.2.4 Ionic Liquids (ILs)

Ionic liquids (ILs) are mainly salts with organic cations and inorganic anions having variable properties depending upon the proportion of the cation and anion present. The ionic liquids are having one remarkable properties of dissolving the cellulose and lignin forming a multitudinous product mixture (Zavrel et al. 2009). Their property of being non-toxic, less volatile, odor free and easy recovery make it an important green solvent for pre-treatment purposes (Ibrahim et al. 2015). The cellulose generated as a result of the dissolution is more amorphous and hence more susceptible to the enzymatic degradation (Zhao et al. 2009). Various ionic liquids which have been used for different lignocellulosic substrates and different outcomes in term of biomass degradation have been observed. For instance [EMIM]Ac (1-ethyl-3-methylimidazolium acetate) is one of the most widely exploited ionic liquid for the pre-treatment purpose when applied to the wheat straw and wood chips, which showed an enhancement of cellulose digestion by 50% (Fu and Mazza 2011) and lignin dissolution by 50% (Moniruzzaman and Ono 2012). Another important ionic liquid [(BMIM)Cl] [(1-butyl-3-methylimidazolium)Cl] has also showed better pre-treatment properties when rice straw was treated for 5 h at 120 °C, complete conversion of glucose was observed. Whereas, the pre-treatment of rice straw with NMMO (N-methyl-morpholine-N-oxide) results in 96% glucose conversion (Fu and Mazza 2011). Similarly, [EMIM]-OAc (1-ethyl-3-methylimidazolium acetate) pre-treatment of sugarcane bagasse resulted in 32% of lignin removal (Qiu et al. 2012), resulting in significant enzymatic hydrolysis. To get a deeper insight of IL efficacy in their native or aqueous form, it is assumed that that the aqueous solutions of ILs have better pre-treatment capacity than that of native form. For instance, the aqueous solution of [BMIM]-$MeSO_3$ (1-butyl-3-methylimidazolium methanesulfonate) has shown better pre-treatment capacity for switchgrass (Gary et al. 2014). Therefore, it could be concluded that the application of ILs in lignocellulosic biomass pre-treatment is quite impressive.

2.2.5 Surfactant

An important chemical mean of the pre-treatment is usage of surfactants (polyethylene glycol, Tween 80, Tween 20, sodium dodecyl sulfate, Triton, dodecyltrimethylammonium bromide etc.) which could be assisted with other

pre-treatments. Surfactants cause structural alterations in the lignocellulosic biomass, which enable the enzymes to hydrolyze the cellulose and hemicelluloses without their unwanted adsorption on lignin (Putro et al. 2016). Combination of surfactants with microwave pre-treatment has also been applied for a significant hydrolysis (Akhtar et al. 2016a). A 33% increase in cellulose content was observed in pre-treated biomass of *Eucalyptus globulus* using surfactant-assisted microwave pre-treatment as compared to native state (Akhtar et al. 2016a).

2.3 Thermo-Physical

Thermo-physical pre-treatment is considered as an effective combined pre-treatment method in biomass processing. Physical disruption of the biomass in temperature-dependent manner has very high efficiency in lignocelulose-based biorefineries. A high temperature with sudden release of pressure effectively degrades the biomass and permits an easy enzymatic hydrolysis.

2.3.1 Hydrothermal Pre-treatment

Steam Explosion

The most common type of thermo-physical pre-treatment is steam explosion, where the lignocellulosic biomass is subjected to saturated steam under high pressure and temperature for few seconds to minutes. The sudden release of pressure triggers instant explosion of the lignocellulosic biomass, thereby increasing the surface area and porosity for effective enzymatic hydrolysis (Pan et al. 2005). At elevated steam temperature (210 °C for 10 min), enhanced enzymatic hydrolysis reaction was observed (Horn et al. 2011). In a recent study, pre-treatment of canola straw using steam explosion technique resulted into increase in glucose yield by 153.22% as compare to native biomass (Garmakhany et al. 2014).

Hot Compressed Water

The use of hot compressed water by keeping the temperature below critical (above 200 °C) at different pressure is one of the recent pre-treatment approaches. It is having two important properties of acting as both solvent and reaction medium. The hot compressed water pre-treatment is a two step-process since cellulose and hemicelluose dissolve under different conditions. There have been various investigations where the efficiency of this method has been explored (Abdullah et al. 2014). For instance, in a two-step hydrolysis of biomass from *Fagus crenata*, *Nypa fruticus*, Japanese cedar and rice straw yielded 92.2, 92.4, 82.3 and 97.8% biomass hydrolysis, respectively, when treated at 230 °C and 10 MPa pressure for 15 min

followed by 270–280 °C and 10 MPa pressure for 15 min (Ogura et al. 2013; Rabemanolontsoa and Saka 2016).

Sub-and Super-critical Water

When temperature and pressure of water is increased from critical level, it becomes super-critical and non-condensable gas like stage. However, when the temperature and pressure of water remains near the critical point it is called as sub-critical water. Due to change in the temperature and pressure at sub- and super-critical levels, the ionic product of water changes. In turn, the ionic products increase the hydrolytic activity of it. Therefore, it can be employed in pre-treatment applications (Saka 2001). The ester and ether linkages in lignocellulosic biomass can be degraded and economically important low molecular weight chemicals can be recovered using this method. In super- and sub-critical water, the hydrolysis reaction is very fast and it require a very precise control of time (in seconds) to avoid any degradation. Almost 100% of hemicelluloses and 65% of cellulosic hexoses were recovered when the pre-treatment was applied for 0.19 and 0.22 s in wheat bran substrate (Cantero et al. 2015). While the drawback with this method is that prolonged treatment yield the various inhibitory products like HMF (5-Hydroxymethyl-2-furaldehyde), erythrose, glycoaldehyde etc. (Antal et al. 2000). Therefore, more precision is required for the development of technology to avoid generation of inhibitory products.

Hydrothermal pre-treatment is widely applied to the lignocellulosic biomass usually at temperature range of 160–240 °C. Whereas, temperature above 240 °C usually results in severe cellulose degradation (Sun et al. 2014). When hydrothermal pre-treatment was given to the bamboo biomass at 200 °C for 120 min, 75% of the cellulose was hydrolyzed into glucose which was only 15.7% with untreated one (Xiao et al. 2014). Also, the hydrothermal treatment increases the surface area in beechwood, therefore, increasing the enzymatic hydrolysis to 70% of the weight than 7% hydrolysis in untreated biomass (Nitsos et al. 2013). The hydrothermal pre-treatment is of more interest as it is of relatively low cost and known to generate very low concentration of inhibitors in direct hydrolysis of lignocellulosic material.

2.3.2 Ultrasound

Ultrasonication has profound effect on loosening the structure of biomass in order to increase the surface area. A high intensity of the ultrasonic waves has a cellulolytic effect and therefore generates the ultra or nano particles for enhanced enzymatic hydrolysis (Siqueira et al. 2010). Studies have shown that the pre-treatment process of ground saw dust using ultrasonic treatment of 10 W/kg of saw dust for 5–10 min increased glucose yield to 61%. Therefore, an increase in the enzymatic hydrolysis by ultrasonic treatment is evident and could be further investigated for its economic viability (Revin et al. 2016).

2.4 Thermo-Chemical

Unlike other pre-treatment, thermo-chemical treatments have also shown very promising results in hydrolysis of the lignocellulosic biomass. The action of heat brings the chemical changes in the feedstocks to enhance the hydrolytic tendency.

2.4.1 Wet Oxidation

Amongst different oxidative pre-treatment methods, wet oxidation is the one which employs oxidation at high temperature. In this method the lignocellulosic biomass is treated at high temperature in presence of water for a very short span of time. The pre-treatment is usually very effective in dissolution of hemicelluloses and lignin. Lignin is likely to be oxidized in various phenolic compounds and other aliphatic carboxylic acids while hemicellulose forms various monomeric sugars and oligosaccharides (Martín et al. 2007). However, direct oxidation using oxidative reagents have also resulted in the potential delignification, and nearly 89% enzymatic hydrolysis was obtained after O_3 pre-treatment of wheat straw, while in case of rye straw it was 57%, making the process vital (Sun et al. 2016b).

2.4.2 Ammonia Recycle Percolation (ARP)

Ammonia recycle percolation (ARP) is a pre-treatment method which employs the use of ammonia in a fixed-bed reactor operated in a flow-through mode. It was mainly developed for pre-treatment of hardwood and herbaceous biomasses (Kim et al. 2006). The ARP is advantageous as it flows out the dissolved lignin and prevents its deposition on the surface of the biomass and therefore prevents the inhibition. The shortcoming with this process is greater loss ($\sim$50%) of the hemicelluloses, which need to be addressed.

2.4.3 Ammonia Fiber Explosion (AFEX)

The usage of liquid ammonia at high temperature and pressure for pre-treatment fails to remove hemicelluloses and lignin completely, however, it reduces the cellulose crystallanity and triggers high enzymatic hydrolysis even at low enzyme loading. Other than this it relocate the lignin and hemicelluloses which further provide more exposure to cellulose for hydrolysis. The AFEX pre-treatment at 100 °C to bermudagrass resulted in 98% of the cellulose hydrolysis by enzymes while the structural component remained the same (Lee et al. 2010). Similarly, the pre-treatment of switch grass yielded 93% glucan conversion (Alizadeh et al. 2005). Also, a 100% cellulose hydrolysis was seen when corn strover was pre-treated by AFEX (Alizadeh et al. 2005). The CO_2-added ammonia explosion pre-treatment of

rice straw is another novel approach for biomass hydrolysis and it resulted in 93.6% glucose recovery when treated at 14% ammonia concentration and 2.2 MPa CO_2 pressure for nearly 70 min (Cha et al. 2014). Therefore, ammonia-mediated pre-treatments provide another promising pre-treatment approach with greater enzymatic hydrolysis and high sugar liberation from the lignocellulosic biomasses.

2.4.4 Supercritical CO_2

Supercritical CO_2 (SC-CO_2) has emerged as an important industrial solvent because of its availability at lower cost and non-toxic nature. The SC-CO_2 behaves both like gases and liquid, and can diffuse through inter-spaces and can solublize the solids too. It gets into the cellular spaces of the biomass and release the CO_2 in an explosive therefore increases the surface area and increases the enzymatic hydrolysis. Also the formation of carbonic acid in the presence of water further hydrolyses the lignocellulosic biomass (Alvira et al. 2010). Further, the presence of moisture also enhances the enzymatic hydrolysis of the biomass. Use of ethanol as co solvent further increases the lignin dissolution in turn increases the enzymatic hydrolysis of corn stover (Lv et al. 2013). Use of SC-CO_2 in comparison to ionic liquids is a relatively cheaper route for the pre-treatment technologies and both of them are green liquid therefore produces no harmful chemicals and safe for the pretreatment purpose. However, the application of high pressure is needed in SC-CO_2 treatment which it is not required in case of IL-assisted technique (Gu et al. 2013).

2.5 Biological Pre-treatment

Biological pre-treatment is considered to be an advantageous approach in order to achieve a low-cost conversion process in eco-friendly manner. The use of various fungi such as white-rot, soft-rot and brown-rot have been employed for this purpose. White-rot fungi are the saprophytic fungi which cause lignin degradation and forms white-rot in wood, various species like *Phenerochaete crysosporium*, *Pycnoporus cinnabarinus*, *Ceriporia lacerate* etc. are being used for the lignocellulosic pre-treatment. Similarly, soft-rot fungus *Daldinia concentric* and brown-rot fungus *Serpula lacrymans, Coniophora puteana* have also been mentioned for the lignocelulosic biomass pre-treatment (Akhtar et al. 2016b). The most commonly used fungi fall under the white-rot category, which are known to produce cellulolytic and lignolytic enzymes (Alvira et al. 2010). The production of lignin degrading enzymes from these fungi result in the selective degradation of lignin. There are various enzymes produced from different organisms which are capable of degrading lignin for example laccase (Lac), lignin peroxidases (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP) etc. (Akhtar et al. 2015; Kumar and Wyman 2009). When rice straw was treated with white-rot fungus *Pleurotus ostreatus,* 41% of lignin, 48% of hemicelluloses and 17% of cellulose was degraded after 60 days

of incubation, out of which, 52% of the holocelluose (cellulose and hemicellulose) and 44% of cellulose was hydrolyzed by the enzymatic treatment (Taniguchi et al. 2005). The only drawback of this process is its longer duration of the pre-treatment which limits its usage in large-scale industrial applications. Similarly, many bacterial species like *Bacillus* sp. AS3 (Akhtar et al. 2013), *Bacillus circulans* (Singh et al. 2008) are capable of saccharifying different lignocellulosic biomass which could substitute use of fungi to obtain fermentable sugars. Also, there is development of genetically modified organisms like *Pichia pastoris* with the enhanced enzymatic activity for the bioethanol production have been studied (Shin et al. 2015). Other than bacteria and fungi, actinomycetes have been long known for their cellulolytic and lignolytic potentials, for instance *Streptomyces badius* 252 and *Thermomonospora mesophila* DSM 43048 are few of the important species with huge cellulose degrading potential (Godden et al. 1992).

Although all the pre-treatments are having their unique properties in terms of product yield and biomass recovery, while their combined effect may trigger even better results. For instance, the hydrothermal pre-treatment with alkaline peroxide increased the lignin degradation by 60% in comparison to the hydrothermal pre-treatment (Cuevas et al. 2014). Whereas several other pre-treatments have been employed in combination to others, like SC-CO_2 and ultrasonic combined pre-treatment, biological pre-treatment with mild acid, dilute acid with alkali etc. have resulted higher lignin degradation and sugar recovery than when applied alone (Sun et al. 2016b).

3 Enzymatic Hydrolysis of Lignocellulosic Biomass

A direct enzymatic hydrolysis of the lignocellulosic biomass is not efficient enough for transition of a technique from lab-to-industry. Therefore, pre-treatment strategies become a pre-requisite for effective hydrolysis of the biomass and subsequent fermentation processes. In addition, there are several factors which may be structural or enzymatic that affects the hydrolytic mechanism of the lignocellulosic biomass (Alvira et al. 2010).

3.1 Cellulose Crystallinity and Degree of Polymerization

The cellulosic component of the biomass exists in two different forms in the lignocellulosic biomass i.e. crystalline and amorphous. An amorphous nature of the cellulose is more suitable for enzymes to act upon it, while higher crystalline cellulose is less prone to digestion. The size of the crystalline cellulose ranges from 3 to 18 nm as it is only digested from the surface which results in lesser degradation (Zhao and Chen 2013). The initial hydrolysis is strictly limited due to crystallinity

of the cellulose while the regenerated cellulose is more prone to enzymatic hydrolysis due to the loss of the same after pre-treatment.

3.2 Hemicelluose-Lignin Matrix

The close association of hemicelluloses and lignin with cellulose contributes towards hindering the enzymatic hydrolysis of lignocellulosic materials. The content and distribution of them mainly affect hydrolytic mechanism involved. Lignocelluosic substrate with lesser proportion of hemicelluose and lignin are easily acted upon by cellulose degrading enzymes and vice versa (Alvira et al. 2010). The enzymes usually bind non-specifically to the non-cellulosic moieties like hemicelluloses and lignin which ultimately reduces the hydrolytic efficacy (Pan et al. 2005). Therefore, lignocellulosic biomass necessitates pre-treatment to remove the hindrance, making cellulose available for enzyme hydrolysis (Mussatto et al. 2008).

3.3 Surface Area and Pore Size

Accessibility of large surface area for the enzymes to act upon certainly enhances the biomass hydrolysis. Availability of more accessible surface area entirely depends upon the particle and pore size. A fine lignocellulosic particle certainly enhances the glucose yield (Yeh et al. 2010b). When the particle size is reduced to nano-scale, available surface area for hydrolytic enzymes for its degradation significantly increases which resulted in the enhanced digestion of the biomass in a shorter period of time (Martin-Sampedro et al. 2012). On the other hand, the larger pore size of the substrate also enables the easy entrance of the enzyme and its other component for the hydrolytic activity therefore enhances the overall deconstruction of the biomass (Sun et al. 2016b).

3.4 Enzymes Inhibitors

The presence of various oligosaccharides, glucose, cellobiose, soluble phenolic compounds, weak acid and furfurals act as potent enzyme inhibitors (Andric et al. 2010), which reduce the enzymatic degradation. The binding of cellobiose and cellulose at their active binding sites for cellobiohydrolase and cellulase, respectively is affected owing to steric hindrance (Zhao et al. 2004). The pre-treatment-induced various compounds are somehow related to inhibition of enzymes and/or microbes (Table 1).

Table 1 Pre-treatment-induced inhibitory products from different biomasses and their impacts

Biomass type	Pre-treatment	Impact	Reference(s)
Soft wood	Acid	– Generation of phenylic compounds, furans, aliphatic carboxylic acids, furfurals and aliphatic aldehydes – Inhibits the enzymatic activity and microbial growth	Larsson et al. 2000, Fengel and Wegener 1989)
Wheat straw	Alkali	– Generation of lactic acid, dihydroxy acids, dicarboxy acids and formic acid – Microbial growth inhibition during fermentation	Ben'ko et al. 2013
Wheat straw, sugarcane bagasse	Wet oxidation	– Generation of phenolic compounds, vanillic and syringic acid, furonic acids and carboxylic acids – Inhibition of enzymatic hydrolysis	Martín et al. 2007, Ben'ko et al. 2013
Populus nigra and *populus maximowiczii*	Organic solvent	– Generation of furfurals – Inhibition of enzymatic hydrolysis	Kim and Pan 2010
Wood chips	Ozonolysis	– Generation of acetic acid, formic acid, oxalic acid, glyoxalic acid and tartaric acid – Microbial growth inhibition	Ben'ko et al. 2013

4 Pre-treatment-Induced Inhibitory Products

The role of pre-treatment strategy is to trigger the hydrolysis and fermentation of the lignicellulosic biomass by removing lignin and hemicelllose or by reducing the crystallanity of the feedstock. But there is a generation of various other inhibitory chemicals generated as a result of lignin and hemicelluloses dissolution put serious hindrance in the bioconversion process. In this segment, we will discuss the various inhibitory products generated as a result of different pre-treatments.

4.1 Acid Pre-treatment-Based Inhibitory Products

The acid-based pre-treatment methods result in the formation of various by-products as result of hemicelluloses and lignin degradation. The hydrolysis of hemicelluloses give rise to pentose sugars and uronic acids, which thereafter undergoes dehydration, resulting into formation of furfural, while dehydration of hexose sugars gives rise to the HMF, which further converted to formic acids and levulinic acids (Jönsson and Martín 2016). Like HMF, furfural also gets degraded into formic acid and resins (Danon et al. 2013). The studies have shown that the concentration of these acids come around 50–100 mM, which results in the

inhibition of microbial growth during saccharification of the biomass. The presence of furfural and HMF resulted in inhibition of the microbial growth at the concentration of 40 and 50 mM, respectively (Larsson et al. 1999). The acid pre-treated lignin results in the formation of various phenolic compounds, most common of them are vanillin, 4-hydroxybenzoinc acid, coniferyl aldehydes and Hibbert ketones (Mitchell et al. 2014). These lignin-derived aromatic compounds mainly contribute towards significant inhibition of the microbial growth as compared to the other aldehydes generated. Their presence in small concentration significantly affects the microbial growth during saccharification process. There are other aromatic compounds released during the acid pre-treatment which are mainly the ones already present in the plants and get extracted. Secondary metabolites such as tannins, benzoic acids, benzoyl alcohols, cinnamic acids mainly contribute towards the inhibition of the cellulases (Jönsson and Martín 2016). The formation of quinines and small aliphatic aldehydes as a result of acid pre-treatment, is a potent microbial growth inhibitor even in small concentrations (Larsson et al. 2000).

4.2 Alkaline Pre-treatment-Based Inhibitory Products

As discussed in the alkali pre-treatment techniques, it mainly works for dissolution of the lignin, which results in the formation of carboxylic acids. In addition, the formation of saccharinic acid takes place as results of peeling reaction of different polysaccharides present. The formation of lactic acid, dihydroxy acids, dicarboxy acids and formic acids (Fengel and Wegener 1989; Klinke et al. 2002) have also been reported. These acids adversely affect the growth of microorganisms during fermentation process.

4.3 Oxidative Pre-treatment-Based Inhibitory Products

The sole idea of oxidative pre-treatment is the reduction of cellulose-lignin linkages and reducing crystal nature of cellulose. There are different pre-treatment methods like alkaline peroxide method, ozonolysis and wet oxidation which all fall under this category of the pre-treatment. Oxidation of the lignin and carbhydrates give rise to different phenolic compounds and furan aldehydes which are the inhibitors of enzymes and microbial growth, respectively. The phenolics compounds are further oxidized to furonic acids and carboxylic acids. The more oxidation results in the formation of vanillic and syringic acid (Martín et al. 2007; Klinke et al. 2002; Ben'ko et al. 2013). Although the extent and type of inhibitory by-products depend upon the type of biomass and pre-treatment method opted.

5 Feasibility to Counteract the Product Inhibition and Recovery Issues in Biorefineries

In order to tackle the formation of inhibitory products after pre-treatment of the lignocellulosic biomass various strategies has been employed. In this section we will discuss the different approached to minimize the pre-treatment-induced inhibitory products and counter act the product inhibition.

5.1 Selection of Appropriate Feedstock

The composition of different components of the lignocelluloses (cellulose, lignin and hemicelluloses) varies with nature and ecological niche of the biomasses. Therefore, the lignocellulosic biomass with low recalcitrance could be used for potential substrate to obtain green fuels and bio-based co-products. For instance, the mild pre-treatment of the Miscanthus grass (Chiaramonti et al. 2012) and wheat straw (Larsen et al. 2012) results into lower generation of aldehyde (17 g/kg) and phenols (5.1 g/kg), respectively. The biomass from *Populus trichocarpa* tree with low recalcitrance level can also be characterized in search for potential substrate (Studer et al. 2011).

5.2 Detoxification

One of the ways to solve the inhibition problems of the lignocellulosic biomass after processing is detoxification or conditioning. Various techniques are involved for detoxification is use of alkali, reducing agents, polymers, liquid-solid extraction (ion exchange and treatment with activated charcol) and enzymatic treatment (Jönsson and Martín 2016). The drawback with these methods is that one needs to incorporate another step in the process which may further increase the cost and time involved in the process. However, addition of reducing agent (Alriksson et al. 2011) and adsorbent (Cannella et al. 2014) did not hinder the enzymatic activity during the bioconversion process but have been found to enhance the simultaneous saccharification and fermentation (SSF). For an instance the use of sulphur oxyanions and sulphite dithionite is reported to improve the enzymatic hydrolysis (Jönsson and Martín 2016). However the detoxification studies are still a big challenge and needs techno-economic consideration before they enter into the industry.

5.3 Microbial Treatment (Bioabatement)

The microbial treatment for degradation of inhibitors is a viable option and can greatly improve both enzymatic hydrolysis and fermentation of the lignocellulosic biomass (Cao et al. 2015). The major hurdle for the implementation of bioabatement is the time required to counteract the degradation of inhibitory products. Further, the consumption of sugar by the microbes used in bioabatement can reduce the overall yield of the process.

5.4 Culturing Schemes

There are various approaches as discussed earlier are being explored to tackle the inhibition problem, the one is applying various culture schemes. The strategies such as SSF (Hoyer et al. 2010), fed-batch (Olofsson et al. 2010) and consolidated bioprocessing (Olson et al. 2012) are being employed for simultaneous hydrolysis and fermentation. However, the cost involved in designing fermentation process, enzyme loading, inoculums size and time of fermentation under the inhibitory conditions remain the prime concern.

5.5 Microbe Selection

Another possible approach for tackling the inhibitors in processing of lignocellulosics is the screening of a potential microbe which can sustainably act in adverse conditions even in presence of inhibitors. A highly resistant *Saccharomyces cerivisiae* for aliphatic carboxylic acids and furan aldehydes was isolated from the grape winery (Favaro et al. 2013) for efficient fermentation.

5.6 Genetically Modified Microbial Strains

Genetic modification of the microbes for developing the resistance against potential inhibitors generated seems to be a vital option. An ethanologenic *S. cerivisiae* strain was reported with a transgene for laccase from white-rot fungus *Trametes versicolor,* which was found to be highly resistant for pre-treatment-induced inhibitory products (Larsson et al. 2001). Similarly, the genetically modified *Escherichia coli* was found resistant towards furfural compounds and sugarcane bagasse hydrolysate (Wang et al. 2013). The genetically modified *Pichia pastoris* strain was reported to produce more efficient enzymatic complexes and resistant to phenolic inhibitors,

yielded 2.7-folds higher bioethanol than that wild-type strain (Shin et al. 2015). There are several instances where the engineered organisms develop capacity to counteract inhibitors while the chemical toxicity of them is still a very big challenge to attain an product yield.

6 Economic Viability: A Challenge for a Sustainable Biorefinery

The very idea of the biorefinery is the production of an array of economically important intermediate or final products from a process. The lignocellulosic feedstock based biorefinery approach is under rigorous research for various value-added products i.e. primary products or energy driven products. The effective implementation of the biorefinery-based process and product generation is still not very much of a real thing. The high cost of the pre-treatment process, generation of inhibitors, and product recovery are the major roadblock in the technology development. Till date, there is no single pre-treatment process has been found suitable for commercialization (Rabemanolontsoa and Saka 2016). The development of economically viable pre-treatment approaches, effective management of the inhibitors and adequate recovery of the products at lesser cost is more desirable. The current technology which covers all of the three aspects stated above incurs a higher cost. For instance, the microwave pre-treatment of lignocellulosic biomass is having high degree of cellulose hydrolysis while the cost involved in the electricity to generate the microwave is exceedingly high, which makes it economically less desirable. Similarly, the cost of biomass processing in case of ultrasonication is very high in term of equipment cost. Therefore, it can only be employed for high value products. Though some of the pre-treatment process using oxalic acid, alkali at high temperature has been successfully commercialized (Inamdar et al. 2011). The intermediate products during pre-treatment like C5 sugars and lignin- based chemicals, which acts as inhibitor but are important precursors for several industrial products. The challenges in the recovery process and cost involved in the pre-treatment are still a matter of concern for industrial implementations. Hence the one of the most important aspect for practical implementation of the biorefinery is the cost reduction and simultaneous production of wide range of value added products. The direct conversion of lignocelluosic biomass in different value-added products by means of chemical catalysis is also one of an unexplored area of integrated biorefinery approach.

7 Conclusion

Delineation of structural and molecular properties of the biomass remains a bottleneck for a suitable pre-treatment technique owing to diverse nature of the lignocellulosic biomasses. Therefore, it becomes imperative to scout a suitable and low-cost pre-treatment technique for biomass hydrolysis in an integrated biorefinery. Accurate prediction of pre-treatment-induced inhibitory products becomes a toolkit to counteract the mechanism involved in product inhibition to obtain a greater product yield. Furthermore, robust techno-economic evaluation is necessary to make a lab-to-industry transition of a biorefinery process. Lignocellulose-based biorefinery is still in infancy stage for establishment of a concrete technology for biomass-to-green fuels concept. Biorefining process utilizing a zero-value cellulosic biomass as a feedstock for production of advanced biofuels, biochemicals and biomaterials is a valuable insight for market-driven strategy to mitigate the future energy crisis.

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Optimization of Media Components for Production of Polyhydroxyalkanoates by *Ralstonia eutropha* Using Paddy Straw as Cheap Substrate

J. Aravind and M. Sandhya

Abstract To reduce the production cost of polyhydroxyalkanoates (PHAs), the feasibility of using a lignocellulosic substrate—paddy straw as carbon source was examined. The design of an optimum and cheap medium for high level production of PHAs by *Ralstonia eutropha* was attempted by using response surface methodology. Based on the Plackett Burman design, K_2HPO_4 and $MgSO_4$ were selected as the most critical mineral salts. Subsequently the optimum combination of the selected factors and the major carbon source, hydrolysed paddy straw were investigated by the Box–Behnken design.

Keywords Polyhydroxyalkanoates · Straw · *Ralstonia eutropha* · Response surface methodology · Box–behnken design

1 Introduction

Polyhydroxyalkanoates (PHAs) are polymers of esters accumulated in form of intracellular granules by a wide variety of bacterial strains. Only bacteria are reported on synthesis and accumulation of PHA converting the excess carbon under conditions of the nutrient limitation especially nitrogen starvation. Under the influence of carbon source starvation, PHAs are degraded intracellularly by depolymerases and subsequently metabolized (Ojumu et al. 2004). Polyhydroxyalkanoates for several decades have attracted much attention as candidates for biodegradable polymers, because they reflect material properties similar to the conventional petrochemical sourced synthetic thermoplastics and elastomers currently in use (Du et al. 2001a, b). The characteristics of pure Polyhydroxybutyrate (PHB), include in its metric as flexible thermoplastic

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processability and or as blended plastic, hydrophobicity, complete biodegradability and biocompatibility with optical purity suggest that PHB as an suitable alternative to the conventional plastics (Sharma and Mallick 2005). *Ralstonia eutropha* was found to achieve maximum accumulation of PHA, especially Polyhydroxybutyrate (Wu et al. 2001). The large scale industrial production of Polyhydroxyalkanoates is still under slow pace due to the need of high investment. The high cost of production of Polyhydroxyalkanoates render it as an expensive endeavor for larger scale production in spite of its other merits. To make larger scale production of PHA economically viable, the focus gains important on the identification and utilization of renewable carbon sources especially harnessing agricultural residues for viable industrial production of PHA. This work was carried out with an aim to utilize paddy straw (Lignocellulosic substrate) as a cheap carbon substrate and optimize PHA production by *Ralstonia eutropha*. A non-enzymatic, chemical method of straw hydrolysis was performed to remove the lignin content and release the carbon content as simple sugars. The residual white mass obtained was used as the carbon source in the Mineral Salt Media (MSM). Maximal bacterial growth and PHA accumulation were achieved after 72–96 h. In order to optimize the production of polyhydroxyalkanoates by *Ralstonia eutropha* using paddy straw as substrate, various fractional factorial methods was employed to zero in on the compatible contractions of substrates for maximized yield of PHA. In comparison with earlier studies, it was concluded that a notable bacterial growth and PHA accumulation could be achieved by utilizing straw as the carbon source for bacterial growth. The different parameters of PHA production, observed on using straw were near to those observed on supplementation with pure glucose. The statistical model was helpful in determining the optimal conditions of the mineral salt media components for higher yield of PHA.

2 Materials and Methods

2.1 Hydrolysis of Straw

The hydrolysis of straw was carried out by the methods derived from Jenkins (1930), Chen and Yang (1984), Nagle (1985). Paddy straw was dried and coarsely pulverised. The pulverised straw (25 g) was boiled with 100 ml of 10% sodium hydroxide solution; the residue was filtered and washed. The same treatment was followed with 100 ml of 10% hydrochloric acid. This alkali-acid treatment was repeated for about 3–4 times. The washed residue was chlorinated twice using 50 ml of sodium hypochlorite and kept in dark for 15 min. The content was filtered and washed with water and with 250 ml of 2% hydrogen per-oxide. The residue obtained was treated with 100 ml of 80% zinc chloride solution containing 4% hydrochloric acid. The residue was boiled, cooled, filtered, washed thoroughly and dried. The final white mass (hydrolysed straw) obtained was used as the substrate for bacterial growth.

2.2 Bacterial Growth in Modified Mineral Salt Media

Pure culture of *Ralstonia eutropha* MTCC 1472 was obtained from IMTECH, Chandigarh, in freeze dried form. The culture was revived in nutrient broth initially and then grown in a simple medium containing 10 g hydrolysed straw, 5 g glucose and 5 g sodium chloride in 1 L of distilled water. It was finally cultured in a modified mineral salt media containing 10 g hydrolysed straw, 5 g glucose, 5 g sodium chloride, 5 g di-potassium hydrogen phosphate, 1 g potassium chloride, 1 g magnesium sulphate and 1 g ammonium sulphate in 1 L of distilled water. The pH of the media was initially adjusted to 7.5 ± 0.5. The culture flasks were kept in shaker at 150 rpm at 35 °C for 4 days (Du et al. 2001a, b; Amirul et al. 2008; Yamanaka et al. 2010).

2.3 Extraction of PHA

The bacterial culture was centrifuged at 2,800 g to obtain the cell pellets. The cells were treated with sodium hypochlorite for 2 h, whereby, lipids and proteins were degraded. The cell lysate was washed with 1:1 (v/v) mixture of acetone: methanol and finally precipitated with chloroform. The chloroform was evaporated by air drying, to yield dry powder of PHA (Santhanam and Sasidharan 2010).

2.4 Analytical Methods

The extracted PHA was assayed spectrophotometrically by the method of Law and Slepecky (1961) in comparison with the standard PHB acid which was treated with concentrated sulphuric acid, boiled for 10 min and cooled, to yield crotonic acid. Crotonic acid yields absorbance maximum at 235 nm. The PHB mass in the extracted PHA sample was determined by the above method. The intracellular PHA accumulation (%) was estimated as the percentage composition of PHA present in the DCW. The PHB content (%) was estimated as the percentage composition of PHB mass in the dry weight of extracted PHA (Zakaria et al. 2010). The bacterial culture was centrifuged at 2,800 g to obtain the cell pellet which was dried to estimate the DCW in units of gl^{-1} (Du et al. 2001a, b). Residual Biomass was estimated as the difference between DCW and dry weight of PHA extracted (Zakaria et al. 2010) to determine the cellular weight and accumulations other than PHA.

2.5 Plackett Burman Design

The most significant off the seven components in the modified mineral salt media were identified by Plackett Burman Design (Stanbury et al. 1984). The variables for the design were the seven media components (X^{-1}) and the number of runs (X) was thus eight. Hence eight experimental flasks of different high and low combinations of the media components were run in duplicate taking potassium chloride as the dummy variable. The dry weight of PHA was taken to be the yield. The yields of each trial were determined. The F-test values for each component were estimated and those components giving higher F-test values were taken up for further studies.

2.6 Response Surface Methodology

The significant factors for PHA accumulation were identified from Plackett Burman design and Response Surface Methodology was used to determine the optimum concentration of these factors for PHA production. A three level Box Behnken experimental design of 17 experiments was formulated using the Design Expert 8.0 software statistical package, combining response surface modelling and quadratic programming. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The behaviour of the system is explained by the following polynomial equation,

$$\mathbf{Y} = \boldsymbol{\beta}_{\mathbf{o}} + \boldsymbol{\Sigma}\,\boldsymbol{\beta}_{\mathbf{i}}\mathbf{x}_{\mathbf{i}} + \boldsymbol{\Sigma}\,\boldsymbol{\beta}_{\mathbf{ii}}\mathbf{x}_{\mathbf{ii}}^{\mathbf{2}} + \boldsymbol{\Sigma}\,\boldsymbol{\beta}_{\mathbf{ij}}\mathbf{x}_{\mathbf{i}}\mathbf{x}_{\mathbf{j}} + \boldsymbol{\varepsilon} \tag{1}$$

where, Y is the predicted response, i.e. the dry weight of PHA, β_0 is the constant coefficient, β_i is the ith linear coefficient of the input factor x_i, β_{ii} is the ith quadratic coefficient of the input factor x_{ii}, β_{ij} is the different interaction coefficients between the input factors x_i and x_j, and ε is the error of the model (Box and Behnken 1960). For this study, the independent variables were coded as A, B and C, and thus the equation could be described as,

$$\mathbf{Y} = \boldsymbol{\beta}_{\mathbf{o}} + \boldsymbol{\beta}_{\mathbf{i}}\mathbf{A} + \boldsymbol{\beta}_{\mathbf{i}}\mathbf{B} + \boldsymbol{\beta}_{\mathbf{i}}\mathbf{C} + \boldsymbol{\beta}_{\mathbf{ii}}\mathbf{A}^{\mathbf{2}} + \boldsymbol{\beta}_{\mathbf{ii}}\mathbf{B}^{\mathbf{2}} + \boldsymbol{\beta}_{\mathbf{ii}}\mathbf{C}^{\mathbf{2}} + \boldsymbol{\beta}_{\mathbf{ij}}\mathbf{AB} + \boldsymbol{\beta}_{\mathbf{ij}}\mathbf{AC} + \boldsymbol{\beta}_{\mathbf{ij}}\mathbf{BC} \tag{2}$$

Experiments were conducted in 250 ml erlenmeyer flask containing 100 ml of media (pH 7.0) prepared according to the design. The culture flasks were kept in shaker at 150 rpm at 35 °C for four days and PHA was extracted. The dry weight of PHA extracted (gl^{-1}) was taken as the response.

Table 1 Results of analysis of bacterial growth, PHA accumulation and PHB quantification

S. No.	Parameters observed at the end of fourth day	Inference
1	Dry cell weight	19.2 gl^{-1}
2	Dry weight of extracted PHA	7.21 gl^{-1}
3	Intracellular PHA accumulation	37.55%
4	PHB mass in the extracted PHA sample	5.19 gl^{-1}
5	PHB content in the extracted PHA sample	71.98%
6	Residual biomass	11.99 gl^{-1}

3 Results and Discussion

3.1 Bacterial Growth and PHA Accumulation

The results of *R. eutropha* DCW (gl^{-1}), dry weight of extracted PHAs (gl^{-1}), intracellular PHA accumulation (%), PHB mass in the extracted PHA sample (gl^{-1}), PHB content in the extracted PHA sample (%) and residual biomass (gl^{-1}) were given in Table 1. It was shown by Du et al. (2001a, b) that a DCW of 27.1 gl^{-1} was achieved in *R. eutropha*, on the supplementation with pure glucose. The DCW obtained in the current study, using straw was 19.2 gl^{-1}. Further, the PHA accumulation in the same bacteria by utilising pure glucose was found to be 47–55% (Du et al. 2001a, b) whereas in this study it was observed that the utilisation of straw as carbon source to grow *R. eutropha* has led to a reasonable PHA accumulation of 37.55% (Sandhya et al. 2013).

3.2 Evaluation of Important Mineral Salts Influencing PHA Accumulation from Plackett Burman Design

It was inferred (Tables 2 and 3) that the hydrolysed straw, with a f-test value of 23.8, made to be the most significant factor in the Mineral Salt Media upon bacterial growth and PHA accumulation. Next to which was di-potassium hydrogen phosphate (11.15) making the second most significant factor. The third level of significance was attributed to magnesium sulphate (4.66). The nitrogen source ammonium sulphate did not show much influence. Hence the response surface methodology was designed with the three factors, hydrolysed straw, di-potassium hydrogen phosphate, magnesium sulphate. In general *R. eutropha* under nitrogen limiting condition accumulates carbon in the form of PHA (Keshavarz and Roy 2010; Khosravi-Darani et al. 2013).

Table 2 Plackett Burman experimental design matrix for evaluating factors influencing PHA accumulation by *Ralstonia eutropha*

Run	Factors	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Dry weight of PHA (gl^{-1})
	Variables	HS	G	NaCl	KCl	K_2HPO_4	$(NH_4)_2SO_4$	$MgSO_4$	
	Levels of variables (gl^{-1})	$H^{-1}0$ L^{-4}	H^{-5} L^{-1}	H^{-5} L^{-1}	H^{-1} L^{-1}	H^{-1} $L^{-0.5}$	H^{-1} $L^{-0.5}$	H^{-5} $L^{-2.5}$	
1		H	H	H	H	H	H	H	7.62
2		H	L	L	L	H	L	H	7.12
3		L	L	H	L	L	H	H	5.24
4		L	H	L	H	L	H	H	5.5
5		H	H	H	L	H	H	L	6.96
6		H	L	L	H	L	L	L	6.36
7		L	H	H	H	H	L	L	4.9
8		L	L	L	L	L	L	L	3.16

HS—Hydrolysed straw; G—Glucose; H—High; L—Low

Table 3 Statistical analysis of Plackett Burman design

	X_1	X_2	X_3	X_4	X_5	X_6	X_7
$\sum$ (H)	28.06	24.98	24.72	24.38	26.6	25.32	25.48
$\sum$ (L)	18.8	21.88	22.14	22.48	20.26	21.54	21.38
Difference	9.26	3.1	2.58	1.9	6.34	3.78	4.1
Effect	2.315	0.775	0.645	0.475	1.585	0.945	1.025
Factor mean square	10.71	1.201	0.832	0.45	5.02	1.78	2.10
f-test	23.8	2.66	1.848	1	11.15	3.95	4.66

3.3 Response Surface Methodology

The Box Behnken three factor experimental design matrix with actual and predicted responses were shown in Table 4. In the present work, only two-way interactions were investigated. Linear, two-factor interaction (2FI), quadratic and cubic models were used to analyze the experimental data in order to obtain the appropriate regression equations. For determination of the adequacy of models depicting PHA accumulation and selection of a satisfactory model for the same, the sequential model sum of squares and the model summary statistics, were studied, as tabulated in Table 5. It could be seen that the p values for quadratic model was lower than 0.01 and Design Expert 8.0 suggested the model to be significant. As a natural log transformation was applied to the experimental data, the interaction of two factors (2FI) and the linear model were suggested to be insignificant using the response surface methodology.

Table 4 Experimental Box–Benken three factor design matrix with response

Run order	Factor A: hyd. straw (gl^{-1})	Factor B: K_2HPO_4 (gl^{-1})	Factor C: $MgSO_4$ (gl^{-1})	Response: PHA (gl^{-1}) Actual	Response: PHA (gl^{-1}) Predicted
1	1	5	0.55	3.72	1.72
2	5.5	5	1	8.3	4.74
3	5.5	1	1	4.28	3.8
4	10	5	0.55	7.66	8.14
5	10	3	1	8.14	2.72
6	10	1	0.55	4.82	5.93
7	1	3	1	3.56	4.04
8	5.5	3	0.55	3.8	8.21
9	5.5	1	0.1	2.66	3.21
10	1	1	0.55	2.2	5.23
11	5.5	5	0.1	5.24	4.29
12	5.5	3	0.55	2.64	7.75
13	5.5	3	0.55	2.58	2.89
14	1	3	0.1	2.781	2.89
15	10	3	0.1	6.4	2.89
16	5.5	3	0.55	2.88	2.89
17	5.5	3	0.55	2.56	2.89

Table 5 Selection of a satisfactory model for PHA accumulation—sequential model sum of squares

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Mean versus total	324.0445	1	324.0445			
Linear versus mean	48.72191	3	16.24064	10.50247	0.0009	
2FI versus linear	1.18488	3	0.39496	0.208776	0.8880	
Quadratic versus 2FI	16.27544	3	5.425147	14.37174	0.0022	Suggested
Cubic versus quadratic	1.54673	3	0.515577	1.882217	0.2736	Aliased
Residual	1.09568	4	0.27392			
Total	392.8692	17	23.10995			

3.4 Regression Analysis

Regression analysis was performed to fit the response functions, i.e. percentage PHA accumulation. The regression models developed represent responses as functions of hydrolysed straw (A), K_2HPO_4 (B), $MgSO_4$ (C). An empirical

relationship between the response and input variables in coded terms was expressed by the following response surface reduced 2nd order polynomial equation:

$$\begin{aligned}\mathrm{PHA} = & 2.892 + 1.844875 * \mathrm{A} + 1.37 * \mathrm{B} + 0.899875 * \mathrm{C} + 0.33 * \mathrm{A} * \mathrm{B} \\ & + 0.24025 * \mathrm{A} * \mathrm{C} + 0.36 * \mathrm{B} * \mathrm{C} + 0.904125 * \mathrm{A}^2 + 0.803875 * \mathrm{B}^2 + 1.424125 * \mathrm{C}^2 \end{aligned} \quad (3)$$

This equation revealed how the individual variables or double interactions affected PHA accumulation. Negative coefficient values indicated that individual or double interaction factors negatively affect PHA accumulation (i.e. accumulation percentage would decrease), whereas positive coefficient values meant that factors increase PHA accumulation in the tested range. From the equation, it was observed that all individual factors had a positive effect on PHA accumulation.

3.5 *ANOVA for Response Surface Quadratic Model*

The ANOVA suggested that the equation and the actual relationship between the response and the significant variables represented by the equation were adequate. The larger the value of F and the smaller the value of p, the more significant would be the corresponding coefficient term. The value of p was lower than 0.0004, indicating that the model could be considered as statistically significant. For the accumulation of PHA, the ANOVA results (Table 6) indicated that the F-value for the model was 19.48, implying that the model was significant. Considering each factor, factor A—hydrolysed straw has the greatest F value of 72.13095 and the least p-value < 0.0001, indicating it to be the most significant factor for PHA accumulation. In this study, A, B, C, A^2, B^2, C^2 were significant factors. The other model terms whose p-values were listed as being greater than 0.1000 in Table 6 were not significant factors. The "Lack of Fit F-value" of 1.88 implies the Lack of Fit is not significant relative to the pure error. There is a 27.36% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good and hence the model was found to be significant.

3.6 *Diagnostic Plots*

The data were analyzed to check the normal probability plot and a dot diagram of residuals correlation as shown in Fig. 1. The data points on this plot lay reasonably close to a straight line, lending support to the conclusion that A, B, C, A^2, B^2, C^2 contribute to highly significant effects, A^2, B^2, C^2 contribute to significant effects and that the underlying assumptions of the analysis were satisfied. The data were also analyzed to check the correlation between the experimental and predicted

Table 6 Analysis of variance

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	66.18223	9	7.353581	19.48035	0.0004	Significant
A-Hyd. Straw	27.22851	1	27.22851	72.13095	<0.0001	
B-K_2HPO_4	15.0152	1	15.0152	39.77672	0.0004	
C-$MgSO_4$	6.4782	1	6.4782	17.16138	0.0043	
AB	0.4356	1	0.4356	1.153946	0.3184	
AC	0.23088	1	0.23088	0.611624	0.4598	
BC	0.5184	1	0.5184	1.373292	0.2796	
A^2	3.441861	1	3.441861	9.117823	0.0194	
B^2	2.720905	1	2.720905	7.207941	0.0313	
C^2	8.539503	1	8.539503	22.62197	0.0021	
Residual	2.64241	7	0.377487			
Lack of fit	1.54673	3	0.515577	1.882217	0.2736	Not significant
Pure error	1.09568	4	0.27392			
Cor total	68.82464	16				

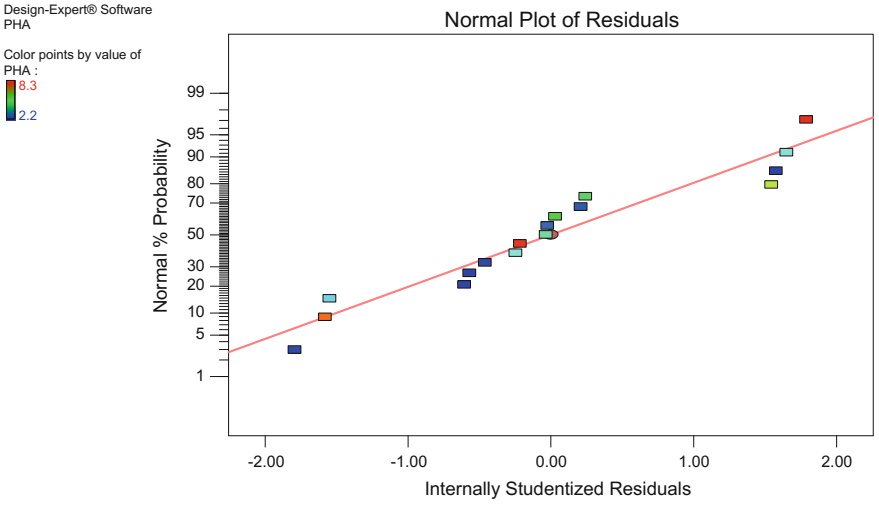

Fig. 1 Normal plot of residuals

percentage removal, as shown in Fig. 2. The experimental values were the result obtained, while the predicted values were obtained by calculation from the quadratic equation. It can be seen from Fig. 2 that the data points on the plot were distributed near to the straight line, indicating a good relationship between the

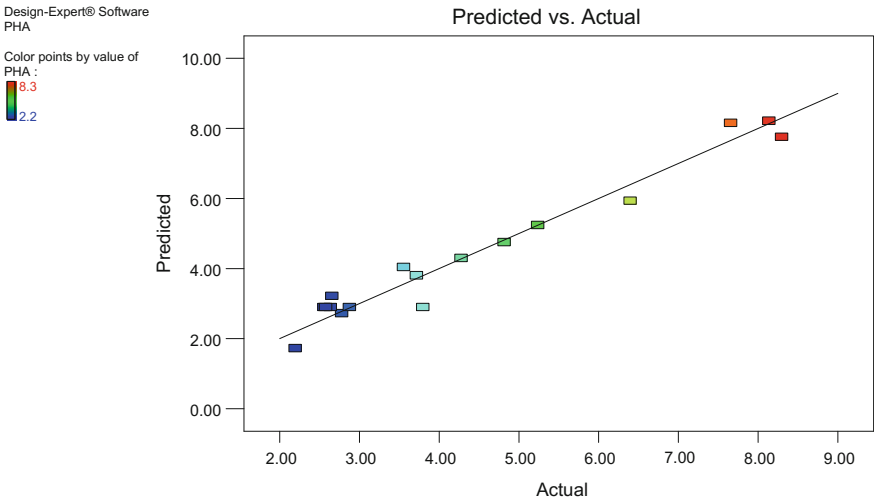

Fig. 2 Plot of experimental and predicted values

experimental and predicted values of the response. The result also suggests that the selected quadratic model was adequate in predicting the response variables for the experimental data.

3.7 Response Surface Plots

The response surface plots of interaction of Hydrolyzed straw with K_2HPO_4 on PHA production, interaction of Hydrolyzed straw with $MgSO_4$ on PHA production, interaction of $MgSO_4$ with K_2HPO_4 on PHA production were shown in Figs. 3, 4 and 5 respectively. Each plot represents a number of combinations of two test variables with the other variable maintained at constant levels. The maximum PHA accumulation was indicated by the surface confined in the smallest curve (circular or elliptical) of the contour plot. In response surface contour plots, the optimum values of variable factors could be analyzed by the saddle point or by determining the maxima formed by the x- and y-coordinates. As seen from the Figures the PHA increases with increasing values of Hydrolyzed straw, K_2HPO_4 and $MgSO_4$. From the fig, it was seen that increase in hydrolysed straw interacted positively with K_2HPO_4 and $MgSO_4$ and increased the PHA accumulation. K_2HPO_4 and $MgSO_4$ did not show to have had such an interaction on each other upon PHA accumulation. The optimal levels of these factors were found to be, 8.5–10.0 gl^{-1} for Hydrolysed straw, 4.0–5.0 gl^{-1} for K_2HPO_4 and 0.75–1.0 gl^{-1} for $MgSO_4$.

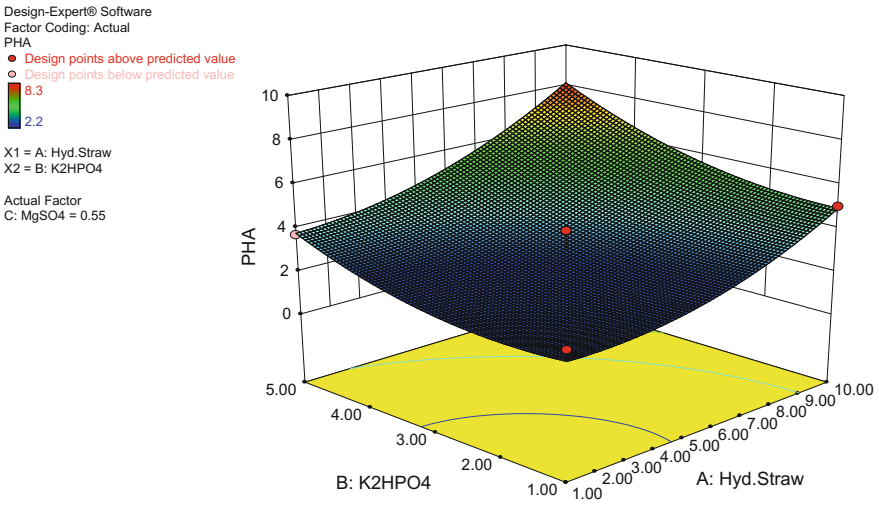

Fig. 3 Interaction of hydrolyzed straw with K_2HPO_4 on PHA production

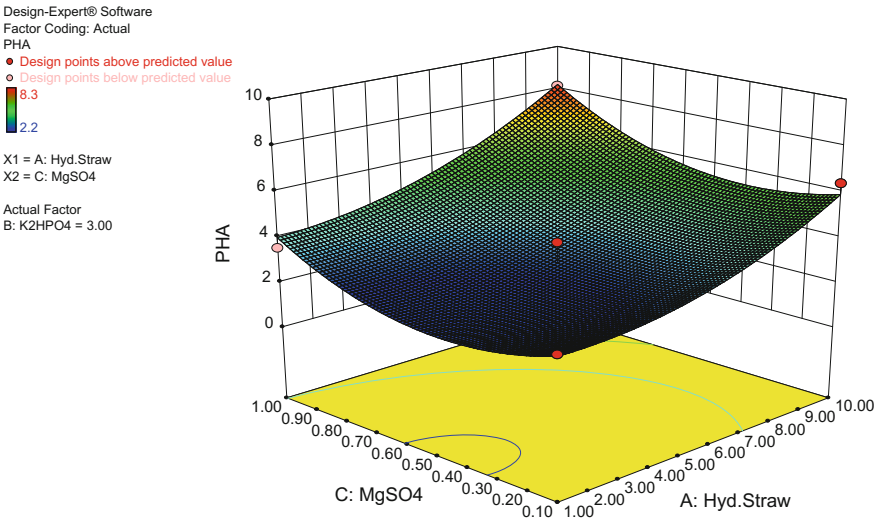

Fig. 4 Interaction of hydrolyzed straw with $MgSO_4$ on PHA production

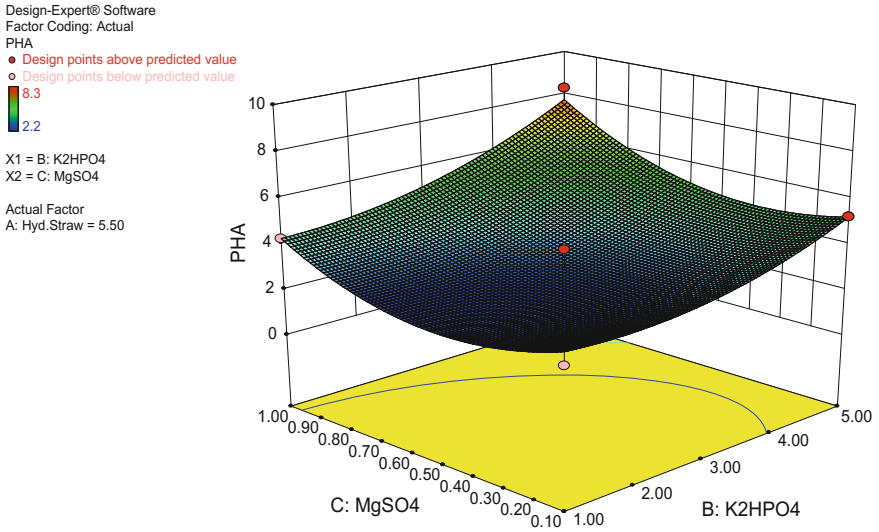

Fig. 5 Interaction of $MgSO_4$ with K_2HPO_4 on PHA production

4 Conclusion

Plackett–Burman design and Box–Behnken design were adopted to screen the key factors and identify optimal culture conditions for polyhydroxyalkanoate production by *R. eutropha*. The results suggested that statistical design methodology offers an inexpensive and reasonable approach for polyhydroxyalkanoate production medium optimization. The proposed model equation illustrated the quantitative effect of variables and also the interactions among the variables on polyhydroxyalkanoate production. Under the optimal medium condition (10 gl^{-1} hydrolysed straw, 4.76 gl^{-1} K_2HPO_4 and 0.69 gl^{-1} $MgSO_4$), the experimental PHA production of 8.3 gl^{-1} perfectly matched with the predicted value of 8.3 gl^{-1}, which verified the practicability of this optimum strategy.

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Statistical Modeling and Optimization of Bioethanol Production from *Parthenium hysterophorus*

Selvarajan Vanitha, Sundaram Vidhya Bharathi and Selvaraju Sivamani

Abstract Potential of producing liquid transportation biofuels is demanding because of depleting fossil fuels. The aim of this study is to produce bioethanol from *Parthenium hysterophorus* by statistical modeling and optimization. Response surface methodology based Box–Behnken design was employed to optimize the pre-treatment of *P. hysterophorus* using tartaric acid. Pre-treatment of plant tissue was confirmed through Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM) and visualization in photographic image. Then, cellulases enzyme was used for hydrolysis of cellulose in cellulignin (cellulose and lignin) and then detoxified with lime. Finally, co-fermentation of *Kluyveromyces marxianus* with *Saccharomyces cerevisiae*, *Fusarium oxysporum*, *Zymomonas mobilis* and *Klebsiella oxytoca* was performed for bioethanol production. Ethanol concentrations of 1.248, 1.195, 1.879 and 1.616% (v/v) were obtained for mixed culture of *K. marxianus* with *Z. mobilis, K. oxytoca, F. oxysporum* and *S. cerevisiae* respectively after 72 h of fermentation. Hence, it is inferred that, the mixed culture of *K. marxianus* and *F. oxysporum* was found to be the best combination for ethanol production from *P. hysterophorus*.

Keywords Pre-treatment · *Parthenium hysterophorus* · Lignocellulosic biomass · Bioethanol

1 Introduction

In the current scenario, fast depletion of the available fossil fuels, the concern about energy security and the threatening global climatic change have led the world market to search for an alternative fuel resource (Wan et al. 2012). Bioethanol has always been considered as a better alternate for petrol as it reduces the dependence

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on crude oil and promises cleaner combustion leading to healthier environment (Ishola et al. 2013). Considering the cost of production and lesser availability of the conventional feedstocks, lignocellulosic materials are found to be promising substrates for the production of bioethanol.

The conventional process of ethanol production from lignocellulosic materials involves pretreatment, hydrolysis and co-fermentation. The pretreatment plays vital role in ethanol production from lignocellulosic materials (Nanda et al. 2014). Hemicellulose in the materials is hydrolysed in pretreatment usually by weak inorganic acids. The hydrolysis of pretreated material is performed by using cellulases (Maeda et al. 2011). Co-fermentation metabolizes hydrolysates of hemicellulose and cellulose to ethanol by pentose- and hexose-fermenting microbes respectively. The widely used pentose fermenting microbes include *Kluyveromyces marxianus, Pachysolen tannophilus*, *Pichia stiptis* etc. *K. marxianus* was found to metabolize hemicelluloses hydrolysate effectively (Grba et al. 2002). Hexose fermenting microbes include *Zymomonas mobilis* and *Saccharomyces cerevisiae*. *Zymomonas mobilis*, a Gram-negative bacterium, produces 1 mol of ATP per mole of glucose consumed by fermentation through Entner-Doudoroff pathway (Rogers et al. 2007). *S. cerevisiae* has a number of disadvantages, despite its extensive use as an ethanologenic organism (Raman and Pothiraj 2008).

Lignocellulosic materials contain hemicellulose, cellulose and lignin in various proportions. *Parthenium hysterophorus* is one of the important lignocellulosic feedstocks because of its perilous nature and abundant availability (Singh et al. 2015). *P. hysterophorus* is an annual plant belonging to the family Asteraceae. It is a noxious weed distributed in many countries which due to its toxic nature poses a serious threat to the society (Singh et al. 2014). Infestations by *P. hysterophorus* include degradation of the natural ecosystem, reduction of pasture growth, allergic responses and respiratory illness in humans (Patel 2011). Thus it is necessary to eradicate the plant from the society in an effective way. Hence, it was attempted in this study to produce bioethanol from *P. hysterophorus.*

The optimization process involves varying one and multiple factors at a time. The former classical design is time consuming and laborious when the number of factors is more than 5 (Myers and Montgomery 2009). The latter statistical design is useful for model development and process parameter optimization in lesser time and experiments (Box et al. 2005). The process parameters can be optimized through screening and response surface designs. The screening design is used to identify significant factors, whereas the response surface method is used to determine the optimal settings of the significant factors.

The objectives of this work are to characterize *P. hysterophorus* to evaluate their potential for the production of renewable bioethanol; and to optimize the production of bioethanol from *P. hysterophorus* by applying response surface methodology (RSM) through batch experiments.

2 Materials and Methods

2.1 Sample Preparation

Fresh plants of *P. hysterophorus* were collected from Cheran Maanagar area of Coimbatore City, Tamil Nadu, India. Green healthy plants were chopped into pieces and dried in a hot air oven at 60 °C to constant weight. Dried biomass was then carefully grounded to about 250 μm size using ball mill and stored in cleaned and dried zip-lock cover for experimental studies.

2.2 Characterization of P. Hysterophorus

Plant material was characterized for total soluble sugars, starch, hemicellulose, cellulose and lignin.

2.3 Pretreatment of P. Hysterophorus Using Organic Acid

Monocarboxylic (Formic), dicarboxylic (oxalic) and hydroxyl (tartaric) acids were selected for pretreatment of *P. hysterophorus.* Tartaric acid produced more total reducing sugars. So, about 2.5 g of dried biomass of *P. hysterophorus* was suspended in 1.5% (w/v) tartaric acid with a solid-to-liquid ratio of 0.20 g/mL, and hydrolysed at 121 °C for 15 min. The resultant mixture was centrifuged at 5000 rpm for 5 min. Sugars were extracted from supernatant by repeated washings with distilled water and the hydrolysate was stored for bioethanol production. The reducing sugar content was estimated from the hydrolysate by dinitrosalicylic acid (DNSA) method (Miller, 1959). The residue containing predominantly cellulose and lignin were subjected to enzymatic hydrolysis. Pre-treatment of plant tissue was confirmed through Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM) and visualization in photographic image.

2.4 Enzymatic Hydrolysis

Enzymatic hydrolysis of the pre-treated biomass was performed using sodium-acetate buffer and cellulase. 0.5 mL of enzyme was dissolved in 5 mL, 50 mM sodium acetate buffer of pH 5 was added to the residue of acid pretreated biomass. The reaction mixture was incubated at 50 °C for 30 min and the reaction was arrested by boiling the mixture at 100 °C for 5 min. Then the mixture was centrifuged at 5000 rpm for 5 min. Supernatant obtained was detoxified before

fermentation. Glucose content in supernatant was measured by glucose oxidase-peroxidase (GOD-POD) method (Trinder 1969). Residue was discarded since it contains biomolecules not required for bioethanol production.

2.5 Detoxification

The acid pre-treated hydrolysate and enzymatic hydrolysate of *P. hysterphorus* were detoxified using 0.18% (w/v) calcium hydroxide solution at 50 °C to remove the acid remains, furfural, hydroxymethylfurfural and other inhibitors of ethanol production. $Ca(OH)_2$ solution was added until the pH reached 10 with continuous stirring. Then, the temperature was maintained at 50 °C for 30 min without stirring. After detoxification, the pH of the hydrolysate was adjusted to 6.5 with 0.1 N HCl and further subjected to fermentation (Chandel et al. 2011).

2.6 Co-Fermentation

For ethanol production, mixed cultures include pentose and hexose fermenting organisms were subjected to fermentation. The four different mixed cultures include *Kluyveromycesmarxianus* with *Saccharomyces cerevisiae*, *Fusarium oxysporum*, *Zymomonas mobilis* and *Klebsiella oxytoca*. The fermentation experiments were performed at 30 °C in 250 mL conical flasks containing 100 mL of the detoxified hydrolysate comprising an equal proportion of hemicellulose and cellulose hydrolysates. The initial pH of the medium was adjusted to 5.5. The pre-cultures, inoculated in 250 mL conical flasks containing nutrient media grown at 30 °C in agitated condition, in its mid log phase were used for fermentation. The cells were harvested by centrifugation at 4 °C and 5000 rpm for 10 min and sugar contents were analyzed.

3 Results and Discussion

3.1 Characterization of P. Hysterophorus

Dried sample of *P. hysterophorus* yielded total sugars of 31.91% (w/w). Total sugars is sum of reducing and non-reducing sugars which through experiments yielded 1.66 and 30.25% (w/w) respectively. Starch content of the samples estimated through anthrone method yielded 6.06% (w/w). Through gravimetric analysis, cellulose, hemicellulose and lignin were found to be 37.11, 10.01 and 0.10% (w/w) respectively. These biomolecules form an integral part of any plant system, the contents of which will greatly influence the yield of bioethanol.

3.2 Pretreatment of P. Hysterophorus Using Organic Acid

In order to optimize the reaction conditions of weak acid hydrolysis for bioethanol production, an independent 3-level-3-factorial Box–Behnken design (BBD) was employed. BBD is a type of response surface methodology, which employs multiple regression and correlation analyses as tools to assess the effect of two or more independent factors over a dependent factor. It reduces the number of experimental runs required to generate statistically significant data.

Considering the deleterious effects of the inorganic acids over the environment, organic acids of high pK_a values were chosen for the present study. Monocarboxylic (Formic), dicarboxylic (oxalic) and hydroxy (tartaric) acids were selected for pretreatment of *P. hysterophorus* and studied the amount of total reducing sugars (TRS) released. Based on the results, it was observed that high sugar content was released by tartaric acid because it consist of a carboxylic acid substituted with a hydroxyl group on the adjacent carbon (Fig. 1).

The main factors influencing weak acid hydrolysis of the substrates are acid concentration, contact time, substrate concentration, temperature and volume of the acid. Optimal thermal conditions (121 °C, 15 psi) were maintained for all experiments since the experiment was performed in autoclave. The two other factors, substrate concentration and volume of the acid were correlated into a single factor as solid to liquid ratio (SLR). Hence time t (min), weak acid concentration A (% (w/v)) and solid to liquid ratio S (g/mL) were chosen as the factors for the experimental design. Table 1 shows the independent factors and levels used in this study.

Based on the independent factors and the levels chosen, a Box–Behnken design matrix was constructed for *P. hysterophorus*. The matrix included 15 experimental runs with different combinations of time, tartaric acid concentration and SLR values. This matrix, along with the experimental and the predicted total reducing sugar (TRS) values (g/L) are shown in Table 2.

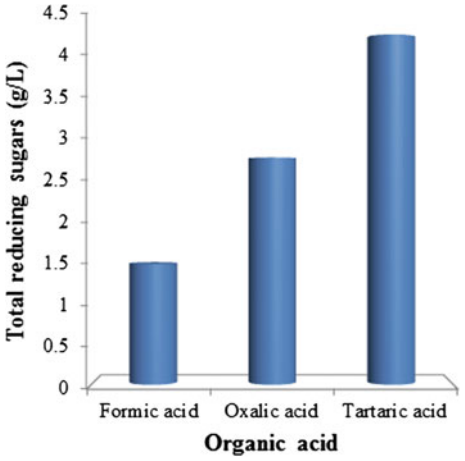

Fig. 1 Effect of organic acids on TRS production for *P. hysterophorus*

Table 1 Independent factors and levels used for Box–Behnken design for *P. hysterophorus*

Factor	Symbol	Unit	Levels		
			−1	0	+1
Time	t	min	5	15	25
Tartaric acid concentration	A	% (w/v)	0.1	0.8	1.5
Solid to liquid ratio	S	g/mL	0.05	0.125	0.20

Table 2 Box–Behnken design matrix for optimization of parameters for *P. hysterophorus*

Std	Run	t(min)	A (% (w/v))	S (g/mL)	TRS (g/L)	
					Experimental	Predicted
2	1	5	1.5	0.125	1.068	0.873
3	2	5	0.8	0.05	0.717	0.721
8	3	15	0.8	0.125	1.183	0.873
7	4	25	1.5	0.125	1.159	0.939
6	5	15	0.1	0.2	0.419	0.806
15	6	25	0.8	0.05	0.624	1.025
5	7	15	0.1	0.05	0.945	0.900
9	8	15	1.5	0.2	1.118	1.116
12	9	25	0.8	0.2	0.924	0.873
1	10	5	0.8	0.2	0.861	0.846
10	11	15	0.8	0.125	1.030	0.780
13	12	25	0.1	0.125	0.809	0.628
4	13	15	0.8	0.125	1.190	1.051
14	14	15	1.5	0.05	0.254	0.694
11	15	5	0.1	0.125	0.786	0.964

Analysis of variance (ANOVA) for the response surface quadratic model for *P. hysterophorus* is provided in Table 3. The coefficients of the response surface quadratic model were also evaluated. The F- and p-values indicate that the linear coefficient of acid concentration is highly significant than the other factors of the model. Thus it can be inferred that tartaric acid concentration is the main factor which influences the overall yield of reducing sugars. ANOVA table of the substrate showed low value for the lack of fit which indicates that the correlation among the reaction parameters lies within the selected range.

The results obtained for the 15 runs were analysed via multiple regression. The coefficients of the full model were evaluated through regression analysis and tested for their significance. Finally, the best fitting model was determined which showed that linear coefficients (A and S), quadratic coefficient (A^2) and cross-product coefficients (tA and AS) were significant.

The surface plots for *P. hysterophorus* are shown in Fig. 2a–c. The effect of different acid concentration and reaction time on weak acid hydrolysis at a constant SLR of 0.125 g/mL is represented in Fig. 2a. At any value of tartaric acid

Table 3 Analysis of variance for selected Box-Behnken design for *P. hysterophorus*

Source	Sum of squares	df	Mean square	F value	Prob > F	
Model	67.20179	9	7.466866	21.3823	0.0018	Significant
t	0.05104	1	0.05104	0.1461	0.7179	
A	27.46887	1	27.46887	78.6605	0.0003	
S	5.589496	1	5.589496	16.0062	0.0103	
t x A	4.9729	1	4.9729	14.2405	0.0130	
t x S	0.227052	1	0.227052	0.6501	0.4567	
A x S	12.58121	1	12.58121	36.0278	0.0018	
t^2	0.06867	1	0.06867	0.1966	0.6760	
A^2	16.26951	1	16.26951	46.5897	0.0010	
S^2	0.254181	1	0.254181	0.7278	0.4325	
Residual	1.746039	5	0.349208			Not significant
Lack of fit	1.311663	3	0.437221	2.0131	0.3489	
Pure error	0.434376	2	0.217188			
Cor total	68.94783	14				

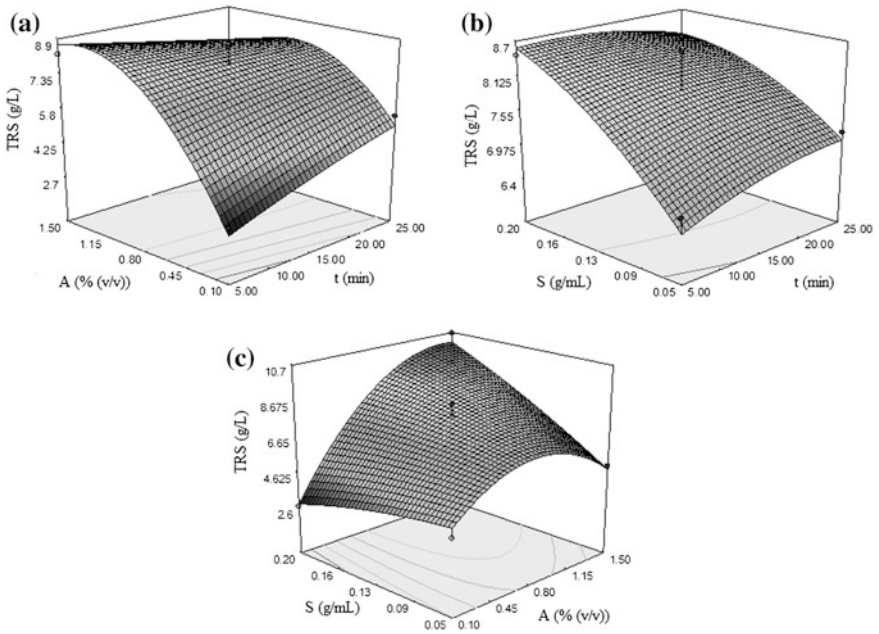

Fig. 2 Response surface plot indicating the effect of **a** tartaric acid concentration and contact time **b** contact time and solid to liquid ratio and **c** tartaric acid concentration and solid to liquid ratio on TRS content in *P. hysterophorus*

concentration between 0.1 and 1.5% (w/v), the total reducing sugar content linearly increased with increase in time. The maximal content of total reducing sugars was observed at an acid concentration of 1.5% (w/v) and low reaction time of 5 min. The effect of different reaction time and SLR on the release of total reducing sugars at constant acid concentration (0.8% w/v) is shown in Fig. 2b. At low reaction time, an increase in SLR resulted in the increased release of total reducing sugars. But with low SLR and less reaction time, TRS yield was significantly low. Figure 2c shows the effect of acid concentration, SLR and their reciprocal interactions on weak acid hydrolysis at a reaction time of 15 min. At the highest acid concentration, 1.5% (w/v), an increase in the SLR enhanced the yield of total reducing sugars.

The final estimated response model for the estimation of TRS content obtained by pre-treatment of *P. hysterophorus* is as follows:

$$[TRS] = 1.45601 + 0.21604 * t + 7.66810 * A + 0.54635 * S - 0.15929 * t * A - 0.31767 * t * S + 33.78095 * A * S - 1.36375E-003 * t^2 - 4.28393 * A^2 - 46.64444 * S^2 \quad (1)$$

From the Eq. 1, TRS is the response factor which is influenced by three independent factors of the experimental design. t, A and S are the values of the factors, reaction time (min), acid concentration (% w/v) and solid to liquid ratio (g/mL) respectively. The model provided proved to be suitable for the adequate representation of the real relationship among the selected factors under the given conditions.

The optimal reaction conditions for which the maximum release of TRS would be obtained was estimated through the Box–Behnken design analysis. For *P. hysterophorus*, the optimal values of 1.35% (w/v), 7 min and 0.19 g/mL corresponding to the tartaric acid concentration, reaction time and SLR respectively were obtained. Table 4 shows theoretical TRS yield as predicted under optimal conditions for *P. hysterophorus* as 10.695 g/L. In order to verify the prediction of the model, the optimal reaction conditions were employed to five independent replicates of pre-treatment. The average TRS concentration for *P. hysterophorus* was 10.921 g/L which is well within the estimated value of the model equation. This demonstrated that the response surface methodology with appropriate experimental design can be effectively applied for the optimization of the process factors in a reaction.

Table 4 Validation of the model for *P. hysterophorus*

S. No.	t (min)	A (% w/v)	S (g/mL)	TRS (g/L)		Mean	Standard deviation	Coefficient of variation
				Experimental	Predicted			
1.	7	1.39	0.19	11.317	10.695	10.921	0.159	2.53%
2.	7	1.39	0.19	10.538				
3.	7	1.39	0.19	10.942				
4.	7	1.39	0.19	10.869				
5.	7	1.39	0.19	10.937				

3.2.1 Fourier Transform Infrared Spectroscopic Analysis of Pre-Treated *P. Hysterophorus*

Fourier transform infrared (FTIR) spectroscopy is an important analytical tool for studying the structure-spectral relationships of the associated molecular vibrations. In this study, the analysis of the molecular vibrations of *P. hysterophorus* reveals the alteration in the functional structure due to pre-treatment.

In the FTIR spectra of *P. hysterophorus* (Fig. 3), the wave numbers 2924.09 cm^{-1} and 2854.65 cm^{-1} correspond to the stretching vibrations of $–CH_3$ and $–CH_2$ groups. The medium peaks of wave numbers 1627.92 cm^{-1} and 1735.93 cm^{-1} reveal the stretching vibrations of the carbonyl groups which is a characteristic of secondary amines and other compounds containing C=O group. Absorption due to the wave numbers 1442.75 and 1427.32 cm^{-1} respectively in non-pretreated and pre-treated samples reveal the bending vibrations of –CH indicating lignin. The difference in the absorption peaks indicates the alteration in lignin content due to pre-treatment.

The sharp peak of 1249.87 cm^{-1} specifies the presence of ester carbonyl groups. The wave numbers 1103.28 and 1049.28 cm^{-1} indicate the fingerprint of C–H deformation or C–O or C–C stretching, pertaining to carbohydrates. The peaks corresponding to the wave numbers 1033.85 and 1049.28 cm^{-1} indicate the presence of starch. The peaks in the range of 532–671 cm^{-1} reveal the transmittance due to benzene rings and other aromatic compounds. Comparing the differences in the transmittance between the wave numbers 700–1100 cm^{-1} which indicate the fingerprint of carbohydrates and starch, structural deformities in the samples due to pre-treatment were revealed.

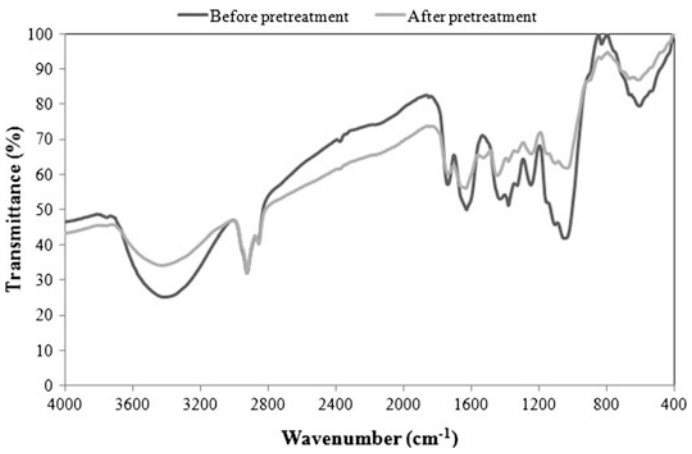

Fig. 3 FTIR spectra of *P. hysterophorus* before and after pretreatment

3.2.2 Scanning Electron Microscopy and Visualization in Photographic Image of Pre-Treated *P. Hysterophorus*

Scanning electron microscopy images comparing the morphology of *P. hysterophorus,* before and after pre-treatment are shown in Fig. 4. The weak acid hydrolysis using 1.5% (w/v) tartaric acid for 15 min shows disruption of the cell wall. This result is in accordance with the FTIR analysis where the modification in peak values signifies the cell wall disruption. Photographic images visualization showed changes in morphology (Fig. 5).

3.3 Enzymatic Hydrolysis

The hydrolysates obtained from pre-treatment and enzymatic hydrolysis were analyzed for their sugar content. This value was compared with the sugar value

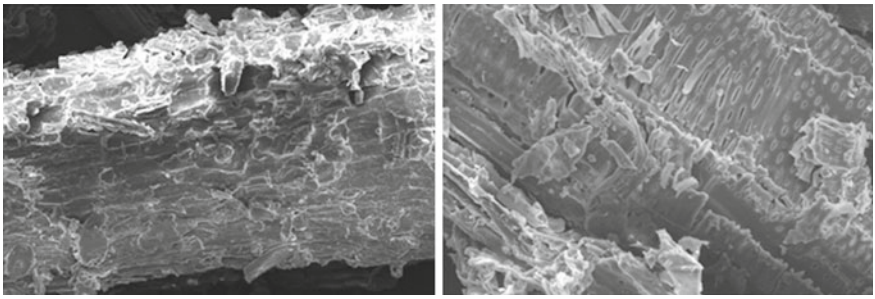

Fig. 4 Scanning electron microscopy images of *P. hysterophorus before* and *after* pre-treatment

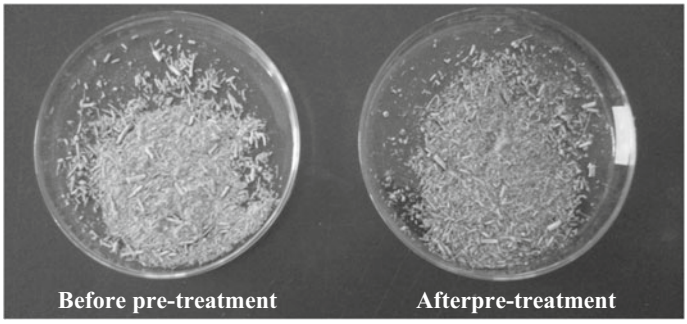

Fig. 5 Morphology of *P. hysterophorus before* and *after* pre-treatment visualized in photographic image

Table 5 Percentage conversion of sugars for *P. hysterophorus* after pre-treatment and enzymatic hydrolysis

Parameters	Percentage removed		% Conversion
	Before conversion	After conversion	
Hemicellulose, starch and sugars	47.97	28.87	39.81
Cellulose	37	14.23	61.54

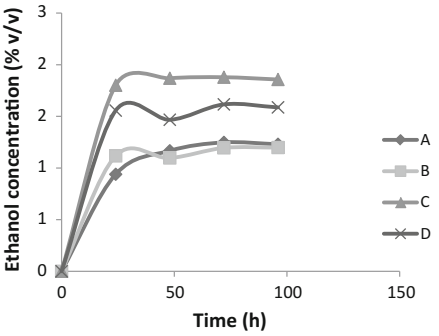

Fig. 6 Ethanol yield from *P. hysterophorus* using mixed cultures of 4 different combinations

A- *K. marxianus* + *Z. mobilis*; B -*K. marxianus* + *K. oxytoca* ;
C- *K. marxianus*+ *F. oxysporum;* D- *K. marxianus* + *S. cerevisiae*

(Table 5) obtained from the initial characterization studies and the percentage conversion value was calculated. The percentage conversion of sugars, starch and hemicellulose was 39.81% and for cellulose it was 61.54%.

3.4 Detoxification and Co-fermentation

The effect of four different combinations of microorganisms on the production of bioethanol from *P. hysterophorus* were studied.

The fermentability of the hemicellulose and the cellulose hydrolysates of *P. hysterophorus* were evaluated using different strains of xylose and hexose fermenting microbes. As evident from the graph shown in Fig. 6, in the fermentation using different combinations of microbes, there was a steep increase in the ethanol concentration during the initial hours and a steady state was reached during 72–96 h of fermentation. The maximum ethanol yield using the mixed culture of *K. marxianus* and *Z. mobilis* was observed as 1.248% (v/v) during 72 h of fermentation. Similarly, maximum ethanol yields of mixed cultures *K. marxianus* and *K. oxytoca* (1.195% (v/v)), *K. marxianus* and *F. oxysporum* (1.879% (v/v)) and *K. marxianus* and *S. cerevisiae* (1.616% (v/v)) were also obtained during the 72 h

of fermentation. Among these, the highest ethanol yield of 1.879% (v/v) was obtained using the mixed culture of *K. marxianus* and *F. oxysporum.* Hence, it is inferred that, the mixed culture of *K. marxianus* and *F. oxysporum* was found to be the best combination for ethanol production from *P. hysterophorus*.

4 Conclusion

In this study, an attempt was made to evaluate the possibility of *P. hysterophorus* as lignocellulosic material for production of bioethanol. *P. hysterophorus* contains 31.91% (w/w) total sugars, 6.06% (w/w) starch, 10.01% (w/w) hemicellulose and 37.11% (w/w) cellulose. After pre-treatment with tartaric acid in autoclave, 10.921 g/L of TRS was produced. 61.54% of cellulose was converted during enzymatic hydrolysis. Maximum ethanol concentration of 1.879% (v/v) was produced from co-fermentation of *K. marxianus* and *F. oxysporum.* Thus, it could be concluded that *P. hysterophorus* has the potential of being commercial lignocellulosic feedstock for bioethanol production.

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Optimization of Fermentative Hydrogen Production by *Klebsiella pneumoniae* KTSMBNL 11 Isolated from Municipal Sewage Sludge

Arivalagan Pugazhendhi and Kaliannan Thamaraiselvi

Abstract In the present study, the fermentative hydrogen-producing bacterial strain KTSMBNL 11 was isolated from municipal sewage sludge and identified as *Klebsiella pneumoniae* based on 16S rDNA gene sequence analysis. Batch experimental studies were performed to determine the effects of initial pH, temperature and substrate concentration on hydrogen production. At pH 6.0 and 36 °C, the maximum hydrogen production rate of 525 ml/l/h was obtained with 30 g/l of glucose, and the highest yield of 2.57 mol/mol G was achieved with an initial glucose concentration of 5 g/l. During anaerobic fermentation, the main soluble metabolites obtained were 2, 3-butanediol and ethanol. Under these optimal conditions, *Klebsiella pneumoniae* has a higher hydrogen yield compared with other pure bacterial cultures reported in the literature. Thus, *Klebsiella pneumoniae* is a promising option as an effective hydrogen-producer.

Keywords Hydrogen · Ethanol · Fermentation · Glucose · *Klebsiella pneumoniae* · Sewage sludge

1 Introduction

Fossil fuels, such as coal, petroleum and natural gas, contain high percentages of carbon and are the world´s primary energy sources. Utilization of fossil fuel has disastrous effects on the environment due to the emission of greenhouse gases,

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which cause climate change and global warming (Peixoto et al. 2011). Currently, over 96% of the world's energy demands are fulfilled by fossil fuels. As forecasted by International Energy Agency (IEA), the fossil fuels are the dominant source of energy. The energy demand is expected to increase by 83% from 2004 to 2030 due to the increasing world population and rapid industrialization and urbanization (IEA 2006). These problems compel scientist to search for efficient alternative energy resources (Das and Veziroglu 2008).

Hydrogen is a viable alternative fuel due its renewability and clean end product (i.e., water) (Nath and Das 2004). This gas is mainly produced from fossil fuels, biomass and water. It is an excellent energy carrier, with a high calorific value of 142 MJ kg^{-1}, which is 2.75 times greater than that of hydrocarbon fuels (Hu et al. 2013). Hydrogen gas can be transported for domestic or industrial consumption by conventional means and is safer to handle than natural gas (Das and Veziroglu 2008).

The technologies used to produce hydrogen from primary non-fossil energy are electrolysis of water, thermochemistry, radiolytic and biological processes (Peixoto et al. 2011). Among these processes, biological hydrogen production is considered the least energy intensive, and unlike conventional chemical methods, it can be carried out at ambient temperature and atmospheric pressure (Das and Veziroglu 2008). Bio-hydrogen gas production from renewable sources is also known as "green technology" (Kapdan and Kargi 2006). Biological methods mainly include photosynthesis and fermentative hydrogen production or dark fermentation. Unlike photosynthesis, fermentative hydrogen production involves a simple, rapid hydrogen production rate, does not rely on the availability of light sources and is more feasible for industrialization (Wang and Wan 2009: Nath and Das 2004). Production of hydrogen via fermentative bacteria (anaerobic organisms) can be performed using either pure or mixed cultures (Hallenbeck et al. 2012; Wang and Wan 2009). Several studies have reported efficient hydrogen production using various substrates and pure cultures of the following bacteria: *Enterobacter* (Khanna et al. 2011; Yokoi et al. 1995), *Clostridium* (Chong et al. 2009; Skonieczny and Yargeau 2009), *Bacillus* (Bala-Amutha and Murugesan 2013; Kotay and Das 2007), *Klebsiella* (Wu et al. 2008; Niu et al. 2010), *Citrobacter* species (Mangayil et al. 2011). Among the fermentative hydrogen producers, *Klebsiella* sp. has been considered the best hydrogen-producing strain, with a high growth rate and valuable by-products, such as 2,3-butanediol and ethanol (Wu et al. 2008; Niu et al. 2010; Xiao et al. 2013). Niu et al. (2010) reported that *Klebsiella pneumoniae* ECU-15, obtained from sewage sludge, showed the highest hydrogen production rate (482 ml/l/h) and yield (2.07 mol/mol G).

Hydrogen production is highly influenced by many physical and chemical factors, such as pH, temperature, substrate concentration and nitrogen sources. These factors have been widely investigated in fermentative hydrogen production studies over the past few years (Das and Veziroglu 2008; Kapdan and Kargi 2006; Chen et al. 2005). During anaerobic fermentation, pH affects hydrogenase activity and governs metabolic flux (Roy et al. 2014). Substrate concentration is considered important because it influences the ability of hydrogen-producing bacteria to increase the net yield of hydrogen. Fermentative hydrogen-producing bacteria can

utilize various carbohydrates, such as hexoses and pentoses (Saripan and Reungsang 2013); however, glucose is the most commonly used substrate due to its efficiency and easy degradation.

During fermentative hydrogen production, glucose is first converted to pyruvate by fermentative bacteria via the glycolytic pathway, generating the reduced form of nicotinamide adenine dinucleotide (NADH). Other sugars can also be used as substrates in the fermentation process. Sugars are broken down into pyruvate, which is converted to acetyl-CoA by the glycolysis pathway via either the pyruvate: ferredoxin oxidoreductase (PFOR) pathway or the pyruvate formate-lyase pathway (PFL). The PFL pathway alone cannot produce more hydrogen because it cannot utilize NADH, but the PFOR pathway can utilize NADH and produce additional hydrogen. Hydrogen can be synthesized by hydrogenases via either an NADH-dependent or ferredoxin-dependent process by oxidizing NADH or FdH, respectively (Hallenbeck et al. 2012). It can be also produced by pyruvate decarboxylation or a formate cleavage process (Wang and Wan 2009). During formate cleavage, the formate is split into hydrogen and carbon dioxide by formate-hydrogen-lyase, and this is the predominant process involved in facultative anaerobes, such as *Enterobacter* and *Klebsiella* (Liu et al. 2011). Currently, many researchers are focusing on improving the hydrogen production rate and yield and converting waste into bioenergy with the help of fermentative bacteria. Among these studies, only a few have been focused on *Klebsiella* sp.

The main objective of this study is to evaluate the feasibility of producing hydrogen using bacteria in municipal sewage sludge. We isolated *Klebsiella pneumoniae* KTSMBNL 11 to better understand and predict fermentative biohydrogen production at different pH values and temperatures using various carbon sources and glucose concentrations. The main goal of the study is to aid cost-effective and environment-friendly production of hydrogen gas.

2 Materials and Methods

2.1 Sample Collection

The sewage sludge used in this study was collected from the primary treatment unit of the municipal wastewater treatment plant in Tiruchirappalli, Tamil Nadu, India. The initial pH of the sludge was 7.2, and the sample was stored at 4 °C.

2.2 Strain Isolation and Identification

The sludge sample was diluted with sterile distilled water and spread onto fresh agar plates containing 15 g/l tryptone, 0.5 g/l L-cystine hydrochloride, 5.5 g/l glucose, 5 g/l yeast extract, 2.5 g/l sodium chloride, 0.5 g/l sodium thioglycollate,

0.01 g/l resazurin, and 0.75 g/l agar. The plates were then incubated at 36 °C in an anaerobic jar. Single colonies were picked and sub-cultured on the same medium several times until the purity of the strain was obtained. The morphology and characterization of the isolated strain was determined according to Bergey's manual (Holt et al. 1994). Gram staining was performed using the Hucker method as described by Doetsch (1981).

2.3 16S RDNA Sequencing and Phylogenetic Analysis

Genomic DNA was extracted according to the Sambrook method (2001). The universal bacterial primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′) were used to amplify the 16S rDNA gene from the isolated bacteria. The reaction mixture consisted of 5 µl of 10x buffer, 0.5 µl of Taq DNA polymerase, 1 µl of dNTPs, 1 µl of $MgCl_2$ (25 mM), 1 µl of each forward and reverse primer, and 2 µl of the template DNA in a 50 µl reaction volume. The PCR program consisted of an initial denaturation at 94 °C for 2 min followed by 28 cycles of repeated denaturation at 95 °C for 1 min, annealing at 52.3 °C for 1.3 min, and extension at 72 °C for 1 min and a final extension at 72 °C for 5 min to ensure full extension of the product. The amplification was performed using a C1000^{TM} Thermal Cycler (Bio-Rad, USA), and the product was purified using a Hiyield^{TM} Gel/PCR DNA extraction kit. The PCR products were loaded on a 1.4% (wt/vol) agarose gel and electrophoresed at 100 V for 30 min. The amplified PCR products were visualized using a UV transilluminator (Gel Doc^{TM} XR +, Bio-Rad, USA) after staining with ethidium bromide. The 16S rDNA sequencing was performed by Synergy Scientific, Chennai, India. The microbial sequence similarity was identified through the NCBI database using basic local alignment search tool (BLAST) analysis. The closest known relatives of the isolates were determined by performing a sequence database search. Bootstrap analysis was performed for the neighbor-joining method using the CLUSTAL X2 1.7 program. The 16S rDNA sequence was deposited in the NCBI nucleotide sequence database.

2.4 Optimization of Serum Bottle Fermentation

The experiments were conducted in 120 ml serum bottles with a working volume of 70 ml. The serum bottles contained 63 ml of the fermentation medium that is, sterile modified Malt Yeast Glucose (MYG) medium (10 g/l D-glucose; 5 g/l yeast extract and 10 g/l malt extract). The cells in the mid-exponential growth phase (i.e., 9 h of culture) were used. 3 ml of the precultured broth was inoculated into MYG medium for hydrogen production. The bottles were sealed and flushed with pure nitrogen gas (99.999%) for 10 min to remove oxygen to ensure anaerobic conditions, capped with a rubber stopper and clamped with aluminum foil. The bottles

were incubated at 36 °C and shaken in an orbital shaker (LSI-1005A, Lab Tech) at 150 rpm. After 24 h, the hydrogen gas production was measured by releasing the pressure in the bottles using a water displacement method. To determine the effects of culture conditions on hydrogen production, the effects of the initial pH value, temperature and substrate concentration were investigated using batch fermentation. The initial pH of the culture was adjusted from pH 4.0 to 9.0 using 1 mol/l NaOH or 1 mol/l HCl solution, and the culture temperature was varied from 25 to 45 °C. Glucose, fructose, xylose, maltose, lactose, cellulose and starch, all at 5 g/l, were tested separately as carbon sources. Initial glucose concentrations ranging from 5 to 30 g/l in 5 g/l increments were also tested.

2.5 Analytical Methods

The optical density (OD) of bacterial cultures were determined by measuring the absorbance at 600 nm using a UV-Visible Spectrophotometer (UV—2450, Shimadzu, Tokyo, Japan).The concentration of glucose was analyzed using the 3,5-dinitrosalicylic acid method (DNS) (Niu et al. 2010). The volume of the gas was measured by releasing the pressure from the head space of the serum bottles. A gas-tight glass syringe was inserted into the rubber cap to collect the gas. The volume of gas produced in batch fermentation runs was recorded using the water displacement method. The purity of the hydrogen gas was measured with gas chromatography (GC, Agilent 4890D) equipped with a thermal conductivity detector (TCD) and a Porapak Q stainless column (80/100 mesh). Nitrogen gas was used as the carrier gas with a flow rate of 20 ml/min. The operational temperatures for the injection port, detector and column oven were maintained at 100, 80 and 150 °C, respectively. The concentrations of soluble metabolites were analyzed using high performance liquid chromatography (HPLC; Agilent 1100, USA).

3 Results and Discussion

3.1 Isolation and Identification of Hydrogen-Producing Strain

The strain KTSMBNL 11 was isolated from sewage sludge and screened for the ability to produce hydrogen in MYG medium. The isolated bacteria was a facultative anaerobe, rod shaped (examined under light microscope) and Gram-negative. The physiological and biochemical properties are summarized in Table 1. When the 16S rDNA sequence results were compared with known bacterial profiles, the strain KTSMBNL11 exhibited 98% sequence similarity with *Klebsiella pneumoniae.* The 16S rDNA gene sequence results were deposited in the NCBI database with the accession number JQ248933.

Table 1 Physicochemical properties of *Klebsiella pneumoniae* KTSMBNL 11

Physiological/biochemical characteristics	Isolated strain	Physiological/biochemical characteristics	Isolated strain
Indole test	−	Sucrose	+
Methyl red test	−	$_{D}$-Mannitol	+
Voges-Proskauer reaction	+	Adonitol	+
H_2S production	−	$_{D}$-Sorbitol	+
Urease activity	+	$_{L}$-Arabinose	+
Phenylalanine deaminase	−	Raffinose	+
Lysine decarboxylase	+	$_{L}$-Rhamnose	+
Ornithine decarboxylase	−	Xylose	+
Motility test	−	Fructose	+
Utilization of Citrate	+	Galactose	+
Glucose	+	$_{D}$-Mannose	+
Lactose	+	Maltose	+

3.2 *Effect of Initial pH on Hydrogen Production*

The pH of the solution plays a vital role in controlling various factors (hydrogenase activity, duration of lag phase and ultimately the metabolic pathway of hydrogen production) during anaerobic biohydrogen production. The effect of pH on hydrogen production was investigated within the range of 4.0–9.0. Table 2 shows that the highest hydrogen yield (1.05 mol/mol G) was obtained with an initial pH of 6.0. At pH 4.0, the cell concentration and hydrogen production were limited because the low pH destroys a cell's ability to maintain its internal pH (Liu and Fang 2007; Niu et al. 2010). At pH 5.0, the cell concentration and hydrogen yields were 0.90 g/l and 0.53 mol/mol G, respectively. When the initial pH was increased to 9.0, the yield of hydrogen decreased drastically. Our results also indicate that the pH is decreased during the fermentation, which is most likely due to the formation of the acidic metabolites, mainly acetic acid and lactic acid (Wu et al. 2008). The

Table 2 Effect of initial pH on hydrogen production by *Klebsiella pneumoniae*

Initial pH	Final pH	Cell concentration (g/L)	H_2 yield (mol/mol G)
4.0	4.0	0.15	0
5.0	4.2	0.90	0.53
5.5	4.6	1.12	0.87
6.0	5.2	1.33	1.05
6.5	5.5	1.47	0.81
7.0	5.8	1.68	0.64
7.5	6.0	1.82	0.54
8.0	6.1	1.96	0.38
9.0	6.2	2.15	0.17

decrease in the pH could also be attributed to the metabolic activities of the cell. The cell concentration increased as the initial pH rose from 4.0 to 9.0. At pH 9.0, the maximum cell concentration of 2.15 g/l was reached, and the hydrogen yield was 0.17 mol/mol G. These findings demonstrated that the maximum cell concentration was obtained at pH 9.0, and the maximum hydrogen yield was obtained at pH 6.0. These results are in agreement with previous reports that the pH of 5.5–6.5 is most favorable for hydrogen production by anaerobic fermentative bacteria (Liu and Fang 2007; Kotay and Das 2007; Mangayil et al. 2011). In fermentative hydrogen production, a low pH interferes with the hydrogenase activity as well as the anaerobic metabolic pathway of microbial strains (Wang and Wan 2009).

3.3 *Effect of Temperature on Hydrogen Production*

Temperature is an important factor that affects cell concentration and the enzymatic reactions required for hydrogen production. Figure 1 shows the effect of temperature on hydrogen production. The results demonstrate that the cell concentration and hydrogen yield increased as the temperature increased from 25 to 36 °C; when the temperature was further increased from 36 to 45 °C, the cell concentration and hydrogen yield decreased. No hydrogen production was observed at 45 °C (data not shown). The lowest yield of hydrogen (1.5 mol/mol G) was observed at 45 °C. Maximum hydrogen production was obtained at 36 °C at an initial pH of 6.0. Wang and Wan (2009) have reported that fermentative hydrogen production by bacteria is temperature-dependent. An increase in temperature increases the production of hydrogen up to a certain point, but beyond that temperature, hydrogen-producing bacteria are not active, resulting in a reduction of both bacterial growth and hydrogen production. This observation is due to the denaturation of

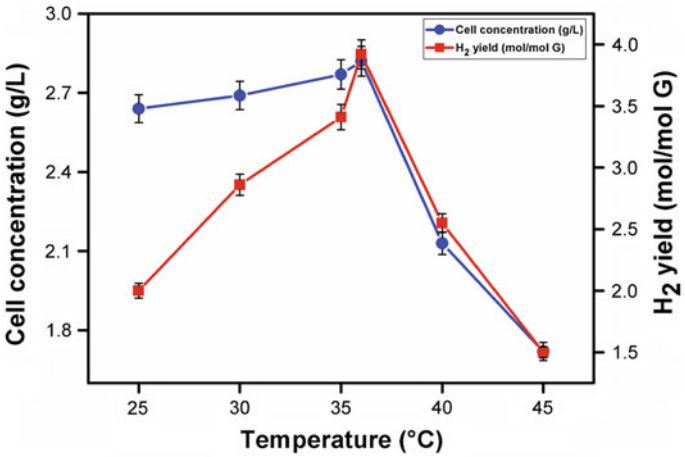

Fig. 1 Effect of temperature on hydrogen production by *Klebsiella pneumoniae*

enzymes or inactivation in the metabolic pathway at high temperatures (Chittibabu et al. 2006). These results are consistent with previous studies demonstrating that the optimal temperature for hydrogen production using anaerobic bacteria is between 36 and 37 °C (Chittibabu et al. 2006; Kotay and Das 2007; Chong et al. 2009; Oh et al. 2003; Niu et al.2010).

3.4 *Effect of Different Carbon Sources on Hydrogen Production*

As shown in Fig. 2, the isolated *Klebsiella pneumoniae* was able to grow and produce hydrogen using a variety of monosaccharides, disaccharides and polysaccharides as carbon sources. Among the tested carbon sources, glucose was associated with the maximum hydrogen yield (1.96 ± 0.05 mol/mol G), followed by fructose (1.07 ± 0.03 mol/mol G), xylose (0.92 ± 0.03 mol/mol G), maltose (0.76 ± 0.06 mol/mol G), lactose (0.53 ± 0.04 mol/mol G), cellulose (0.39 ± 0.05 mol/mol G), and starch (0.35 ± 0.03 mol/mol G). The variation in hydrogen production generated by different carbon sources is due to different substrate transport levels or the metabolism of *Klebsiella pneumoniae* (Niu et al. 2010).

3.5 *Effect of Glucose Concentration on Hydrogen Production*

The effect of initial glucose concentration on cell concentration, hydrogen yield and hydrogen production rate is shown in Fig. 3. As the initial glucose concentration

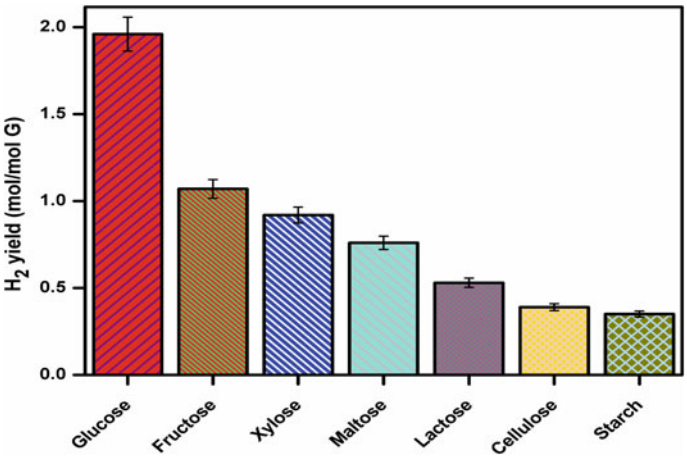

Fig. 2 Effect of different carbon sources on hydrogen production by *Klebsiella pneumoniae*

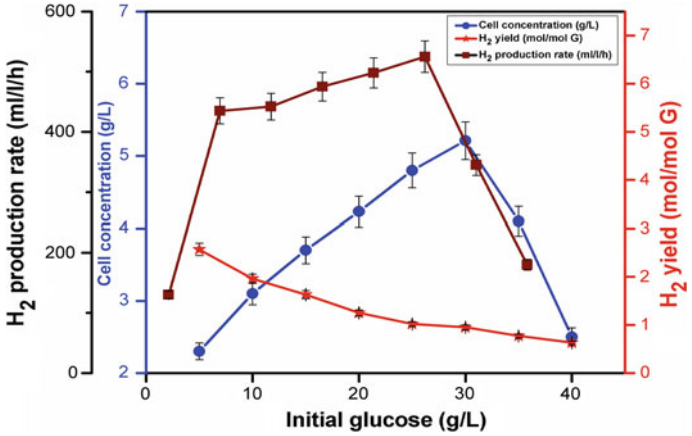

Fig. 3 Effect of initial glucose concentration on hydrogen production by *Klebsiella pneumoniae*

increased from 5 to 30 g/l, the hydrogen production rate increased from 130 to 525 ml/l/h and the cell concentration increased from 2.3 to 5.21 g/l. At initial glucose concentrations above 35 g/l, the hydrogen production rate decreased to 180 ml/l/h, and at an initial glucose concentration of 40 g/l, the cell concentration was 2.5 g/l. The hydrogen yield decreased from 2.57 to 0.63 mol/mol G as the initial glucose concentration increased from 5 to 40 g/l. The decrease in hydrogen yield at higher initial glucose concentrations was due to changes in the by-product distribution. Furthermore, at high glucose concentrations, an organism will consume more NADH, which leads to decreased hydrogen production (Wu et al. 2008). The undissociated organic acids will accumulate at higher substrate concentrations and permeate the cell membrane, thereby leading to a lower intercellular pH (Akutsu et al. 2009). As a result, bacterial growth and hydrogen production will be inhibited. At high substrate concentrations, the carbon flux is directed to the production of reduced by-products such as 2, 3-butanediol and alcohol (Chittibabu et al. 2006). Many studies have reported that substrate inhibition may occur at higher concentrations of glucose, resulting in an inhibitory effect on microorganisms (Chen et al. 2005; Skonieczny and Yargeau 2009). Table 3 compares the maximum hydrogen gas yield of *Klebsiella pneumoniae* KTSMBNL 11 in the presence of glucose with that of other pure bacterial cultures reported in the literature.

Table 3 Comparison of hydrogen yields of using glucose by different pure bacterial cultures using glucose

Organism	Cultivation method/glucose concentration	pH/temperature	Maximum H_2 yield (mol/mol glucose)	Soluble metabolic products	References
Klebsiella pneumoniae KTSMBNL 11	Batch—5.0 g/l	6.0/36 °C	2.57	2,3-BDL, EtOH	Present study
Clostridium butyricum EB6	Batch—15.7 g/l	5.6/37 °C	2.21	N/A	Chong et al. (2009)
Bacillus coagulans IIT-BT S1	Batch—2% (w/v)	6.5/37 °C	2.28	HBu, HAc	Kotay and Das (2007)
Bacillus sp. B2	Batch—20 g/l	7.0/37 °C	1.65	EtOH, HAc	Liu and Wang (2012)
Klebsiella oxytoca HP1	Batch—10–200 mM	7.0/35 °C	1.0	N/A	Minnan et al. (2005
Klebsiella pneumoniae ECU-15	Batch—5.0 g/l	6.0/37 °C	2.07	2,3-BDL, EtOH	Niu et al. (2010)
Citrobacter sp. Y19	Batch—1.0 g/l	7.0/36 °C	2.49	EtOH, HAc	Oh et al. (2003)
Clostridium beijerinckii Fanp3	Batch—5.0 g/l	6.4/35 °C	2.52	HAc, HBu	Pan et al. (2008)
Bacillus sp. FS2011	Batch—10 g/l	6.98/35 °C	2.26	HAc, HBu	Song et al. (2013)
Enterobacter aerogenes Strain HO-39	Batch—1.0 g/l	6.0 − 7.0/38 °C	1.0	HAc, HLa	Yokoi et al. (1995)

4 Conclusion

The strain *Klebsiella pneumonia* KTSMBNL11 isolated from municipal sewage sludge is capable of producing hydrogen. It prefers glucose over other substrates for H_2 production. The maximum hydrogen production (525 ml/l/h) and H_2 yield (2.57 mol/mol G) were obtained at initial glucose concentrations of 30 and 5 g/l, respectively. For maximum hydrogen production, the optimal initial pH was 6.0, and the optimal temperature was 36 °C. The metabolites 2, 3-butanediol and ethanol were generated during hydrogen production.

The results of this study provide insight into the process involved in the optimization of hydrogen production by *Klebsiella pneumoniae* KTSMBNL 11.

Compared with other hydrogen-producing strains reported in the literature, KTSMBNL11 exhibits the highest hydrogen yield and production rate. Thus, *Klebsiella pneumoniae* could be a highly effective alternate hydrogen producer isolated from municipal sewage sludge.

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Part IV
Microbial Isolation for Contamination Cleanup

Microbial Bioremediation of Hazardous Heavy Metals

Preeti Kanwar, Tulika Mishra and Gunjan Mukherjee

Abstract With the passage of time, and with the rise in demands of population Industrialization and new technologies has also augmented. But this rise is now affecting the various ecosystems and thus contaminating the environment. Accumulation of heavy metals has now become a serious concern. Nature has provided us enormous ways for the depletion of these heavy metals viz: leaching, plant uptake, erosion and deflation. But as contaminants are now reached beyond the limit of nature and thus requires alternative ways with lesser or no side effects. The best way out to treat these contaminants is bioremediation. Bioremediation is a process that utilizes plants and microbes for the transformation of heavy metals. There are many microbes that have developed specialized mechanism for heavy metals. Some microbes are found to develop a mechanism through which they are able to sequester and immobilize metals, while some are found to enhance the solubility of metals, some of them oxidizes or reduces them to non toxic or comparatively lesser toxic forms. Now the genetic engineering is also used so that the traits of one organism can be transfer to other and thus one microbe can simultaneously detoxify more than one contaminant. In this chapter, efforts have been made to simplify the causes, effects, possible treatment, mechanism and the future aspect of bioremediation.

Keywords Bioremediation · Heavy metals · Genetically engineered microbes

1 Introduction

The development of any country to some degree is dependent upon the Industrialization. To cover this gap of fast life almost all the countries are rushing after the Industrialization and to the more advance techniques. As a result of fast Industrialization, world is now facing the problem of heavy metal in environment.

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With the rapid growth of industrialization, there is substantial rise in the discharge of industrial waste to the environment mainly soil and water. As a result, there is an accumulation of heavy metals in rural as well as urban areas. Natural ways of depletion of heavy metals include leaching, plant uptake, erosion and deflation, but these are very slow process and are not able to compete with the rate of accumulation of heavy metals. The capricious release of heavy metals into environment is a major health concern worldwide, as they cannot be broken down to non-toxic forms and therefore has long-lasting effects on the ecosystem.

Metals play a very important role to sustain life. Some metals like calcium, cobalt, chromium, copper, iron, potassium, magnesium, manganese, sodium, nickel and zinc comes under the essential metal category as they are involved for redox-processes, to stabilize molecules through electrostatic interactions; as components of various enzymes; and for regulation of osmotic pressure. There is another category called non-essential metals which include silver, aluminium, cadmium, gold, lead and mercury and are not required for any metabolic process, these non essential metals causes toxicity. Many of these metals are known as heavy metals. An excess unrestricted flow of these heavy metals in the environment by Industrial waste, fertilizers are the major concern in nature as these are non-biodegradable and mount up in the environment. There are at least 20 metals that are classified as toxic with half of them released in environment poses immense risk to human health (Akpor and Muchie 2010). The common heavy metals like Cd, Pb, Co, Zn and Cr etc. are phototoxic and possess very harmful effects whether they are at low concentration or high concentrations. They are usually release in natural water resources causing water pollution and with the passage of time these gets sediments in the soil. This sedimentation helps them to seep in the soil and thereby entering in the food chain through plants and aquatic animals. At larger amounts they cause acute toxicity or chronic toxicity. The accumulation of these heavy metals in the water depends upon many factors including industries in region, people's way of life and awareness of the impacts done to the environment by careless disposal of wastes. Their accumulation also affects the microbial community present to detoxify such hazardous waste and as a result affecting biological wastewater treatment process. The pollution occurs both at the level of industrial production as well as end use of the products and run-off. For the healthy environment, detoxification of heavy metals and trace elements from water bodies and soil is required. There are several techniques that are available for the removal of heavy metals and it includes chemical precipitation, oxidation or reduction, filtration, ion exchange, reverse osmosis, membrane technology, evaporation and electrochemical treatment. But these are ineffective at the concentration lower than 100 mg/l (Ahluwalia and Goyal 2007). As most of the heavy metals are water soluble, thus there removal is not possible by physical separation methods (Hussein et al. 2004). Not only the industrial process, but the natural sources are also adding up the accumulation of heavy metals which includes seepage from rocks into water, volcanic activity, forest fires etc.

Bioremediation could be the answer to these heavy metals accumulation.

Bioremediation is a promising, innovative and natural technique that can be helpful in the removal of heavy metals. It is a natural process that offers the possibility to finish or render harmless various contaminants using plants and microbes. Use of microbial species and plants for the remediation of heavy metals can be helpful to detoxify the water or soil. The rejoinder of microbial population depends upon many factors that include type of metal, the nature of the medium and microbial species, concentration and availability of heavy metals.

Data revels that due to heavy toxicity and ubiquity of the metals in the environment, microbes have urbanized inimitable and some times uncanny ways of dealing with unwanted metals. Some microbes are found to develop a mechanism through which they are able to sequester and immobilize metals, while some are found to enhance the solubility of metals, some of them oxidizes or reduces them to non-toxic or comparatively lesser toxic forms. Microorganisms have developed various strategies for the survival in heavy metal contaminated environment, including biosorption, bioaccumulation, biotransformation and biomineralization.

Microbial cell walls consist of polysaccharides, lipids and proteins those posses many functional groups like carboxylate, hydroxyl, amino and phosphate groups, which facilitates the binding of heavy metals. So far many microorganisms have been reported showing the successful bioremediation of heavy metals. Based upon the literature this chapter defines the various possible approaches for heavy metal bioremediation.

2 Sources of Heavy Metals

Before discussing the various sources, it's important to discuss the bioavailability of metals, because understanding the fate of metals in soil and sediments defines the effect of metals on various ecosystems. Bioavailable metals are those that are soluble, nonsorbed, and mobile where as Non-bioavailable are those that get precipitated, complexed, sorbed and nonmotile. Depending upon the Bioavailability, metals get leached to ground water, transfer to food chain and impose hazardous effects. The bioavailability of metal is greatly affected by cation exchange capacity (CEC) of soil, the capacity of a soil to exchange positively charged ions at the soil-soil solution interface. Metals are usually retained to greater extent in soil than water. The cation exchange capacity (CEC) of soil allows the soil particle to hold metals. In addition there are other factors like metal chemistry, sorption to clay minerals and organic matter, pH, redox potential and the microorganisms present which determines the toxicity of metal in a biological system.

The rise in heavy metals in environment is either by the natural sources or man kind activities. Heavy metals occur naturally from the pedogenetic processes and anthropogenic sources. Naturally it can be from weathering of minerals, Erosion and volcanic activities, Forest fires and biogenic sources and particles released by vegetation. Where as anthropogenic sources depends upon the human activities and includes pesticides, wood preservatives, fertilizers, ore mining, smelting paints and

pigments, plastic stabilizers, electroplating, mining, coal combustion, surgical instruments, insecticides, battery waste etc.

The accumulation of heavy metals in soil and water are disturbing geochemical cycle and thus increasing risk to human health, plants and aquatic biota (D'Amore et al. 2005). Discharge of heavy metals from industries and through other man kind activities is now alarming and causing many side effects, some of them is summarize in Table 1.

The ecological effects of toxic heavy metals and their biological manifestation is not a serious problem for only plants and animals but to various ecosystems. They are threat to the microbial population also that are the key players of the different nutrients turnovers in the soils, causing fracas to the soil fertility also and thus affecting agriculture also. It's a call of an hour to get rid of these heavy metals. Heavy metal contamination has led to various medical problems like cancer, birth

Table 1 Various harmful trace elements, their sources and side effects (Donald 2003)

Trace elements	Various side effects	Source
Cadmium	Causes high blood pressure and kidney damage, destroys testicular tissue and red blood cells, toxic to aquatic biota	Industrial discharge, mining waste, metal plating, water pipes
Lead	Leads to anemia, kidney disease, nervous system disease, Excess exposure in children causes impaired development, reduced intelligence	Industry, mining, plumbing, coal, gasoline
Chromium	Essential trace element, (glucose tolerance factor), possibly carcinogenic as Cr(VI)	Metal plating, cooling-tower water additive (chromate), normally found as Cr(VI) in polluted water
Arsenic	Toxic, possibly carcinogenic, causes skin disease	Mining by-product, pesticides, chemical waste
Mercury	Autoimmune diseases, depression, disturbance of vision, tremors, temper outbursts, drowsiness, hair loss, insomnia, loss of memory, restlessness, brain damage, lung and kidney failure	Industrial Waste, mining, pesticides, coal
Nickel	Allergic skin diseases such as itching, immunotoxic, neurotoxic, genotoxic, affects fertility, hair loss cancer of the lungs, nose, sinuses, throat through continuous inhalation	Industrial Waste, mining
Zinc	Dizziness, fatigue, toxic to plants at higher levels	Industrial wastes, metal plating, plumbing
Copper	Essential trace element, not very toxic to animals, toxic to plants and algae at moderate levels	Metal plating, industrial and domestic wastes, mining, mineral leaching

defects, liver and kidney disease and the list is endless. There are three major molecular mechanisms that are involved in causing various health effects viz: Production of reactive oxygen species (autooxidation and fenton reaction) Blocking of essential functional groups in biomolecules (non redox reactive heavy metals) and Displacement of essential metal ions from biomolecules. These mechanisms inhibit or change various cellular metabolic processes that are injurious to the cell. Like exposure to Mercury, Lead, Copper, Cadmium, Zinc and nickel leads to cell membrane disintegration, inhibits transcription, translation, damages DNA, causes protein denaturation and even inhibits cell division. Microbial bioremediation aims to immobilize metal, to reduce its bioavailability and mobility. Metals get precipitated or undergo biotransformation resulting into lesser toxic form and thereby reducing metal pollution.

3 Microbial Bioremediation

Remediation of water and soil loaded with heavy metals can be achieved through biologically encoded changes in oxidation state (Dixit et al. 2015). Bioremediation is the microbe-mediated process for clearance or immobilization of the contaminants, including all possible toxins like hydrocarbons, agrochemicals and other organic toxicants (Dixit et al. 2015). The strategy for bioremediation can be accomplished, by taking care of specification of microbes. As several microbes have varying requirement for various metals, some of them require heavy metals as micronutrients for their growth and metabolism, while others just adsorb these metals. For the complete removal of heavy metal usually more than one technique is used. In In situ treatment method the contaminated site is not excavated, it includes volatilization, biodegradation, phytoremediation, leaching, vitrification etc. while for ex situ method usually the soil is excavated and then treated at some other place; it includes land treatment, thermal treatment, stabilization, chemical extraction etc. The microbiological aspect of bioremediation involves biostimulation (stimulating viable native microbial population), Bioaugmentation (artificial introduction of viable population), bioaccumulation (sequestration and accumulation of heavy metals by microbes) and biosorption (adsorption by living or dead microbes). There are certain microorganisms that are great metal accumulator; the genetic study of such microorganism can be helpful by transferring their traits in the microbes not having such traits through microarray, which produces differently expressed microbes.

The methodology to be implicated for bioremediation greatly depends upon the interaction between metal and microbe. Metal ion binds to microbial surface by electrostatic interactions, redox interactions, covalent binding, vander wall forces, or extracellular precipitation and sometimes by the combination of these processes (Blanco 2000).

4 Possible Mechanisms for Bioremediation

As microbes are all pervading and so are present in heavy metal contaminated soil, for their survival they have developed various mechanisms where they can convert heavy metals to either lesser toxic forms or to metabolic intermediates. They use these metabolic intermediates as primary growth substrates for cell growth and normal function. There are some microbes that have evolved as metal tolerant and some are evolved as metal resistant, this is due to either their early exposure with heavy metals as their life started or due to their long time continuous exposure to heavy metals. There are mainly two mechanisms underlying the metal resistance: General mechanism and metal dependent mechanism.

4.1 General Mechanism

This mechanism is based on the fact that cationic metals bind themselves to anionic cell surfaces and it is attributed to following methods:

(a) Siderophore complexation—Siderophore are low molecular weight iron chelating molecules. Their biological function is to concentrate iron in the environment and transportation of that to cell. Metals like chromium, alluminium etc. that are similar to iron gets binds to Siderophore and thus reduces their bioavailability and thereby reducing metal toxicity (Roane and Pepper 2000).
(b) Biosurfactants complexation—These are produced by many bacteria's and they also binds with metals like cadmium, lead and zinc. They increase their water solubility and in complexed become non-toxic to cells.
(c) Exoploymer binding—It includes substances like polysaccharides, carbohydrates nucleic acids and fatty acids. These are negatively charged functional groups that easily bind with positively charged metals. With this method metals get immobilize and thus their entry inside the cell is restricted.
(d) Precipitation by metal reduction—in this method soluble metals are reduced by microorganisms to less soluble metal salts and thus precipitates.

4.2 Metal Dependent Mechanism

To the best of our knowledge, not much data is available on this but it is believed that this mechanism involves metal binding and sequestration by metallothioneins or similar proteins. Another metal dependent system is efflux system, which involves ATPases and ion/proton pump.

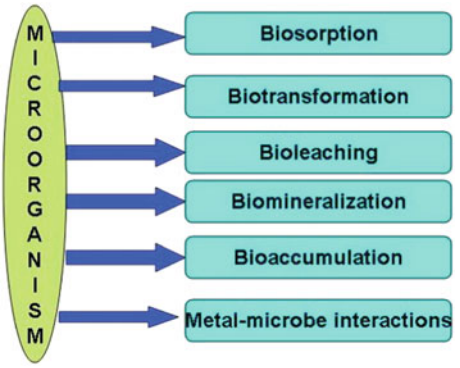

Fig. 1 Various mechanisms followed by microbes for bioremediation

For the target pollutant, microbes have two way defense system, they either produce degenerative enzymes or develop resistance to heavy metal. As shown in Fig. 1 there are various mechanisms for bioremediation. To restore the environment different methods that are followed by microbes includes oxidizing, binding, immobilizing, volatizing and transformation of heavy metals. By utilizing designer microbe approach, understanding the mechanism of growth and activity of microbe, microbe response to environment, implication of bioremediation can be better. Sometimes there is presence of certain organic solvents that can disrupt the cell membrane, for such situations microbes uses its efflux system (Sikkema et al. 1995). Broadly the mechanism will be discussed under adsorption mechanism, Physico-chemical mechanism and genetic mechanism.

4.3 Biosorption

As discussed earlier, there are binding sites (like hydroxyl group, carbonyl group etc.) present on the cell surface through which metals get attached with these microbes without involvement of energy. These extracellular polymeric substances are influenced by acid-base conditions and there by affects metal adsorption (Guiné et al. 2006). Studies have shown that binding of metals with extracellular polymeric substances occurs through various mechanisms including proton exchange and micro-precipitation of metals (Fang et al. 2010, 2011).

Biosorption has major advantages over the conventional treatment method; it is low cost, minimization of biological and chemical sludge, high efficiency, and possibility of metal recovery. But simultaneously it has some disadvantages also which includes the potential of biological process is limited because cells are not metabolizing and once the metal has occupied its space on cell surface it need to be undergo desorption before further use (Ahluwalia and Goyal 2007). There are four factors that affect the biosorption: pH, Temperature, biomass and initial metal ion concentration. Out of these four the pH causes maximum effect, as it affects the metal

chemistry, activity of functional group and competition of metallic ions (Friis and Keith 1998). In addition there are many reports showing the reduction of metal ion to another state by microorganism. Various microbes have been reported to reduce toxic chromium (Cr^{6+}) to harmless state Cr^{3+} which includes *Klebsiella oxytoca, Acenatobacter calcoaceticus*, *Aureobacterium esteroaromaticum*, *Pseudomonas putida*, *Bacillus licheniformis*, *Achromobacter* sp., *Aeromonas* sp., *Enterobacter* sp., *Escherichia* and *Micrococcus* species this reduces metal mobility and thus toxicity (Wang et al. 1989; Clausen 2000; Batool et al. 2012). Now a days, bioaugmentation is also under various trials. In this technique new population having transforming ability is introduced in the already existing population. For this method usually metal-immobilizing population is introduced to stabilize metal movement and availability in a soil, as observed with exopolymer-producing and metal-reducing bacteria.

4.4 Physico-chemical Mechanism

In the biosorption process binding of metal ions continues until the equilibrium is reached (Das et al. 2008). *Cunninghamella elegans* is a promising sorbent for the heavy metal released from textile industry (Tigini et al. 2010). *Saccharomyces cerevisiae* has been reported as good sornet for, Zn (II) and Cd (II) through the ion exchange mechanism (Chen and Wang 2007; Talos et al. 2009). Degradation of heavy metals involves energy for cell metabolic cycle. Among the various microbes fungi is found to be potential biocatalysts that can access and transform them to lesser toxic forms (Pinedo-Rivilla et al. 2009). *Aspergillus parasitica* and *Cephalosporium aphidicola* have been found to degrade lead (Tunali et al. 2006; Akar et al. 2007). *Hymenoscyphus ericae, Neocosmospora vasinfecta* and *Verticillum terrestre* are reported as sorbent that can easily transform mercury (Thavasi et al. 2011).

Bioremediation may occur through aerobic or anaerobic microbial activities. In the aerobic degradation the oxygen is introduced into the reaction mediated by various enzymes (hydroxylases, oxidative dehalogenases, monooxygenases, dioxygenases). In anaerobic degradation initial activation reactions followed by oxidative catabolism mediated by anoxic electron acceptors (Dixit et al. 2015) and helps in the immobilization of toxic metals from the contaminated site. Heavy metals cannot be destroyed but they can be transformed into lesser toxic forms, water soluble form and precipitated (Garbisu and Alkorta 2001). Microorganisms take away heavy metals through the mechanisms, which they employ to derive energy from metals redox reactions (oxidation and reduction). Mechanism for development of resistance is already discussed.

In the soil for oxidation-reduction reactions, microorganisms act as an oxidizing agent and cause the heavy metals to lose electrons, which are accepted by other

Table 2 Microorganism following various mechanisms to treat heavy metals

Metal	Organism involved	Mechanism followed	Reference
Lead (Pb)	*Pseudomonas aeruginosa* BS2, *Enterobacter* sp. J1	Biosurfactant, extracellular sequestration	Nair et al. (2007)
Cadmium (Cd)	*Staphylococcus aureus, Fusarium oxysporum, Pseudomonas* sp. H1, *Pseudomonas azotoformans*,	Intracellular and extracellular sequestration, efflux, reduction	Nies and Silver (1989), Ahmad et al. (2002), Roane et al. (2001), Nair et al. (2007)
Mercury (Hg)	*Clostridium glycolicum* AS1-1	Volatilization	Meyer et al. (2007)
Arsenic (As)	*Lactobacillus* sp. As-1	Reduction, efflux, intracellular sequestration	Nair and Pradeep (2002)

electron acceptors (nitrate, sulphate and ferric oxides). Under aerobic conditions oxygen act as electron acceptor, while under anaerobic conditions microbes oxidize contaminants. Microbes take energy for their growth by oxidizing compound with Fe (III) or Mn (IV) as an electron acceptor (Lovley and Phillips 1988). More the Fe (III) present higher will be the rate of anaerobic degradation (Spormann and Widdel 2000). Microbes change the state of heavy metals like *Geobaccter* species reduces Uranium soluble state (U^{6+}) to insoluble state (U^{4+}) (Lovley et al. 1991). To reduce the stress developed due to heavy metals microbes have various defence mechanisms (exclusion, compartmentalization, complex formation and synthesis of binding protein and peptides) (Gómez et al. 2011). The decision for accumulation of heavy metals in microbes is mediated by metal binding proteins and peptides which include phytochelatins and metallothionein (Cobbett and Goldsbrough 2002), these are either naturally present or induced by genetic engineering. It has also been found that metalloregulatory protein in microorganism is responsible for natural resistant pathways (Singh et al. 2008). In Table 2 we have tried to summarize few microorganisms with varying mechanisms.

4.5 Genetic Engineering Mediated Mechanism

Genetic engineering has blessed a bioremediation process in a more convenient way. Although the technique has not been found to be successful against all the heavy metals. *E.coli* possess mer operon due to which it has the capacity to reduce Hg^{2+}, now this gene is incorporated in *Deinococcus geothemalisi* and at high temperature *Deinococcus geothemalis*, also exhibited reduction of Hg^{2+} (Brim et al. 2003). Similarly, *Cupriavidus metallidurans* (strain MSR33) is a mercury resistant bacteria and after the introduction of pTP plasmid there is a expression of merB and merG, which is helpful in regulating Mercury biodegradation with the synthesis of

organomercurial lyase protein (MerB) and mercuric reductase (MerA) (Rojas et al. 2011). *Pseudomonas putida* posses' *tod* and *xyl* operons that is helpful in complete degradation of toluene when introduced in radiation resistant bacteria *Deinococcus radiodurans* (Brim et al. 2006). In addition, coenzymes and siderophores are also involved in degradation pathway (Penny et al. 2010).

5 Role of Recombinant DNA Technology in Bioremediation

Genetic engineering is one such tool with the help of which microbes can be designed as per the requirement. The traits of one microbe are transferred to another through recombinant DNA technology to engender character specific efficient strain for bioremediation of soil, sludge or contaminated water (Sayler and Ripp 2000). This has advantage over all the other techniques, as microbial strains can tolerate adverse stressful condition and can act as good bioremediator for even more than one heavy metal. With the advancement in this field formulation of microbial biosensors has also emerged that can measure the degree of contaminants in contaminated sites. Biosensors for mercury (Hg), cadmium (Cd), nickel (Ni), copper (Cu) and arsenic (As) are already in use (Verma and Singh 2005). One of the recent approaches includes the genetic engineering of endophytes and rhizospheric bacteria, which is found to be very effective and accurate in contaminated site (Divya and Deepak Kumar 2011). In the previous section, few microbes have been discussed in which additional character of other microbes has been introduced, Table 3 is putting glance on some more genetically engineered microbes.

Table 3 Some of the genetically modified microbes and the responsible gene

Heavy Metal	Genetically engineered bacteria	Gene expressed	Reference
Mercury	*E. coli* strain	Organomurcurial lyase	Murtaza et al. (2002)
	Achromobacter sp. AO22	mer	Ng et al. (2009)
	Pseudomonas K-62	Organomercurial lyase	Kiyono and Pan-Hou (2006)
Cadmium	*E.coli* strain	SpPCS	Kang et al. (2007)
Cadmium and Mercury	*Ralstonia eutropha* CH34, *Deinococcus radiodurans*	merA	Valls et al. (2000); Brim et al. (2000)
Arsenic	*E. coli* strain	Metalloregulatory protein ArsR	Kostal et al. (2004)

6 Future Prospects

Advancement in technology and fast growing industry has put a burden on environment in the form of release of heavy metals and thereby contaminating soil and water. As the conventional methods are complex and slow, microbes are adding help to overcome such situation. Microbial bioremediation has some limitations but still providing a safer and faster way out for treatment of contaminated sites. The most important turning point is genetically engineered microbes that can simultaneously work on more than one heavy metal. More work is required to be done in order to have more efficient GEM's. Manipulation in the outer member of microbes can be more helpful or even the expression of particular transforming gene is the likely way to enhance the bioremediation capacity of toxic metals.

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Screening, Isolation and Development of Fungal Consortia with Textile Reactive Dyes Decolorizing Capability

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Abstract Five different samples (3 effluent samples and 2 soil samples) were collected from CETP in SIPCOT, Perundurai, Erode District, Tamil Nadu and were serially diluted and cultured. 8 different fungal colonies were isolated. Of these fungal isolates, 4 colonies were selected for their ability to decolorize Reactive Red 120, Reactive Black 5 and Direct Red 81 which are synthetic dyes. These selected isolates were identified as *Pleurotus ostreatus, Aspergillus niger, Penicillium simplicissimum* and *Penicillium chrysosporium*. Among the selected isolates, *Pleurotus ostreatus* revealed the maximum percentage of the reactive dye removal of about 76, 64 and 62% for Reactive Red 120, Reactive Black 5 and Direct Red 8 respectively. Further, Four different fungal consortium (FC) were developed, of which FC1 consisting of *Pleurotus ostreatus*, *Aspergillus niger*, *Penicillium simplicissimum*, *Penicillium chrysosporium* exhibited the highest dye removal percentage of 78% for Reactive Black 5.

Keywords Effluent · Fungal consortia · Reactive red 120 · Reactive black 5 · Direct red 8 decolorization

1 Introduction

The decolorization of the textile dyes with the microbial isolates from the dye contaminated sites is widely studied for preserving the environment. Various microbial communities have been utilized for decolorization of textile dyes. During dyeing in textile industries around 2% of textile dyes are discharged into aquatic ecosystem while 10–50% of used dyes are lost in textile effluents, and its leads to ground water contamination (O'Neill et al. 1999). Previously, the textile and dyestuff industries have used the microbes for degradation of dyes (Knapp et al. 1995).

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Similarly, *Aspergillus* sp. isolated from the soil sample near textile industry has effectively decolorized Reactive Blue and other structurally different synthetic dyes (Mohandass et al. 2007). Decolorization of the textile dyes (Orange 3R, Blue 3R, Yellow GR, Black RL and T blue) was carried out with indigenous soil fungi isolated from the soil samples around textile distillery industries (Raju et al. 2008).

Microbial isolates both bacterial and fungal obtained from the contaminated sites were used to decolorize three different azo dyes [Acid navy blue (Acid blue 120), Fast red A (Acid red 88) and Acid sulphone blue (Acid blue 89)] and one triarylmethane dye [Acid magenta (Acid violet 19)] (Prachi and Anushree 2009). Indigenous fungus, *Penicillium simplicissimum* isolated from the sediment collected from the industrial contaminated sources have proven to decolorize the three reactive dyes very effectively (Bergsten-Torralba et al. 2009).

In general, biological methods using various microbes like bacteria, fungi and algae for removing dye could be a viable option as a low-cost and eco-friendly decentralized wastewater treatment system for small-scale industries. Various marine derived fungal isolates has been used to degrade textile dyes from raw effluents (Ashutosh et al. 2010). Many of the industrially used textile dyes were decolorized in aerobic and anaerobic conditions. Reactive red 11 and 152 azo dyes were decolorized by aerobic fermentation conditions (Kodam and Gawai 2006). Apart from fungal degradation and decolorization of textile effluent dyes, many fungal based enzymes has been used in the discoloration of azo dyes. One of main enzymes Manganese Peroxidase from White-Rot Basidiomycete *phanerochaetesor* did was used to decolorize azo dyes (Koichi et al. 2003). Similarly, Colour removal of textile dyes were studied by using culture extracts of white rot fungi (Mehmet et al. 2009). Also, white rot fungus *Coriolus versicolor* IBL-04 can decolorize dyes in various textile industry effluents (Muhammad et al. 2009).

This study focuses on the biological decolourization of textile effluents through fungal isolates obtained from contaminated sites. Then the isolates with significant ability to decolorize the synthetic reactive dyes were selected and the microbial consortia were developed with the isolates and the cumulative effect was studied.

2 Materials and Methods

2.1 Dyes

Reactive Red 120, Reactive Black 5 and Direct Red 81, commercially available reactive dyes were collected from Department of textile Technology, Kumaraguru college of Technology, Coimbatore—641049.

2.2 Sample Collection

The dye effluent samples and soil sample from the dye contaminated sites were collected from CETP in SIPCOT, Perundurai, Erode district, Tamil Nadu. All 3 effluent samples and 2 soil samples were collected in sterile polythene bag and stored at 4 °C for further use.

2.3 Isolation of Fungal Colonies from Effluent and Soil Samples

The dye effluent samples and soil sample were serially diluted and spreaded over the Potato Dextrose Agar (Hi-Media) plates and plates were then incubated at 28 ± 2 °C for 48–72 h. A mixture of fungal colonies was obtained on these plates after incubation. From these, distinct colonies were chosen and pure culture of each distinct colony was obtained using streak plate method. The pure cultured fungal colonies were then maintained at 4 °C as slant for further studies.

2.4 Development of Fungal Consortia and Evaluation of Its Dye Decolorizing Efficiency

In this study, four different types of fungal consortium (FC) were developed and their commercial dye decolorizing efficiency was studied. These four consortia consist of different sets of fungi and they were named as FC1 (*Pleurotus ostreatus*, *A. niger*, *P. simplicissimum*, *Penicillium chrysosporium*); FC2 (*A. niger*, *P simplicissimum*, *P. chrysosporium*); FC3 (*P. ostreatus*, *A. niger*, *P. chrysosporium*) FC 4 (*P. ostreatus*, *P. simplicissimum*, *P. chrysosporium).*

The textile dyes, Reactive Red 120 (λm = 520 nm), Reactive Black 5 (λm = 574 nm) and Direct Red 81 (λm = 510 nm) were used at 50 mg/100 ml concentrations. These dyes were prepared at a concentration of 50 mg/100 ml and the decolourization assay mixture consists of 10% of dye. Decolourization experiments were carried out with the medium (containing Glucose—0.1%, yeast extract—0.05%, Peptone—0.5%, NaCl—0.5%, $(NH_4)_2SO_3$—1%, K_2HPO_4—0.02%, KH_2PO_4—0.5% and $MgSO_4.7H_2O$—0.5% amended with 50 mg of 3 synthetic dyes) prepared in 100 ml were used in the present study to investigate their effect on the decolourization rate by 4 different fungal consortium for a period of one week.

Dye removal (%) was calculated as

$$\text{Dye removal } (\%) = \frac{OD_i - OD_f}{OD_i} \times 100$$

Where, OD_i is the initial absorbance of dye mg L^{-1} and OD_f is the final absorbance of dye concentration mg L^{-1}at different time intervals.

3 Results and Discussion

3.1 Isolation of Fungal Colonies from the Effluent and Soil Samples

About 8 different fungal colonies were isolated from the effluent samples and soil sample from the dye contaminated sites at CETP in SIPCOT, Perundurai, Tamil Nadu. These 8 fungal colonies were named based on the source from which it was isolated. Table 1 shows the total number of fungal colonies isolated from the samples.

3.2 Decolourization Studies

About 8 different fungal isolates were selected and subjected to decolourization studies. Figure 1 shows the synthetic dye removal % by the fungal isolates from CETP samples. Among 8, CETPF1 and CETPF8 shows maximum percentage of dye removal range from 55 to 75%. CETPE1 shows higher rate of dye decolorization percentage for Reactive Red 120 (75%), Reactive Black 5 (65%) and Direct Red 81 (63%), whereas CETPF4 shows minimal level of dye removal percentage for Reactive Red 120 (23%), Reactive Black 5 (18%) and Direct Red 81 (25%). Based on the decolorization efficiency, 4 fungal isolates were screened and identified as *P. ostreatus, A. niger, P. simplicissimum* and *P. chrysosporium*.

Table 1 Name and source of the fungal colonies isolated from CETP samples

Source of sample for isolation of fungal isolates	Source and culture name
CETPS1	CETPF1 and CETPF2
CETPE1	CETPF3
CETPE2	CETPF4
CETPE3	CETPF5 and CETPF6
CETPS2	CETPF7 and CETPF8

CETPE1—CETP Effluent sample1; *CETPE2*—CETP Effluent sample 2; *CETPE3*—CETP Effluent sample 3; *CETPS*—CETP Soil sample; *CETPF*—CETP sample fungi

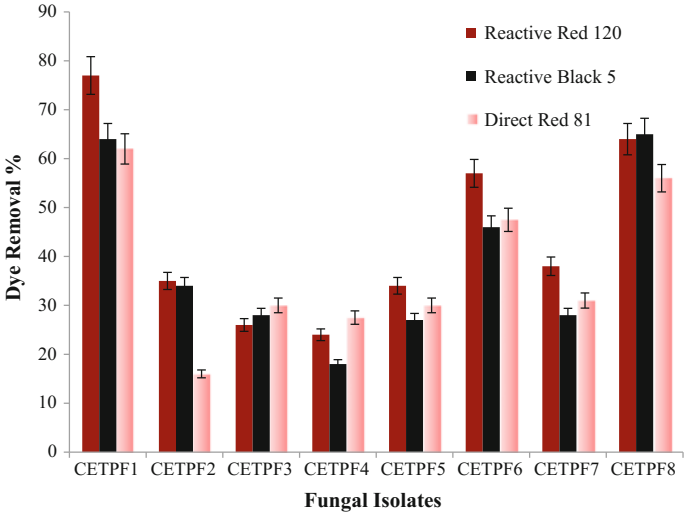

Fig. 1 Synthetic dye removal % of the fungal isolates from CETP samples

3.3 Dye Decolouration Studies of the Indigenous Fungi and Microbial Consortia

In these studies, Fig. 2 shows Synthetic dye removal % of the screened fungal isolates from CETP samples. Among four fungal isolates, *Pleurotus ostreatus* was more effective and which was followed by *P. simplicissimum*, *P. chrysosporium* and *A. niger*. The dye removal percentage is 76% for Reactive Red 120, 64% for Reactive Black 5 and 62% for Direct Red 8, in case of *Pleurotus ostreatus*. Whereas *Aspergillus niger* shows 35% dye removal for Reactive Red 120, 34% dye removal for Reactive Black 5 and 15% dye removal for Direct Red 8. In case of two different species of *Penicillium* shows 42–70% of dye removal for all three reactive dyes. Among the other fungi studied, *Trametes versicolor, Pleurotus* sp, *Bjerkandera* sp, *Ischnoderma resinosum, Irpex lactus* and *Pycnoporus cinnabarinus* isolates also shown higher rate of decolourization against some synthetic reactive dyes, such as Reactive black 5, Reactive blue 19 and Reactive yellow (Roy and Archibald 1991; Shin et al. 1997; Swamy and Ramsay 1999; Maxima and Costa 2004; Eichlerova et al. 2005).

With respect to fungal consortium, FC1and FC3 (Figs. 3, 5) shows maximum dye removal % for reactive Black 5 (76%), Reactive Red 120 (74%) and for Direct red 8 (72%). Similarly, FC2 (Fig. 4) shows maximum dye removal % for reactive Black 5 (74%), Reactive Red 120 (72%) and for Direct red 8 (65%). Whereas FC4 (Fig. 6) shows lower level of dye decolourization when compared to all other 3 fungal consortium.

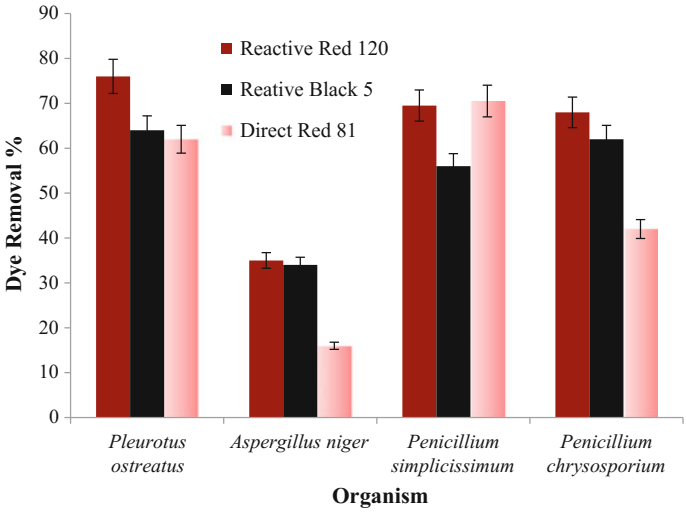

Fig. 2 Synthetic dye removal % of the screened isolates from CETP samples

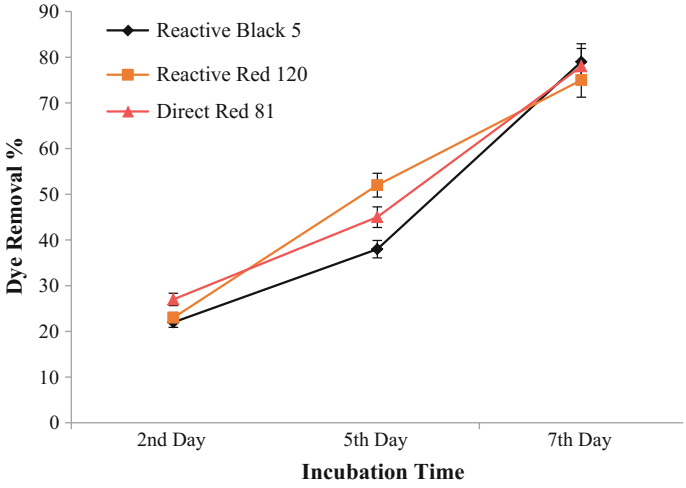

Fig. 3 Synthetic dye removal % by Fungal Consortium 1 (FC1)

The decolourization efficiency for reactive Black 5, Reactive Red 120 and direct red 8 was observed to be 52, 18, and 44% respectively for a period of one week. Previously, reported that, most of the white rot fungi which require 7–20 days period to decolorize various synthetic dyes with 90% decolourization efficiency (Kirby et al. 2000) and other mixed microbial cultures (Adedayo et al. 2004; Senan

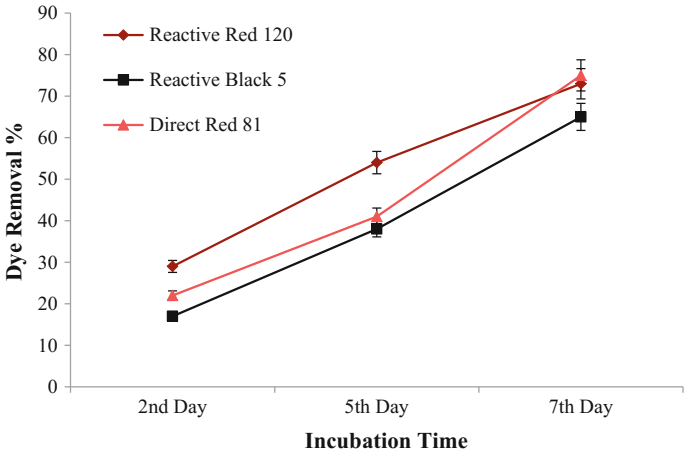

Fig. 4 Synthetic dye removal % by Fungal Consortium 2 (FC2)

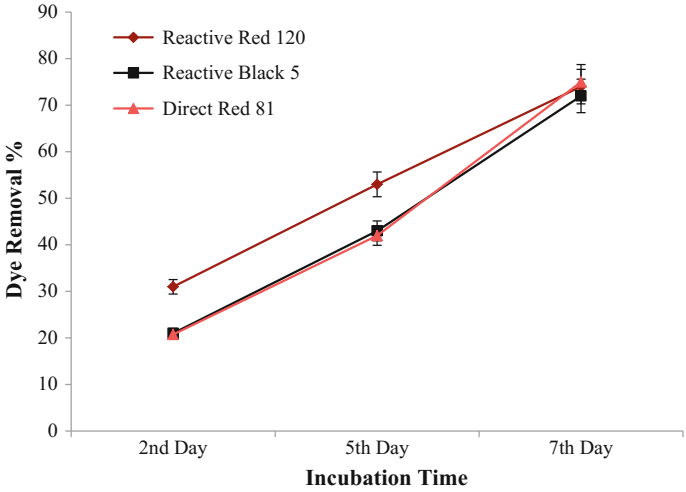

Fig. 5 Synthetic dye removal % by Fungal Consortium 3 (FC3)

and Abraham 2004). These consortia were tested for the ability to decolorize the synthetic dyes and it was found that they showed about 55–80% dye removal within a considerable period of time. The fungal consortia were found to degrade the synthetic dyes.

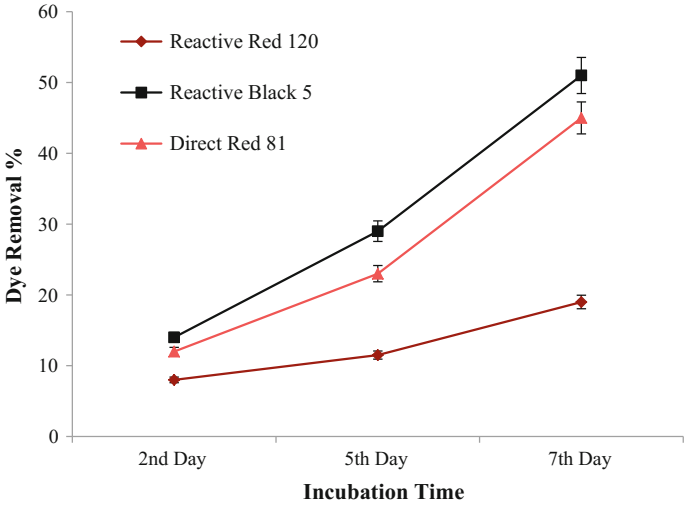

Fig. 6 Synthetic dye removal % by Fungal Consortium 4 (FC4)

4 Conclusion

Textile dyes are one of the most prevalent chemicals used in various sectors, which will emit huge volume of pollutant both in the form of effluent and sludge. With increasing usage of the wide variety of dyes in the industries, pollution from the effluents has become increasingly alarming. For degradation, microbial decolourization and degradation is an eco-friendly and cost-effective process when compare with all other processes. The fungal isolates were isolated from the effluent and soil samples from the contaminated sites. It was concluded that, the fungal consortia obtained by grouping the fungal species isolated from the samples of the dye contaminated sites showed higher synthetic dye removal % than the individual isolates. Thus, the microbial consortia can be used for the decolourization of textile dyes efficiently.

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Optimization of Biosurfactant Production and Crude Oil Emulsification by *Bacillus* Sp. Isolated from Hydrocarbon Contaminated Soil Sample

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Abstract Biosurfactants, the surface-active compounds produced by microorganisms, have bright prospects in the bioremediation of oil spills. They can also serve as substitutes to synthetic surfactants in food and cosmetic industries. In this study, bacterial strains from soil samples exposed to petroleum hydrocarbons were screened using drop collapse, oil displacement and hemolytic activity tests. Six isolates showed encouraging results in these tests. Based on further assessment of emulsification activity and surface tension reduction, three of the isolates AS03, AS09 and AS01 were judged to be good biosurfactant producers. 16S rRNA gene sequencing confirmed the identity of the best isolate AS03, to be *Bacillus licheniformis*. Optimization of biosurfactant production and crude oil emulsification by this isolate was attempted using response surface methodology. The optimum levels of three significant variables viz., glucose, yeast extract and toluene were ascertained to be 10.5 g/L, 10 g/L and 5% (v/v), respectively, which resulted in 54.79% emulsification activity ($E_{24\%}$). The biosurfactant was characterized to be of lipopeptide nature based on the FTIR spectrum. These bacterial strains and the produced biosurfactant hold potential for oil spill clean-up and related environmental applications.

Keywords *Bacillus licheniformis* · Biosurfactant · Emulsification index · FTIR spectroscopy · Response surface methodology

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1 Introduction

Biosurfactants are amphiphilic biomolecules that display marked surface activity and are produced by certain microorganisms as by-products during their metabolism (Banat et al. 2010). They are capable of reducing surface tension and interfacial tension between molecules, at the surface and interface, respectively. They may be glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids, or neutral lipids (Cappello et al. 2012). They have excellent detergency, emulsifying, dispersing, penetrating and metal sequestering traits, there by making them viable alternatives to synthetic surfactants. They are non-toxic and less persistent in the environment. They have extensive industrial applications, especially in the food, cosmetic and pharmaceutical sectors (Pastewski et al. 2006).

Biosurfactants find extensive application in the bioremediation of oil spills (Emitazi et al. 2005). Pristine marine environments are increasingly becoming polluted with crude oil and petroleum products that gain entry through accidental spills. Synthetic surfactants are often used to emulsify and disperse the oil. However, they have detrimental environmental impacts owing to their toxicity and lack of biodegradability. Hence, biosurfactant producing bacteria or the recovered surface-active compound from these organisms could be used for this purpose (Hasanshahian and Emtiazi 2008). These compounds play a prominent role in environmental clean-up and it has been well documented in literature (Sayakti et al. 2013).

Biosurfactant producing microorganisms are present in both terrestrial as well as aquatic environments and they are especially known to inhabit extreme environments on account of their pH, temperature and halo tolerance. *Bacillus subtilis* (Nitschke and Pastore 2006), *Pseudomonas aeruginosa* (Thanomsub et al. 2007), *Candida rugosa* and *Rhodococcus* sp. (Gesheva et al. 2010) are some important biosurfactant producing bacteria. These microorganisms are able to solubilize the hydrophobic compounds present in their environment and utilize them as substrates for growth. Substrates used for biosurfactant production range from common carbohydrates such as glucose and sucrose to hydrocarbons such as crude-oil and diesel (Kappeli and Finnerti 1980). Biosurfactants may be synthesized by the bacteria via the de novo pathway or from assembly of the substrates (Syldatk and Wagner 1987).

In the present work, bacterial strains were isolated from hydrocarbon contaminated soil samples and screened for biosurfactant production. The isolate showing the highest activity was identified by 16S rRNA gene sequencing and its biosurfactant production was optimized by response surface methodology (RSM). The compound was recovered and characterized using FTIR analysis.

2 Materials and Methods

2.1 Materials

Soil sample exposed to petroleum hydrocarbons was collected from the area around a gas station in Periyanaikanpalayam locality of Coimbatore District, India. All chemicals and reagents used were of analytical grade and purchased from standard sources (Sigma Aldrich, USA and HiMedia, India).

2.2 Isolation of Bacterial Strains

The soil samples were serially diluted and plated on mineral salts (MS) agar medium. The culture medium consisted of (per L): KH_2PO_4—1.0 g, Na_2HPO_4—2.0 g, $(NH_4)_2SO_4$—3.0 g, $MgSO_4.7H_2O$—0.5 g, NaCl—0.10 g, $CaCl_2.7H_2O$—0.05 g and agar—15 g. 2 ml of trace element solution was added to it, whose composition was (per L): $ZnSO_4$—0.30 g, $CuSO_4$—0.25 g, $FeSO_4.7H_2O$—0.20 g and $MnSO_4$—0.17 g. This culture medium was supplemented with 2% (v/v) crude oil. The inoculated plates were incubated for 24 h at 35 °C. Discrete colonies showing observable morphological variation were repeatedly sub-cultured in the same medium to obtain pure cultures and subsequently maintained as agar slants and glycerol stocks.

2.3 Screening for Biosurfactant Production

The isolated bacterial strains were screened for biosurfactant production after cultivation in MS broth (similar composition as stated above, excluding agar).

2.3.1 Hemolytic Activity

The ability of the bacterial strains to induce hemolysis in blood agar plates was checked (Carrillo et al. 1996). Sheep blood agar (base) was sterilized at 121 °C for 15 min. The agar temperature was brought down to 50 °C and 7% (v/v) sterile sheep blood was added to it. Pure cultures of the bacterial strains were streaked and the plates incubated at 35 °C for 48 h. The colonies were observed for the presence of clear zones around them.

2.3.2 Oil Displacement Test

The diameter of the clear zone formed upon placing a drop of biosurfactant containing solution on an oil-water surface was measured (Morikova et al. 2000; Youssef et al. 2004). 50 ml of distilled water was taken in a petri dish followed by the addition of 20 µl of crude oil to the water surface. 10 µl of culture supernatant was then added to the oil surface. The diameters of the clear zones obtained for different strains were measured and compared.

2.3.3 Drop Collapse Test

It depends on the destabilization of liquid droplets by the surfactant. 2 µl of crude oil was applied to the wells of a microtiter plate and equilibrated for 24 h. 5 µl of culture supernatant was transferred to it and the size of the drop was observed with the aid of a magnifying glass after 1 min (Adamu et al. 2015).

2.3.4 Surface Tension Measurement

The surface tension of the culture supernatant was measured (K20 Force Tensiometer, Orbit, India) based on the principles of Wilhelmy plate method (Fernandes et al. 2007). The validity of the readings was checked against pure water (70.78 ± 0.02 mN/m).

2.3.5 Emulsification Activity

To determine the emulsification index (E_{24}), equal volumes of culture supernatant and crude oil were taken in a test tube. The mixtures were vortexed for 2 min at very high speed and left undisturbed for 24 h. The heights of the stable emulsified layers were measured and emulsification indices calculated (Emtiazi et al. 2009):

$$E24\,(\%) = \frac{Height\ of\ the\ emulsion\ layer}{Total\ height\ of\ the\ liquid} * 100$$

2.3.6 Biosurfactant Detection by Methylene Blue Method

To 1 ml of the bacterial cultures, 0.003% methylene blue was added and shaken vigorously for 30 s. Equal amount of chloroform was added to the tubes and left for 20 min to allow extraction of methylene blue anionic surfactant ion pair into the chloroform layer. After the extraction, the tubes were centrifuged at 3000 g for

5 min and absorbance values measured at 625 nm, using pure chloroform as the reference (Jones and Esposito 2000).

2.4 Identification of the Bacterial Isolates

All the isolates that showed appreciable biosurfactant production were identified by MALDI-TOF Mass Spectroscopy (Aitken 2005). In this analysis, the match patterns of the samples were compared using Bruker Taxonomy Tree. Ten closest matches were considered and the bacteria identified.

Genotypic identification of the best isolate was performed using 16S rRNA gene sequencing. Single colony from freshly streaked agar plate was inoculated into nutrient broth. The cells were harvested, DNA isolated and checked on agarose gel for purity and quantity. 16s rRNA gene primers 27F (5'-AGAGTTTGATC (A/C) TGGCTCAG-3') and 1492R (5'-ACGG (C/T) TACCTTGTTACGACTT-3') were used to amplify ~1.4 kb gene from the genomic DNA (Giovanoni 1991). The amplified product was gel purified, quantified and sequenced using ABI prism 3100 Genetic Analyzer (Applied Biosystem). Consensus sequence was generated from the forward and reverse sequences using 'Aligner' software. It was subjected to BLAST in the NCBI GenBank database (Altschul et al. 1997). Based on the maximum identity scores, the cultures were identified and phylogenic tree constructed (Kimura 1980).

2.5 Optimization of Biosurfactant Production

Box—Behnken Design developed by Design Expert 9.0 (Stat Ease Inc. Minneapolis, USA, trial version) was used for this purpose. The three significant parameters whose levels were optimized through RSM were glucose (**A**), yeast extract (**B**) and toluene (**C**). They were inferred to be the best carbon source, nitrogen source and inducer in preliminary one-factor-at-time (OFAT) studies. Each one of the above independent variables was studied at three different levels (-1, 0, +1). A matrix of 17 experiments involving these three factors was generated by the software. Accordingly, production was carried out in 250 ml Erlenmeyer flasks containing 100 ml culture medium of pH 7.0. The flasks were sterilized, inoculated under aseptic conditions and incubated at 35 °C for 48 h, in an incubator shaker (Orbitek, India) set at 100 rpm. All experiments were carried out in triplicates and the data given represent the mean. At the end of the incubation period, emulsification index ($E_{24\ \%}$) was calculated using the cell-free supernatant and the value recorded as the response (dependent variable).

The response data were analyzed by feeding into the software. Contour plots and standard analysis of variance (ANOVA) were generated. A quadratic polynomial regression model was assumed for the predicted response. Regression analysis was

performed on data obtained from the designed experiments. For a three-factor system, the model equation was as follows:

$$Y = \beta 0 + \beta 1 * A + \beta 2 * B + \beta 3 * C + \beta 11 * A^2 + \beta 22 * B^2 + \beta 33 * C^2 + \beta 12 * AB + \beta 13 * AC + \beta 23 * BC$$

where, Y is the predicted response, *β0* is the intercept, *β1, β2* and *β3* are the linear coefficients, *β11, β22* and *β33* are the squared coefficients, and *β12, β23* and *β13* are the interaction coefficients.

2.6 Biosurfactant Recovery and Characterization

The pH of the culture supernatant was adjusted to 2.0 using 6 M HCl and left overnight at 4 °C for precipitation of the biosurfactant. It was collected by centrifugation at 8000 g for 15 min and dissolved in distilled water. Chloroform and methanol mixture (6.5:1.5) was added, shaken well and kept for separation of the layers. The organic phase was evaporated, permitting recovery of the surfactant in powder form (Nitschke and Pastore 2006).

The sample was characterized using FTIR Spectroscopy (Nicolet 6700). 0.01 mg of the sample was taken and ground with 0.02 mg of dried potassium bromide pellets and cast as an ultrathin film on the carrier. The spectra were obtained in the frequency range of 4000—400 cm^{-1}, with a resolution of 4 cm^{-1} for 10 scans.

3 Results and Discussion

3.1 Isolation of the Bacterial Strains and Screening for Biosurfactant Production

Serial dilution and plating of the soil sample in MS agar supplemented with crude oil led to the isolation of 10 bacterial strains. They were screened for biosurfactant production using blood agar hemolysis, oil displacement and drop collapse tests. The isolates responded variably to the screening tests, with some showing strongly positive responses and others showing weak to negative responses. The manner in which a particular strain responded to the different screening procedures also varied, but to a lesser extent. From Table 1, it could be inferred that four of the isolates (AS01, AS03, AS07 and AS09) produced moderate to good hemolytic activity on blood agar plates. These isolates gave good responses to oil displacement and drop collapse tests as well. AS04 and AS08 additionally yielded positive results in the

Table 1 Summary of the screening tests for biosurfactant production

Strain	Screening tests			Surface tension (mN/m)	E_{24} (%)	Methylene blue detection (OD 625 nm)
	Hemolysis	Oil displacement	Drop collapse			
AS01	+++	++	+++	36.3	36.5	0.83
AS02	+	–	+	47.6	07.5	0.19
AS03	+++	++	++	34.6	38.4	0.89
AS04	+	++	++	43.7	31.4	0.45
AS05	–	–	+	47.2	14.5	0.30
AS06	+	–	–	51.3	06.8	0.08
AS07	++	+++	++	39.5	25.9	0.70
AS08	+	++	+	45.9	19.8	0.72
AS09	+++	+++	++	35.4	35.3	0.81
AS10	+	+	–	47.5	12.4	0.09

−Negative; +Weakly positive; ++Moderately positive; +++Strongly positive
The surface tension of uninoculated MS broth taken as blank was 54.2 mN/m

latter two tests. AS02, AS05, AS06 and AS10 showed only weak to negative responses in the screening tests.

The isolates' biosurfactant producing ability was quantitatively assessed through further tests. AS01, AS03 and AS09 caused considerable reduction in surface tension from 54.2 to 36.3, 34.6 and 35.4 mN/m, respectively. *Bacillus methylotrophicus* USTBa had caused a similar decrease in surface tension when grown in MS broth supplemented with 2% (v/v) crude oil (Chandankere et al. 2013). AS01, AS03 and AS09 also exhibited pronounced emulsification indices of 36.5, 38.4, and 35.3%, respectively. Such emulsification of the lipid substrate enhances its biodegradation potential (Minf et al. 2011). AS03 also showed the highest absorbance of 0.89 at 625 nm in the methylene blue method for biosurfactant detection. This was followed by 0.83 and 0.81 for AS01 and AS09, respectively. Biosurfactants from *Pseudomonas* sp. 2B have been detected using CTAB–methylene blue agar medium method (Aparna et al. 2012). Based on these results, the three isolates were concluded to be good biosurfactant producers, with AS03 having an edge over the other two isolates.

3.2 *Identification of the Bacterial Isolates*

The three best biosurfactant producers were subjected to MALDI-TOF Mass Spectroscopic analysis and the Bruker Taxonomy Tree was used to match the patterns and identify the isolates. AS01, AS03 and AS09 were tentatively identified as *Bacillus pumilus, Bacillus licheniformis* and *Bacillus flexus*, respectively.

16S rRNA gene sequencing was performed for the best biosurfactant producer AS03. The sequence was submitted to NCBI GenBank database and was allotted

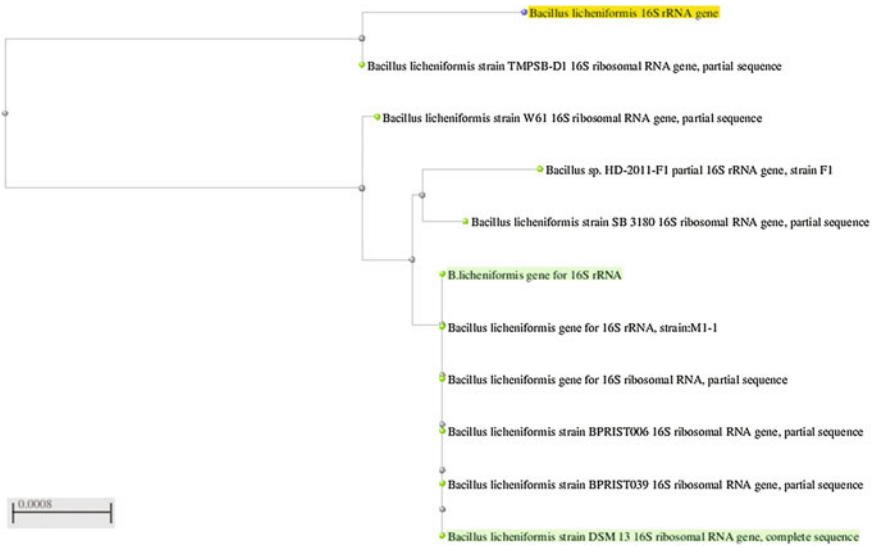

Fig. 1 Phylogenetic tree of *Bacillus licheniformis* AS03

the Accession No. KT236094. Upon performing nucleotide blast, it showed up to 99% similarity to *B. licheniformis* 16S rRNA gene sequences available in the database. The phylogenetic tree depicting sequence relationship between AS03 and others in the database is given in Fig. 1.

3.3 Optimization of Biosurfactant Production

Statistical tools assist in designing experiments for optimizing a process. They incorporate the interaction effects of variables and enable us to arrive at the optimum levels of variables within a minimal number of experimental runs. Three factors (glucose, yeast extract and toluene) were selected and their levels optimized using Box-Behnken design of RSM. The experimental design is given in Table 2. This study was carried out to understand the impact of a range of variables on the response, emulsification index ($E_{24\%}$).

The following second order polynomial equation illustrates the empirical relationship between the independent variables and the response:

$$Y = 45.252 - 0.1975 * A + 3.30625 * B - 3.48875 * C - 2.8775 * AB - 1.2875 * AC + 3.53 * BC + 1.2115 * A^2 + 3.299 * B^2 + 1.804 * C^2$$

It can be inferred from the above equation that the linear coefficient B, interaction coefficient BC and all three squared coefficients have positive impacts on the

Table 2 RSM experimental design and responses obtained for biosurfactant production from *B. licheniformis* AS03

Run	Factor 1	Factor 2	Factor 3	Response
1	20 (+1)	6.5 (0)	5 (+1)	40.98
2	10.5 (0)	6.5 (0)	2.75 (0)	48.88
3	10.5 (0)	6.5 (0)	2.75(0)	45.87
4	20 (+1)	6.5 (0)	0.5 (-1)	52.57
5	10.5 (0)	6.5 (0)	2.75 (0)	43.65
6	10.5 (0)	10 (+1)	5 (+1)	54.79
7	20 (+1)	3 (-1)	2.75 (0)	50.5
8	20 (+1)	10 (+1)	2.75 (0)	51.22
9	10.5 (0)	3 (-1)	5 (+1)	40.98
10	10.5 (0)	3 (-1)	0.5 (-1)	52.98
11	10.5 (0)	6.5(0)	2.75 (0)	45.85
12	10.5 (0)	10 (+1)	0.5 (-1)	52.67
13	1 (-1)	3 (-1)	2.75 (0)	42.55
14	1 (-1)	10 (+1)	2.75 (0)	54.78
15	1 (-1)	6.5 (0)	5 (+1)	46.54
16	1 (-1)	6.5 (0)	0.5 (-1)	52.98
17	10.5 (0)	6.5 (0)	2.75 (0)	42.01

Factor 1—glucose (g/L), Factor 2—yeast extract (g/L), Factor 3—toluene % (v/v), Response—$E_{24\%}$

Coded levels of the variables are specified within brackets

emulsification index. ANOVA was used to check the adequacy of the model and F test was employed for this purpose. Model F value of 5.53 and p-value of 0.0173 indicate significance of the model. p-values less than 0.05 indicate that the corresponding model terms are significant. In general, larger magnitudes of t and F values and smaller p values indicate that the corresponding coefficient terms are significant. In this case, B, C, BC and B^2 are significant model terms. A p-value of 0.4540 for lack of fit means that it is not significant.

The R^2 value lies in the range of 0–1.0 and a value close to 1.0 is desirable, indicating fitness of the model. It gives a measure of how much variability in the observed response can be explained by the experimental parameters and their interactions. The R^2 value of 0.9802 for the quadratic model shows good correlation between the experimental and predicted values. The coefficient of variation (CV) determines the ratio of standard deviation to mean and the result is considered reproducible if the value is not greater than 10%. In this study, CV of 5.4% was obtained. Adequate precision measures signal-to-noise ratio and a ratio greater than 4 is desirable. It detects which experimental parameters generate signals that are large in comparison to the noise. An adequate presision of 12.58 obtained for the model points to a good signal.

The contour plot is a way of expressing the regression equation graphically. It depicts the interactions among the variables and is used to determine the optimum

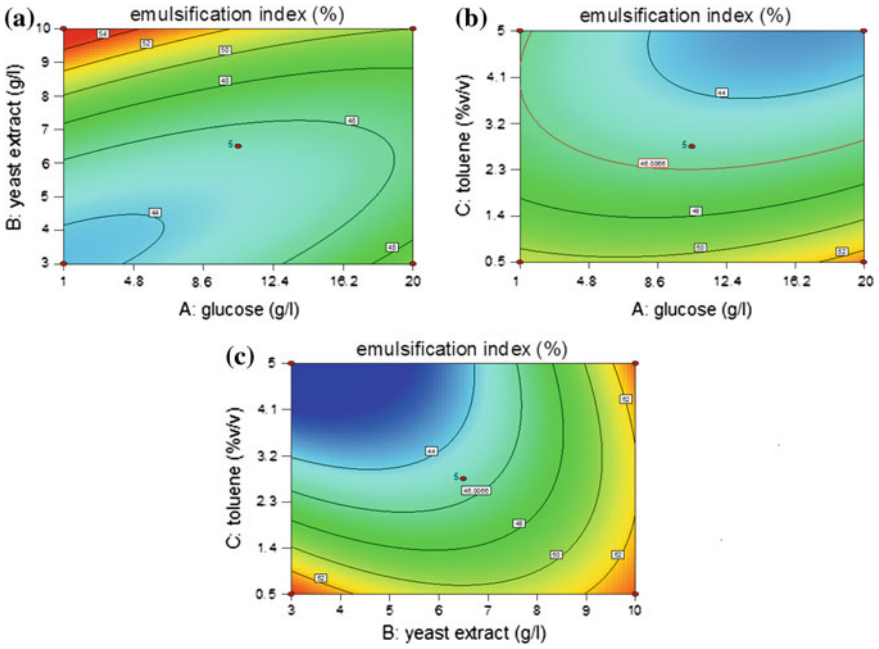

Fig. 2 Contour plots depicting interactions between glucose—yeast extract (AB), yeast extract—toluene (BC) and glucose—toluene (AC)

concentration of each factor for good response. The contour plots illustrating the interactions between the variables are given in Fig. 2.

A maximum of 54.79% emulsification activity (1.42-fold increase upon comparison with the 38.4% prior to optimization) was obtained in a culture medium comprising of 10.5 g/L glucose, 10 g/L yeast extract and 5% (v/v) toluene. Such emulsification of the crude oil in water is advantageous as it facilitates the biodegradation of this environmental pollutant. It enhances the bioavailability of the oil and thus increases the biodegradation rate. Earlier, biosurfactant production by *Stenotrophomonas maltophilia* NBS-11 has been optimized using pH, dextrose, $ZnCl_2$ and $MgSO_4$ as critical control factors (Hemalata et al. 2015). However, the effects of inducers such as toluene on the biosurfactant production have not been investigated. In another study, biosurfactant production by *Bacillus mycoides* has been optimized by the combination of central composite design (CCD) and RSM. Through the analysis, glucose and temperature were found to be the most significant factors, whereas pH and salinity had less effect within the ranges investigated. A maximum reduction in surface tension was obtained under the optimal conditions of 16.55 g/L glucose, 39.03 °C, 55.05 g/L total salt and medium pH 7.37 (Najafi et al. 2010).

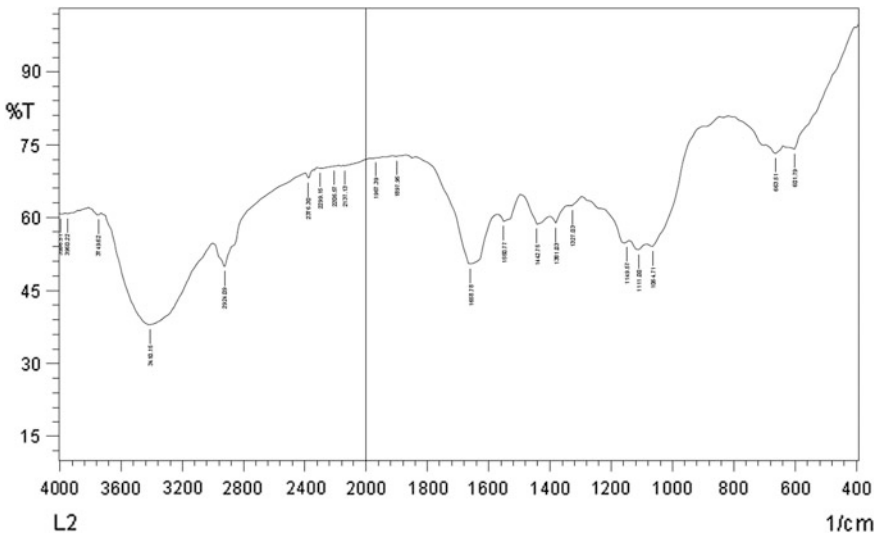

Fig. 3 FTIR spectrum of the biosurfactant produced by *B. licheniformis* AS03

3.4 Biosurfactant Recovery and Characterization

Organic solvent extraction permitted recovery of 1.02 g/L of biosurfactant from the acidified culture supernatant of AS03. It was characterized using FTIR Spectroscopy (Fig. 3) and the results were compared with the spectral data of other biosurfactants. The observed peaks were similar to those commonly found in lipopeptide biosurfactants. The strong peak at 3410 cm^{-1} represents OH, CH, and NH stretching vibrations. This is characteristic of carbon-containing compounds with amino group. Another strong peak was observed at 1658 cm^{-1}, signifying CO–N stretching vibration. Absorption in the region of 1600–1700 cm^{-1} is characteristic of amide I vibration in proteins, thus indicating the presence of peptide groups in the biosurfactant. The presence of aliphatic chains was confirmed by the observation of peak at 2924 cm^{-1}. This could be due to the C–H stretching vibration of CH_3 and CH_2 groups in alkyl chains. The weak band at 1381 cm^{-1} resulted from deformation and bending vibrations of C–CH_2 and C–CH_3 groups in aliphatic chains. The absorption at 1111 cm^{-1} could be attributed to C–O–C vibrations in esters. Therefore, it could be concluded that the biosurfactant produced by *B. licheniformis* AS03 has a lipopeptide composition. Such lipopeptide nature and similar molecular vibrations have also been reported for the biosurfactant partially purified from halophilic *Bacillus* sp. BS-3 (Donio et al. 2013).

4 Conclusions

This study has resulted in the isolation of quite a few bacterial strains with promising potential for biosurfactant production and crude oil emulsification. The surface active agents are likely to have been advantageous to the cells for thriving in the hydrophobic environment. Biosurfactant production by these strains must have given them an edge over other inhabitants by increasing the bioavailability of the hydrophobic substrates, permitting their utilization as carbon source by the cells. Marked emulsification activity and surface tension lowering ability were possessed by three of the isolated bacterial strains. 16S rRNA gene sequencing has confirmed the identity of the best biosurfactant producer. as *B. licheniformis*. Statistical medium optimization has signified the optimum levels and interaction effects of three variables, namely glucose, yeast extract and toluene on the emulsification activity. FTIR spectroscopy has given an insight into the functional groups and molecular vibrations of the recovered biosurfactant and denoted its lipopeptide nature. The data obtained from our studies suggest that the bacterial strains AS03, AS09 and AS01 possess significant biosurfactant activity for possible application in the bioremediation of oil contaminated sites. Further investigations on the characteristics of the biosurfactant are needed in order to unveil its full application potential.

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Prospecting Multiple Enzyme Systems of Mangrove Fungi for Dye Decolourization Potential

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Abstract Decolourisation of effluents is always a major issue in the effluent treatment process. There are many methods for the decolourisation of these effluents. Enzymatic decolourisation is one of the promising methods. Laccase, one of the major enzymes involved in decolourisation mechanism is produced by many microbial sources. In the present study *Trichoderma viride*, a known plant growth promoting fungal isolate obtained from mangrove ecosystem of Valanthakad, Kerala, India was found to produce laccase enzyme. Plate assay was performed for evaluating the decolourising ability using different synthetic dyes. A higher decolourisation was observed at various dye concentrations. The isolate was found to be efficient in decolourization of synthetic dyes effectively through enzymatic method. The fungal dye biosorption was also studied using the isolate and it was found that there was high efficacy for laccase system in dye decolourization.

Keywords Laccase · Decolourization · Fungus · Azo dyes

1 Introduction

Application of dye in textile industries is known since old civilizations. The avenue for the dyeing was further extended to all our day-to-day needs simply for the value addition and making it more pleasing for the mankind. However, there are lots of problems associated with dye usage. Apart from natural dyes which were used

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earlier, there are numerous synthetic dyes of different classes. Anthraquinone, triphenylmethane and azo dyes were extensively used in various industries like food, textile, cosmetics, paper making as well as in pharmaceuticals (Swamy and Ramsay 1999; Padamavathy et al. 2003).

The accumulation of these dyes in washout leads to a troublesome effluent treatment. Decolourisation of effluents from any industrial process is always tedious as it is mandatory to achieve the permissible standards before the effluent disposal. Though, there are many technologies existing for the same, the sludge generated as a result of these effluents treatment is so high. Moreover, these synthetic dyes are proved to be toxic, mutagenic and carcinogenic (Namasivayam and Sumithra 2005; Diwaniyan et al. 2010). Biological or enzymatic decolourization methods are quite promising and will always involve an eco-friendly process and results in less toxic end residues. Hence, the biological decolourisation methods are well suitable for the reduction of toxicity of effluent thereby facilitating its safe disposal.

There are many strategies for treating the colored wastewater through physical and chemical processes, namely flocculation, flotation, coagulation, adsorption, oxidation, precipitation and reduction, ozonation and membrane separation. However, their usage is very limited due to high processing cost and due to many other drawbacks (Azmi et al. 1998; Robinson et al. 2001). As mentioned earlier, the dye degradation is achieved either by biotransformation or adsorption on biomass. The major advantage of these processes is its low-cost biological materials utility, as living or dead microorganisms. Moreover, during the biological processes biotransformation of dye occurs thereby leading to complete mineralization or reduction of toxicity of the final products (Pointing and Vrijmoed 2000; Robinson et al. 2001; Deng et al. 2008).

There are many enzyme systems well studied for its potential for dye decolourization efficacy. However, the laccase is one of the major enzymes which are widely used for decolourization of industrial effluents on a commercial scale. Laccase is a copper containing enzyme system, widely produced by many fungal isolates. These enzymes are also produced by wood-rot fungus and many plant pathogens like *Fusarium* sp. Considering such strains for large scale production of laccase for effluent treatment is thought provoking as it makes us concerned over the bio-safety issues. Fungal laccases are so promising enzymes for the decolourisation mechanism and many previous studies focused on the microbial laccase production for the effluent treatment. Still there is high demand for prospecting new potential isolates producing laccase or lignolytic enzyme system which are safe for large scale production. *Trichoderma viride*, one of the plant growth promoting fungal strain was found to possess laccase producing ability. Considering its non-pathogenecity and low harmful effects, it could be a better source for laccase production for the application of decolourisation (Divya and Sadasivan 2016).

The demand of the enzymatic decolourization is of high priority due to low cost process involved, eco-friendly process and high specificity of enzyme systems. The present work, was to demonstrate the decolourization efficacy of some commonly used dyes. Azo or mordant dyes were compared with other synthetic dyes used for textile industries. These dyes were used as model for decolourising ability of the

Trichoderma viride and further evaluated using its enzyme system as well as using its biosorption potential (Hsueh and Chen 2007).

An efficient system is required for treating the textile effluent resulting in an adequate color removal. There are many reports on highly efficient biological systems including bacterial, fungal and algae in dye removal (Deng et al. 2008; Przystas et al. 2009; Khan et al. 2013). Fungal biomass were found as an efficient sorbents as well as the enzyme produced also contributes towards the biodegradation of these synthetic dyes (Santos et al. 2007; Deng et al. 2008; Godlewska et al. 2009; Przystas et al. 2009; Diwaniyan et al. 2010; Przystas et al. 2013; Hadibarata et al. 2013; Si et al. 2013). Enzyme assisted decolourisation of effluent is well studied. The most widely studied are white rot fungi which produce non-specific enzymes, such as lignin peroxidase, manganese peroxidase and laccase that degrade mostly aromatic compounds (Przystas et al. 2015). However, the major challenge in this is the toxicity of the dye stuff in the effluents. These may inhibit the microbial growth and thereby actually not allowing the sufficient enzyme production for decolourisation process. Another strategy is to use the crude enzymes in decolourization. Still the presence of some inhibitory compounds ceases the enzyme actions and thereby reducing the decolourisation efficiency. Apart from this, direct fungal biomass can also be used in decolourisation, and this involves the biosorption of the dye from effluent.

2 Materials and Methods

2.1 Isolation and Screening of Lignolytic Fungus

Mangrove debris and plant specimens were collected from Valanthakad mangrove ecosystem, Kerala, India. Serial dilutions were made and inoculated on potato dextrose agar (PDA) plate and incubated at 28 ± 2 °C. Pure culture were made and maintained on PDA slants. Laccase activity was screened by inoculating the isolates on PDA plates supplemented with 0.2% tannic acid. Positive isolates showed reddish brown colour on reverse side of the plate. Positive isolates were identified based on colony morphology, lacto phenol cotton blue (LCB) mount and molecular method (D1/D2 region amplification) (Vinod et al. 2014).

2.2 Plate Assay for Decolourisation Ability

Synthetic dyes namely congo red, malachite green, bromophenol blue and crystal violet were used for the dye degradation assay. Potato dextrose agar was supplemented with 1 µg/ml concentration of the above four dyes. *Trichoderma viride*

VKF3 was inoculated on the medium and incubated at 28 ± 2 °C for 5 days. The zone of clearance was observed followed by the incubation (Swamy and Ramsay 1999).

2.3 Decolourisation Using Lignolytic Enzyme Systems

The dyes for which high decolourisation occurred was further tested for determining decolourisation percentage. For this, potato dextrose (PD) broth was supplemented with various concentrations (1, 5 and 10 mg/l) of the selected dyes, inoculated the fungal strain and kept at 37 °C for 7 days. On each day, the decolourisation rate was determined spectrophotometrically. Flasks were maintained in static condition at room temperature (Swamy and Ramsay 1999). After the decolourisation experiment, the biomasses were filtered and wet weight was measured for understanding the lethality of dye for the fungal biomass. Wet weight of biomass was expressed in g/l of the liquid medium in which decolourisation was performed.

2.4 Decolourisation Using Biosorption Mechanism

Effect of heat-killed fungal biomass for the absorption of colour was also checked for most efficiently degraded dyes namely bromophenol blue and Congo red. Biomass grown in PD broth was heat killed at 121 °C for 20 min at 15 lbs pressure. Wet fungal biomass of approximately 1 g was weighed and added into different concentrations of dye (1, 5 and 10 mg/l) dissolved in distilled water. Absorbance was read at regular intervals for understanding decolourization kinetics. Finally, the decolourization efficacy of live and dead fungal biomass was compared. Scanning of filtrate was performed in the range of 400–800 nm using Double Beam UV-Vis Spectrophometer. Scanning of filtrate from experimental tubes added with dead fungal biomass and compared with the spectra to show the absorption kinetics contributing to decolourisation mechanism (Swamy and Ramsay 1999).

2.5 Enzymatic Removal of Dye Thin Film

Effect of enzyme on malachite green was tested by preparing thin film coatings in test tubes by keeping different concentrations of dye poured into test tubes and storing under static conditions for 3–4 days to ensure a good and stable coating. The dye was decanted and the tubes thoroughly washed with distilled water 3 times to remove free residual dye particles. Crude enzyme (3 ml) was added into each tube and 1 ml of phosphate buffer of pH 6.0 was added to ensure optimal enzymatic

reactions. The tubes were kept for 1 h in static condition and poured off the enzyme solution. Buffer alone was used instead of enzyme in the control tubes. The removal of malachite green film was observed visually.

2.6 Multiple Enzyme Production by Fungal Isolate

Multiple enzyme screening for the fungal isolate was performed on potato dextrose agar plates supplemented with 2% Brich wood xylan and carboxymethyl cellulose for xylanase and cellulase respectively. Followed by 3 days of incubation at 28 °C, the plates were flooded with iodine solution to observe clear zone around positive colonies (Charitha and Sunil 2012). Lipase screening for fungal isolates was performed using PDA plates supplemented with 1% olive oil and 0.5% phenol red indicator. Lipase enzyme degrades oil to release free fatty acids which changed the medium pH and the medium colour changed from red to yellow (Singh et al. 2006).

3 Results and Discussion

3.1 Enzyme Assisted Decolourisation of Synthetic Dyes

The isolate *Trichoderma viride VKF3* was able to grow in PDA media supplemented with dyes. There was very high growth visible in crystal violet medium and in bromophenol blue containing medium (Fig. 1). It was observed that the fungal growth was very slow in malachite green containing plate. This shows that some dyes may have inhibitory effect on the fungal growth. Plate containing Congo red had high dense fungal growth and found to decolorize the plate when comparing to control plate. Bromophenol blue was completely decolorized by the fungal strain. Hence, the decolourisation percentage of congo red as well as bromophenol blue was checked. There was a reduction in percentage of decolourisation when dye concentration was increased (Fig. 2). The laccase enzyme was quantified in the decolourisation medium and was found to be 1.67 U/ml.

Fig. 1 Decolourisation of synthetic dyes using laccase producing *Trichoderma viride VKF3* (Crystal violet, malachite green, congo red and bromophenol blue)

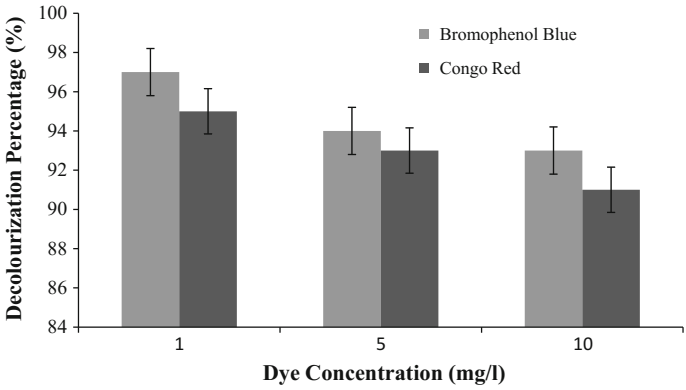

Fig. 2 Decolourisation of bromophenol blue and Congo red using laccase enzyme produced by *Trichoderma viride* VKF3

3.2 Biosorption of Dyes Using Fungal Biomass

To elucidate the biosorption mechanism of fungal biomass, the dyes supplemented media were added with known weight of dead biomass. After the incubation time of 5 days, the decolourization efficacy was expressed in percentage and the supernatant was subjected to scanning in the range of 400–800 nm using UV-Vis Double beam spectrophotometer. When compared to the spectra of the untreated control, there was a tremendous decline in absorbance peak in case of bromophenol blue and Congo red (Fig. 3).

3.3 Stain Film Removal Using Enzyme System

Stain removal is another practical application of microbial enzymes. The malachite green stained tubes were used for the experiment. The thin film coating of malachite green was found to be effectively removed by the laccase enzymes produced by *Trichoderma viride VKF3*. This enzyme could be used in detergents and stain removal formulations for increasing their efficiency (Fig. 4).

3.4 Multiple Enzyme Production by Trichoderma viride VKF3

Trichoderma viride VKF3 was further screened for its ability to produce multiple enzymes namely cellulase, xylanase and lipase which are hydrolytic in nature (Table 1). *T. viride* VKF3 was found to produce all the ligno-cellulosic enzymes as

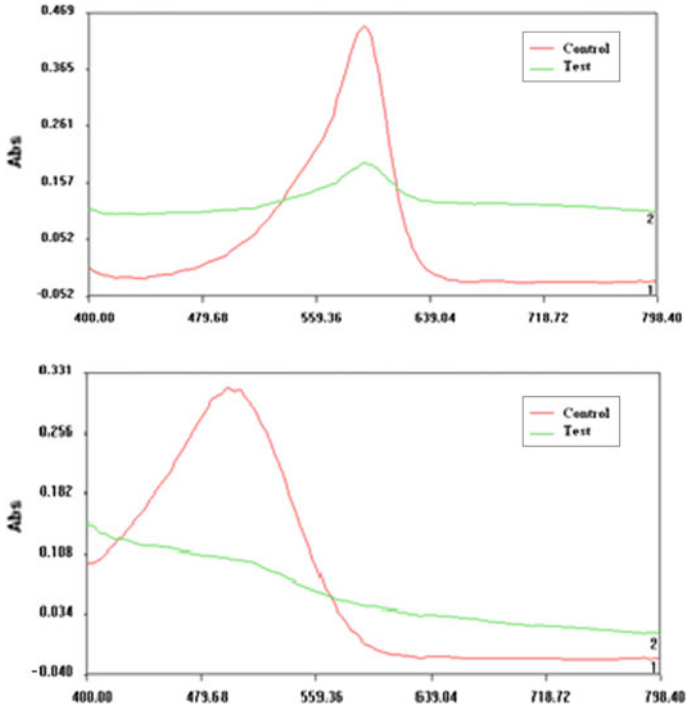

Fig. 3 Absorption spectra of bromophenol blue and Congo red before and after treatment using dead fungal biomass

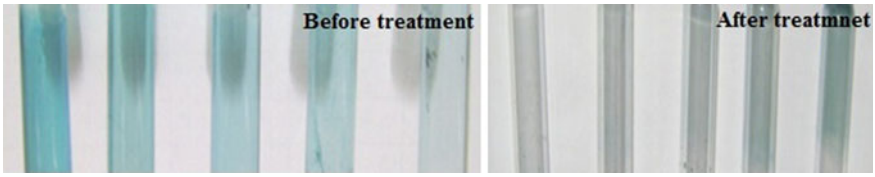

Fig. 4 Thin film of malachite green at various concentrations before and after enzymatic wash. *Below tubes* shows the residual dye leached out by enzyme solution

Table 1 Screening of *Trichoderma viride* VKF3 for multiple enzyme production

Enzymes screened	Status
Cellulase	+
Xylanase	+
Lipase	+

well as lipase enzyme. Hence, it was found very promising as it could effectively be used in diverse substrate and result in a good effluent treatment. *T. viride* VKF3 was found to a potential cellulase producing strain (Vinod et al. 2014); apart from this it was also producing xylanase enzyme.

4 Conclusion

Trichoderma viride VKF3 is a promising fungal isolate with laccase producing ability and could be used for decolourisation of dye effluents in industrial waste treatment processes. The fungal isolate was able to absorb dyes to the biomass in dead condition also. So the dead fungal biomass recovered after enzyme production also could be utilized for the decolurisation of dye effluents. It is a cheap and cost-effective method for dye removal and suitable for formulating stain or dye removing detergents.

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Bacillus tequilensis A Novel Thermotolerant Strain for Effective Bioremediation of Melanoidin Pigment in Its Natural Environment

N. Rameshkumar, S. Dhanapaul, M. Krishnan and N. Kayalvizhi

Abstract The present study was aimed to isolate and characterize a novel thermo tolerant melanoidin degrading bacteria from its natural environment. A total number of 35 bacterial strains were isolated among them 2 strains labelled A and H exhibited a maximum of 10% ethanol tolerance and were able to grow at 60 °C. In which, a novel strain A exhibited maximum decolourization 72%, BOD, COD and was able to grow at higher temperature in comparison with other strains. Further, to confirm the identity, it was subjected to morphological, biochemical and molecular characterization. Based on the analysis the identity of the strain A was confirmed as *Bacillus tequilensis*. The production of MnP and laccase enzymes was observed and decolourization efficiency was analysed through FTIR, the results revealed that most of the predominant compounds present in the control sample were degraded. The reported novel thermo tolerant bacterium *Bacillus tequilensis* was able to decolorize the melanoidin pigment of distillery effluent more efficiently even at higher temperature than the bacterium reported earlier.

Keywords Melanoidin · Waste water · Decolourization bacteria · Molasses

1 Introduction

In India annually, 60 billion litres of molasses spent wash generated by 350 molasses based distilleries producing 3.25 × 109 L of alcohol and generating 40.4 × 1010 L of wastewater annually (CPCB 2011; Pant and Adholeya 2007). Spent wash is a major

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waste containing about 2% of dark brown recalcitrant pigment called melanoidin which exhibit strong odorous, low pH, temperature around 90–110 °C, high in BOD (50,000–60,000 mg/L) and COD (1,10,000–1,90,000 mg/L) content. After conventional treatment, the effluent still contains a considerable high BOD (8,000–10,000 mg/L) and COD (45,000–52,000 mg/L) load and the dark colour persists. Dark brown colour of distillery spent wash is mainly due to the presence of organic compound known as melanoidin (Ohmomo et al. 1985). Melanoidin is main content of spent wash and is formed by the reaction between amino acid and carbohydrate called "Maillard reaction" (Wedzicha and Kaputo 1992). Disposal of spent wash into soil is equally detrimental, causing reduction in soil pH, inhibition of seed germination and depletion of vegetation by acidified soil (Raghukumar et al. 2004).

Although, several physico-chemical methods such as ozonization, flocculation, chemical coagulation, precipitation, activated carbon adsorption and advanced oxidation have been reported for removal of melanoidin from wastewater (Bernardo et al. 1997; Mandal et al. 2003; Pena et al. 2003). In addition, these methods require high reagent dosage and results in large amount of sludge (Tiwari et al. 2013). In contrast, biological treatments with microbial system for biodegradation of melanoidin is more promising and drawing more attention due to its several added advantages such as low cost, eco-friendly approach and cost-competitive to chemical decomposition processes (Mohana et al. 2009).

Several kinds of microorganisms such as fungi *Penecillium decumbens*, *Aspergillus* sp., *Aspergillus niger*, *Flavodon flavus* (Chopra et al. 2004; Raghukumar et al. 2004; Pant and Adholeya 2009) as well as bacterial strains such as *Pseudomonas*, *Bacillus*, *Alcaligenes* and *Lactobacillus* has been discussed by several authors (Kumar et al. 1997; Kumar and Chandra 2006; Mohana et al. 2007). However, growth pattern, culturing, spore formation, and poor growth rate in aquatic systems limit the performance of the fungal system in comparison with bacterial system. Recently, Tiwari et al. (2013) has reported that *Pediococcus acidilactici* B-25 strain poses several advantage its more effective due to its ability to grow on minimum nutrient supply, efficiency to perform in broader pH range and able to grow even in higher temperature, less time consuming and promising for decolourization. Interestingly, an indigenous isolate from its natural ecosystem could perform well than in comparison with a recombinant/genetically engineered organism from different ecological niche. In this direction, our objective was to isolate a novel thermo and ethanol tolerant indigenous bacteria which can potentially degrade the melanoidin pigment in the spent wash of distillery effluent. This is the first report of melanoidin decolourization by *Bacillus tequilensis*. This novel strain can be potentially exploited for large-scale treatment of distillery effluents. Thereafter, distillery effluents can be used for various applications in agronomical practices.

2 Materials and Methods

2.1 Collection of Sample

Distillery spent wash collected from the site near by distillery unit in Tiruchirappalli, South Tamil Nadu, India and samples were stored at 4 °C. Characterization of effluent was done for pH, colour and subjected for the further analysis. The microbes were screened from the distillery effluent for the melanoidin degrading ability.

2.2 Physico-chemical Analysis of Distillery Effluent Used in the Study

The changes in physico-chemical parameters (colour, BOD, COD, total solid (TS), sulphate, nitrogen and phosphate) in distillery effluent before and after bacterial degradation were analysed as per standard methods (APHA 2005). Nitrate, chloride ion and pH were determined using an ion meter (Orion auto analyser model-960) by their respective ion electrode. To measure the colour intensity of bacterial treated distillery effluent media, it was centrifuged at 10,000 rpm for 15 min to remove bacterial biomass. The supernatant was taken and absorbance was measured at 475 nm. The percent decolourisation was expressed as the degree of decrease in absorbance at 475 nm against initial absorbance at the same wavelength.

2.3 Isolation of Thermophilic Bacteria

The molasses sample (10 mL) was taken in a 250 mL conical flask containing 90 mL sterile distilled water. The flask was shaken on an electric shaker to get a homogenous suspension and serially diluted with sterile distilled water to make different dilutions viz. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. One mL of 10^{-2}–10^{-5} dilution was plated in Petri dishes with nutrient agar medium incubated at 60 °C overnight. The representative colonies for each of the observed different colony morphologies were streaked onto another set of such nutrient agar plates to obtain pure cultures.

2.4 Ethanol Tolerance Assay

Single colony of each bacterium were inoculated separately in 5 mL of Luria broth and incubated at 37 °C in a shaker at 200 rpm. Five hundred microliters of the overnight cultures were subcultured to 50 mL Luria broth (supplemented with 5 and

10% ethanol) in closed culture tubes to prevent ethanol volatilization and were incubated at 37 °C on a rotary shaker with an aeration speed of 200 rpm and the density of bacterial culture was measured at 600 nm (Zhou et al. 2006).

2.4.1 Biochemical Identification and 16S rRNA Gene Sequencing

The isolated bacteria A was identified morphologically and biochemically using the standard procedures (Barrow and Feltham 1993). The total DNA was prepared from overnight grown cultures by the alkaline lysis method described earlier (Kapley and Purohit 2001). About 5 µl DNA was used to amplify the 16S rDNA gene using universal eubacterial primers (27F) 5′-AGAGTTTGATCMTGGCTCAG-3' and (1492R) 5′-TACGGYTACCTTGTTACGACTT-3′ and a 1,500 bp product was amplified. The reaction mixture contained 5 µl template, 19 µl PCR buffer, 200 µM of each dNTP, 3.0 mM $MgCl_2$, 25 pmol of primer, and 2.5 units of Amplitaq DNA polymerase (Perkin Elmer) in a final volume of 50 µl. The thermocycling steps (Eppendorf, Germany) used were as follows: 35 cycles of denaturation at 94 °C for 1 min, followed by annealing at 45 °C for 1 min and extension at 72 °C for 2 min. The PCR product was gel purified using QIA gel extraction kit, Qiagen, Germany, and sequenced using primer 27F. The partial sequences were subjected to BLAST analysis (Altschul et al. 1997) this suggests the identity of the isolates.

2.4.2 Phylogenetic Analysis

Sequence contigs were assembled manually and subjected to BLAST program to find similarities with sequences deposited at GenBank (Altschul et al. 1997). Multiple-sequence alignments and phylogenetic analysis were carried out using MEGA version 5.0 software (Tamura et al. 2011). The phylogenetic tree based on 16S rDNA was constructed according to the neighbour-joining method (Gonzalez and Mas 2011). The homology of ethanol-tolerant isolate to other species was compared according to their evolutionary divergence.

2.4.3 Biomass Determination

Bacterial cells in broth were collected by centrifugation (10,000 rpm for 10 min at 4 °C), washed with distilled water, and dried in an oven at 80 °C until getting a constant dried weight reported in the form of dry cell mass (g/L^{-1}).

2.4.4 Test for Degradation of Solids in Effluent

The five selected strains were tested for their ability to efficiently degrade solid content of the biomethanated spent wash. Well mixed 5 mL samples of the wastewater were inoculated with 1, 2 and 5% inoculum sizes of overnight cultures grown in nutrient

broth and then incubated at 60 °C for 3 days. The samples were then centrifuged (8000 rpm, 10 min). The solids thus separated were then oven-dried for 1.5 h and then weighed.

2.4.5 Test for Effluent Decolourization and Melanoidin Degradation

The five strains were subjected to a test for melanoidin degradation, measured as their ability to decolourise wastewater samples. The biomethanated distillery effluent was diluted to 5% using water, 30% inoculum overnight cultures were added to the 5% effluent and incubated with shaking of 140 rpm at 45 °C. The effluent without inoculum served as control. The test was carried out in duplicates. After nine days of incubation, the samples were centrifuged at 6000 rpm for 10 min to exclude solids, biomass and optical density (OD) of supernatant was read at 475 nm, the reported λ_{max} of melanoidin. The decrease in absorbance at 475 nm shows the extent of degradation of melanoidin.

The most effective strain was then inoculated at 10, 20 and 30% inoculum sizes by volume into the 5% biomethanated distillery effluent and incubated with shaking of 140 rpm at 45 °C. The effluent without inoculum served as control. The test was carried out for 9 days, with measurement of OD_{475} on day 6 and the final day i.e. day 9.

2.4.6 Estimation of Decolourization Activity

Melanoidin degradation activity was assayed by measurement of the decrease in optical density as absorbance at 475 nm after diluting with 0.1 M acetate buffer (pH 5.0). The decolourization yield was expressed as the degree of the decrease in absorbance at 475 nm against the initial absorbance at the same wavelength. Decolourization activity was calculated by Eqs. 1 and 2.

$$D\% = 100 \times (A_{fin} - A_{ini})/A_{ini} \quad (1)$$

where, D% = Decolourization percentage, A_{ini} = Initial absorbance, A_{fin} = absorbance after incubation.

$$\text{Decolorization } (\%) = (\text{Initial OD} - \text{Final OD} \times 100)/\text{ InitialOD} \quad (2)$$

where OD = Optical Density at 475 nm.

2.4.7 Analysis of Melanoidin Degradation by FTIR

The changes occurred in the functional groups of compounds present in the biomethanated spent wash were studied through FTIR (Nicolet IR 200 model, Thermo Electron Corporation, Korea) spectral analysis by comparing treated samples with the untreated samples. Spent wash (30%) was inoculated with the selected microbial isolates and incubated for 7 days at 30 °C. After incubation period, 10 mL from each sample was taken separately and centrifuged at 10,000 rpm for 10 min in order to separate microbial debris as pellet. The supernatant was mixed with 20 mL of chloroform in a separating funnel and the chloroform soluble layer collected in a small china dish. The collected samples were kept on a hot water bath till all the chloroform evaporated. The residue remained in the china dish was mixed with potassium bromide (KBr) and the pellet used for FTIR spectrophotometer measurement (Ghosh et al. 2004). The spectra were recorded in the region from 400 to 4000 cm^{-1} with a spectral resolution of 4 cm^{-1} using a Perkin Elmer spectrometer. The data was analysed using eFT-IR software and interpreted with the help of IR absorption spectra tables from the webpage (http://www.science-and-fun.de/tools/) interactive program using information compiled from authenticated references.

3 Results and Discussion

3.1 Physico-chemical Characteristics of Effluent

The detail physico-chemical properties of biomethanated distillery effluent were characterized and the results were summarized in Table 1. The colour, odour,

Table 1 Physico-chemical character of biomethanated distillery effluent

Parameter	Value
Color	Dark brown
Odor	Like molasses
pH	4.4
Temperature	84 °C
Total suspended solid (mg/L)	4786
Total dissolved solid (mg/L)	65,510
Total solid (mg/L)	52,350
Biological oxygen demand (mg/L)	45,322
Chemical oxygen demand (mg/L)	96,145
Calcium (mg/L)	74,000
Magnesium (mg/L)	24,000
Phosphate (mg/L)	183.036
Sulphate (mg/L)	1888.89
Nitrate (mg/L)	685.71
Chloride (mg/L)	6997.83
Iron (mg/L)	15.83

temperature, pH, biological oxygen demand (BOD), chemical oxygen demand (COD) and total dissolved solids (TDS) were over the permissible limits. In addition, to melanoidin the effluent also contained the organic metals such as calcium, magnesium, phosphate, sulphate, nitrate and chloride. The physico-chemical analysis revealed that the biomethanated distillery effluent was bind property with the compounds present in biomethanated distillery effluent. Accumulation of such toxic pollutant is lethal to all living organisms. To overcome such adverse effect it requires a pre-treatment before its safe disposal into the environment. Decolorization of melanoidin containing effluent through chemical methods (Chandra and Singh 1999), flocculation treatments (Migo et al. 1993) and physiochemical treatments such as ozonation (Kim et al. 1985; Ichikawa et al. 1996) and activated carbon adsorption (Chandra and Pandey 2001) has been accomplished but these methods are not economically feasible on a large scale due to cost limitation. In contrast biological system using bacteria, fungi and actinomycetes are more promising and eco friendly, among them fungi is the dominating group. However, bacteria also promise to decolorize a melanoidin up to the same extent which fungi do. To find a new microbial system having ability to degrade these recalcitrant compound is always desirable and could play a key role in environmental cleaning even at industrial level.

3.2 Isolation, Screening and Identification of Isolates

A total number of 35 bacterial isolates were screened from the biomethanated distillery effluent site nearby, Tiruchirappalli, South Tamil Nadu, India. In the pilot study, ten isolates (labelled A-J) which were able to grow at 60 °C were screened. Further to confirm the ethanol tolerance level the isolates were subjected to 5, 10% of ethanol and incubated at 60 °C for 24–48 h. Among them the strains A, B, C, D, F and H were able to grow at 5% of ethanol tolerance. The strains A and H exhibited a maximum of 10% ethanol tolerance in addition to thermo tolerance (Fig. 1). Furthermore, the isolates were checked for decolourization on the biomethanated distillery effluent containing nutrient agar medium (pH 7.0) after 24–48 h incubation at 60 °C. The results revealed that five strains (A, B, C, D and H) exhibited >1.0 cm clear zone around the distillery effluent. Further to ascertain, the effect of temperature in the decolourization efficiency the experiments were conducted at different temperature ranging between 45, 60, and 80 °C respectively in which decolourization was higher at 45 °C. The isolates showing high zone of clearance were considered as effective decolourizers and selected for quantitative screening. During the quantitative screening, the isolates were inoculated individually in 50 mL of basal broth medium (pH 7.0) containing distillery effluent. The flasks were incubated at 45 °C for 24–48 h for selection of efficient thermo and ethanol tolerant decolorizing bacterium. Of the five bacterial isolates, the strain A

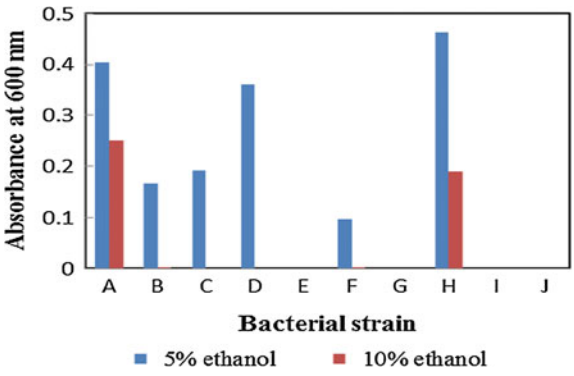

Fig. 1 Ethanol tolerance of isolated thermophiles A–J

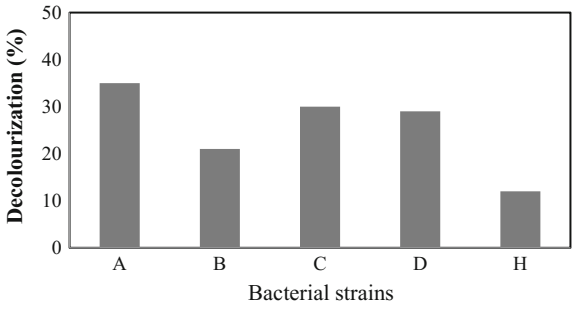

Fig. 2 Decolourization of bacterial strain treatment with 5% effluent

was found to be more efficient decolourizer of distillery effluent with thermo and ethanol tolerance evidenced by maximum decolourization activity (36%) (Fig. 2).

To assess the distillery effluent decolorizing ability of the strain A, preliminary screening was done on agar plate using the basal medium amended with distillery effluent. Decolourization efficiencies obtained in the present study are lower than those attained by Kaushik and Thakur (2009) (85% removal of colorant) with bacteria of the genus *Bacillus*. However, it is worth knowing in the present study non-diluted distillery effluent with a higher initial content of colorants than in the studies reported (Kumar and Chandra 2006; Bharagava et al. 2009). The study results yielded higher decolorization of a non-diluted distillery effluent. It was reported that temperature is an important factor for melanoidin decolorization, COD and BOD reduction (Kumar et al. 1997). The optimal temperature for maximum melanoidin decolorization by our strain A was 45 °C. The remarkable decolorization with a maximum of 75% in the temperature at 45 °C revealed that the strain A is a thermotolerant. Our strain showed better performance in decolourization potential than the earlier study reported by Kambe et al. (1999) which showed a maximum of 35.5% spent wash decolorization at 55 °C by *Bacillus* sp.

3.3 Identification of Bacterial Strain

The morphological, physiological and biochemical characteristics of isolate A has been shown in Table 2. On the basis of biochemical tests, the efficient bacterial strain A was characterized as Gram-positive, long rod-shaped bacteria in chains giving positive tests for motility, catalase activity, casein, starch and gelatin hydrolysis as well as for the V–P test, citrate utilization test, nitrate reduction and for acid production from glucose, cellobiose, mannose, and arabinose. The strain A showed negative for indole production, methyl-red test, H_2S production, as well as for oxidase activity. However, they showed a positive reaction for urease activity and acid production from raffinose. The bacterial strain A was fermentative in nature. Based on these characteristics the bacterial strain A has been identified as *Bacillus* species.

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. Of these, strain A was subjected for morphological, biochemical characterization which identified the strain A belongs to Genus *Bacillus* sp.

Furthermore, to confirm the identity of the strain at the molecular level the PCR-amplified 16S rDNA gene sequence was performed (Fig. 3). Interestingly, 1,500-bp 16S rDNA gene of the strain A has shown closest relatedness with that of *Bacillus tequilensis* EI-34-6 (AJ 293011). The phylogenetic tree shown in the

Table 2 Biochemical analysis of selected isolates

Analysis	A
Colony morphology	Flat, moist, small, lactose fermenting
Gram staining	Long Gram positive bacillus
Oxidase	0
FTM	+/+
Indole	–
Triple sugar iron	R / YNo gas
Citrate	–
Urease	–
Inulin	–
Mannitol	–
Trehalose	–
Arabinose	–
Salicin	–
Egg yolk	–
Starch	–
Skim milk	–
Arginine	–
Organism identified as	Non-reactive *Bacillus* sp.

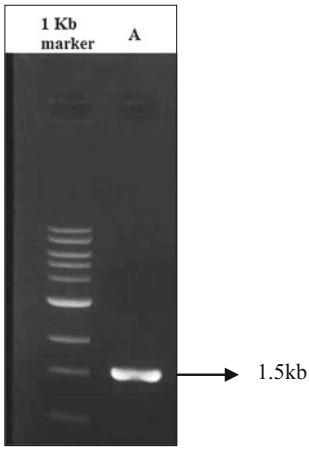

Fig. 3 PCR amplification of 16S rRNA gene of isolated bacteria; Lane M 1500 bp Ladder; Lane 1—Strain-A

Fig. 4 was constructed from evolutionary distance by the neighbor-joining method using MEGA version 4.0 software. The tree was constructed using the reference sequences of 21 different *Bacillus* spp., retrieved from GenBank. The phylogenetic tree resulted in two different clusters sharing their closest sequence homology with the respective sequence used in the analysis. Based on the physiological, biochemical and 16S rDNA sequence analysis confirmed the identity of the strain A as *Bacillus tequilensis*. Following the phylogenetic analysis, strain A was placed in the cluster making up the genus *Bacillus*. The strain A also shared its genetic relatedness with *B. lichenformis* belonging to the same genus. The 1400 bp 16S rDNA sequence was submitted to the gene bank.

Neighbor-joining tree showing the phylogenetic position of melanoidin-degrading bacteria and their related species based on 16S rRNA gene sequences. The GenBank accession number for each bacterium used in the analysis is shown in parenthesis after the species name melanoidin degradation by the promising decolorizing microorganism.

3.4 Changes in Enzyme Activity During Degradation Study

The MnP, MIP and laccase activity in the culture supernatant showed maximum 3.98, 4.06 and 0.95 U mL^{-1} at 96 h, respectively of bacterial growth (Fig. 5). While, further bacterial incubation showed gradual decrease of all enzyme activities. The enzyme activity showed direct correlation with the distillery effluent decolourisation (decrease in absorbance at 475 nm). The induction of MnP and MIP in the culture supernatant was noted at 48 h of bacterial growth and remains active up to 144 h, while the laccase induction was observed at 96 h and its activity also declined after 144 h (Fig. 5). This indicated the initial role of MnP and MIP in distillery effluent degradation. It was also observed that the decolourisation increased with increase in

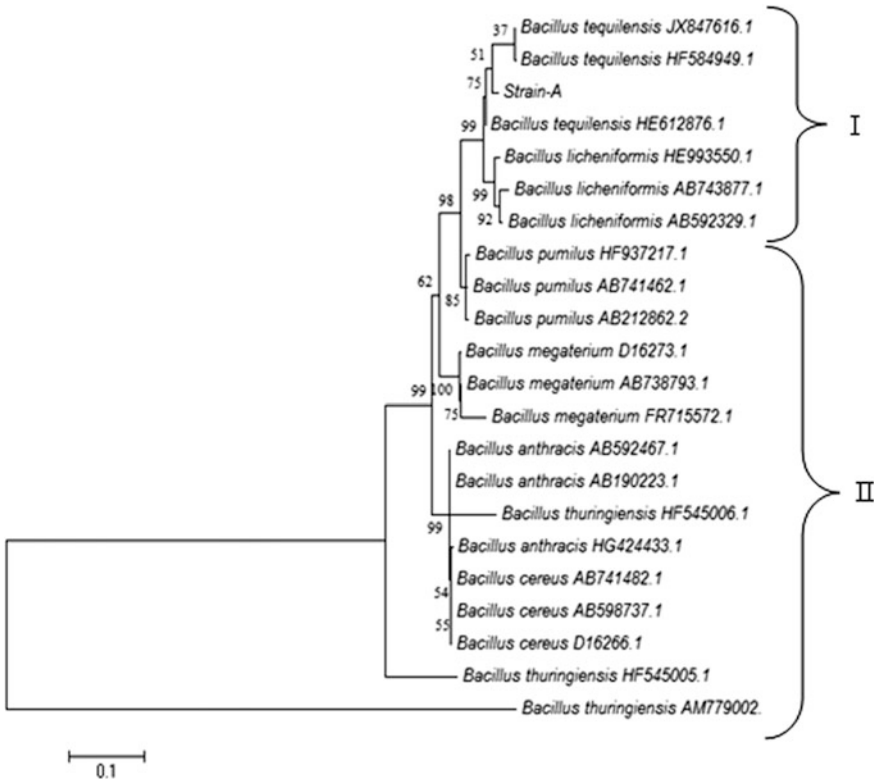

Fig. 4 Phylogenetic analysis of *B. tequilensis*

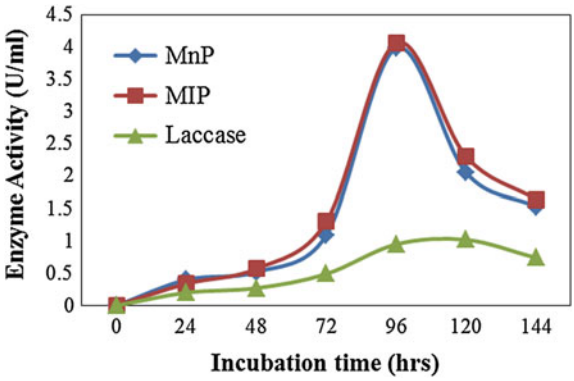

Fig. 5 MnP, MIP and laccase activity by bacterial strain during decolorization of distillery effluent. MnP—manganese-dependent peroxidase, MIP—manganese independent peroxidase

inoculum size of selected bacteria. Maximum 71% decolourisation was observed at 10% (v/v) inoculum size. Further, increase in the inoculum size did not improve the decolourisation (data not shown). The results clearly indicated that *Bacillus*

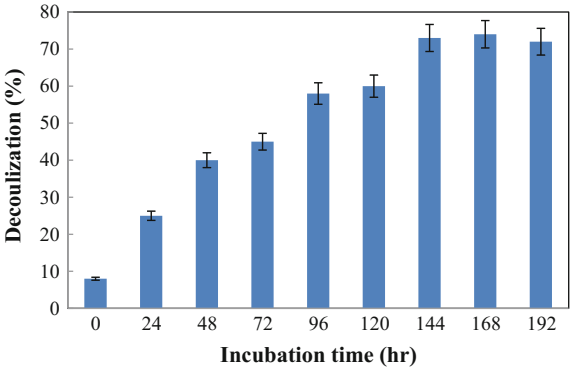

Fig. 6 Effect of different incubation time on melanoidin decolorization

tequilensis showed 72% decolourization in 144 h of incubation. Further increase in the incubation period did not increase the decolourization (Fig. 6). Melanoidin degradation involved in the production of extracellular H_2O_2 and peroxidases, because peroxidase activity requires H_2O_2, which is produced during the glucose oxidation and thus establishing the necessity to add glucose as extra carbon source (Miyata et al. 2000). MnP catalyzes H_2O_2-dependent oxidation of Mn(II) to Mn(III). This Mn(III) catalyzes one-electron oxidation of phenolic and non-phenolic recalcitrant compounds by H_2O_2, promoting the production of corresponding free radicals leading to the degradation of melanoidins. These findings were well supported by the earlier results reported by various workers on the enzymatic activity and melanoidin degradation capability exhibited by the same bacterial genus (Kumar and Chandra 2006; Mohana et al. 2007).

3.5 FTIR Analysis

To monitor the degradation of distillery effluent the sample was subjected to FTIR spectral analysis, reducing levels of these colouring compounds has been shown (Fig. 7). Decrease in optical density of melanoidin in spent wash, is necessary to quantify the concentrations melanoidin at their max (330 and 283 nm respectively) of these specific colorants in the samples. Hence, peak shift in the FTIR spectra with quantities of treated distillery effluent respect to control suggest their degradation. The changes in the peak showed the conversion of complex melanoidin compounds into simple forms i.e. polymers concentration being broken down to monomers or dimers. Compare to the FTIR spectra of control and degraded products formed after degradation revealed the biodegradation of biomethanated distillery effluent by *B. tequilensis*. The spectrum of control biomethanated distillery effluent showed the peaks at 3430.5, 1623.8, 1533.9, 1393.0, 1114.8, 1048.6, 847.4, 753.4, and 619.8 cm^{-1} which represents N–H stretching, N=N stretching as in azo compounds, N–H deformation, C–N stretching, O–H deformation, C–OH

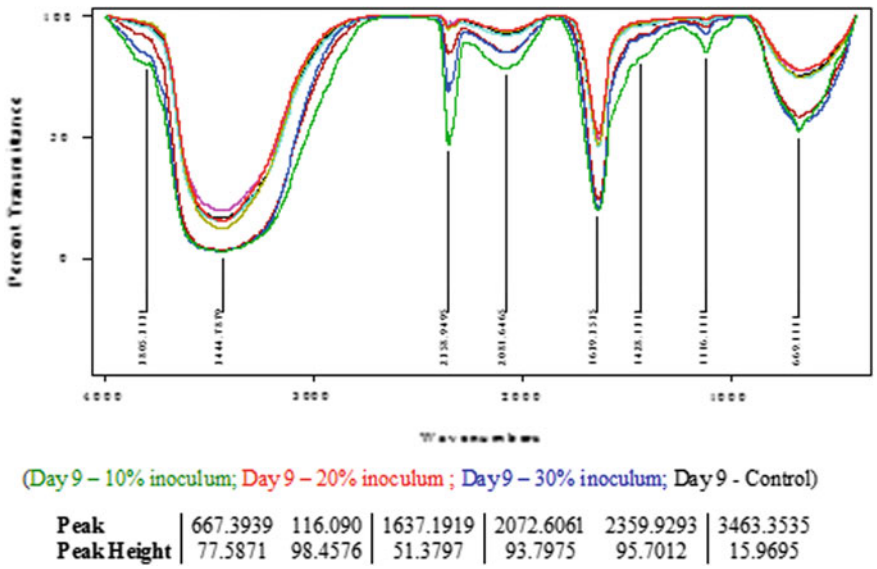

Fig. 7 FT-IR spectra of decolourization by isolate **A** (Day 6—Control; Day 9—Control; Day 6—10% inoculum of *Bacillus tequilensis*; Day 6—20% inoculum of *B. tequilensis*; Day 6—30% inoculum of *B. tequilensis*; Day 9–10% inoculum of *B. tequilensis*; Day 9—20% inoculum of *B. tequilensis*; Day 9—30% inoculum of *B. tequilensis*)

stretching, S=O stretching as in sulfonic acid, C–H stretching and C–H deformation as in benzene ring and C–Cl stretching, respectively. Thus, differential spectra of the untreated and treated dye confirmed its degradation (Table 3). Absence of peak at 1,623.8 cm^{-1} in product spectra confirms removal of azo bond during degradation which also supports the enzymatic pattern of *B. tequilensis*. Removal of the peak at 1,048.6 and 619.8 cm^{-1} for S=O stretching and C–Cl stretching in product spectra confirmed desulfonation and dechlorination of melanoidin compound in biomethanated distillery effluent. An important structural feature of the melanoidin is the presence of conjugated C=C and C=N bonds which impart colour to these polymers. The strain *B. tequilensis* employed during the present investigation might have brought about the cleavage of ethylinic C=C and azomethine C=N linkages. Therefore, conjugation is broken down due to enzymatic oxidation; hence UV intensity is reduced. Such cleavage would generate hydroxyl groups (3400 cm^{-1} band). In conformity with this, intensity of 3400 cm^{-1} band showed a marked increase in the treated samples compared to the control. The FTIR analysis of the bacterial degraded sample showed a shift in the compounds identified in treatment as compared to control. The absence of certain peaks and the presence of new features in the FTIR spectra of the test samples with respect to control indicate the degradation of the distillery effluent. Further, the reduction of peak heights in several regions of the FTIR spectra in treated samples when compared to control indicates the decrease in the amount of substance in the test samples.

Table 3 Identification of FTIR spectral peaks

Observed ν (cm^{-1})	Interpretation	Reference ν (cm^{-1})
662–673	Strong C–O–C deformation vibrations in the fragment H_2C=CH–(C=O)–O–R	660–675
1116–1119	Strong to medium intensity C–O–C symmetrically stretching vibrations in dixanthates and/or strong to medium phthalide ring vibrations and/or very strong C=S stretching vibrations in 4—pyrithiones and/or very strong =C–H in—plane deformation vibrations	1110–1120; 1110–1120; 1105–1120; 1110–1125
1635–1640	Predominantly C=C, C=N and C=O stretching vibrations occur in the ~1660–1800 cm^{-1} region. Strong stretching vibrations in acrylate H_2C=CHCOOR	1635–1640
2066–2084	Strong diazoketone—CO–CN_2—vibrations and/or weak C=C stretching vibrations in conjugated alkenes conjugated with C=C, C=C	2055–2100; 2035–2100
2358–2364	Medium intensity O–H and N–H stretching vibrations in pyrimidinone and/or medium intensity broad signal vibrations in R_2(P=S)SH	2200–3000; 2300–2420
3435–3464	Very strong N–H stretching vibrations of free pyrroles and/or medium to weak asymmetrically stretching NH_2 vibrations in dilute solutions of primary amines and/or strong, broad concentration—dependent vibrations of intermolecular hydrogen bonds	3400–3500; 3330–3550; 3250–3550

(ν—Wavenumber)

The melanoidin acquires its characteristic dark colour from its conjugated C=C and C=N bonds. The FTIR spectral peaks observed in the wave number range of 1635–1640 and 2066–2084 cm^{-1} correspond to these chromophores and are observed to get smaller with respect to the control as the treatment progresses. The breaking down of these bonds is therefore concluded to have been brought about by the bacterial enzymes in the degradation of melanoidin during this process. The degradation of phenolic compounds from the distillery effluent after bacterial degradation was observed previously (Bharagava and Chandra 2010). A similar result has also been reported as melanoidin product of distillery waste after fungal treatment (Gonzalez et al. 2000). Microbial decolorization of distillery effluent therefore shows great promise as a cost-effective, environmentally safe biotechnology for the treatment of high-strength industrial wastewater. Post methanated distillery spent wash cannot be disposal directly for irrigation purpose, as effluent contains high BOD and more water is required to dilute this parameter below 100 mg/L as per CPCB rule and regulation (Wagh and Nemade 2015). This study could open a new avenue to control the environmental pollution and health hazards, causes so many health related problems to people working in and around the effluent distillery unit. This approach can be used to develop a cost-effective, eco-friendly biotechnology package for the bioremediation of spent wash before its disposal.

4 Conclusion

From the present study, it was observed that thermophilic bacteria *B. tequilensis* possess significant ability to decolourize the dark and dangerous distillery effluent at high range of temperature, therefore it is beneficial at industrial level for treatment of distillery effluent at economical level. Interestingly, *B. tequilensis* reported in the present study showed both MP-adsorption and MP-degradation abilities. These attractive features added further dimension to our strain than the strains reported earlier and gives a promising scope for its potential implication in environmental rescue of distillery sites. Therefore, it can be concluded that thermophilic aerobic digestion of biomethanated distillery effluent has great benefits, especially in a tropical country like India where the ambience is more conducive for this type of process.

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Optimization of Growth Medium and Biosorption of Chromium Using Micro Algae and Cyanobacteria

R. Kasimani, R. Seenivasagan and K. Sundar

Abstract Industrial effluents are one of the major sources of heavy metal contaminations and cause significant environmental problems to the aquatic life. Some heavy metals are easy soluble and unable to separate easily using conventional chemical methods. Hence biological methods like biosorption/bioaccumulation were carried out for the removal of heavy metals from the environment. The objective of the present study is to optimize algal growth in various growth media. Additionally the ability of three species of blue green algae, *Chlorella* sp. and *Neochloris* sp. and an unidentified isolate to adsorb chromium was tested. The presence of chromium in the samples was analyzed using AAS, SEM-EDX and FT-IR. The results showed that highest algal growth rate was observed in BG11 medium with solution B. The unidentified cyanobacterial isolate exhibited higher chromium removal capacity (75.63%) followed by *Chlorella* sp. (44.37%) and *Neochloris* sp. (72.5%).

1 Introduction

Rapid industrialization and advance technologies produce large amount of effluent, that contains toxic heavy metals which would combine with water resources and leads to worldwide environmental problems (Esmaeili et al. 2011). Industry effluents primarily contains both trivalent (Cr(III)) and hexavalent chromium (Cr(VI)) which are more hazardous due to carcinogenic and mutagenic effects (Mohanty et al. 2006; Dogan et al. 2011). When compared to hexavalent chromium, trivalent chromium is less toxic and mobile (Anderson 1997). Inorganic toxic chromium is

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used in leather tanning, stainless-steel production, pigment manufacturing, electroplating, textile dyeing, wood preservation industries, nuclear power plants, metal processing, manufacture of alloys, welding and chromate manufacturing industries (Acharya et al. 2009; Sahu et al. 2009). According to World Health Organization 2.00 and 0.05 mg/L total chromium and hexavalent chromium (Cr(III), Cr(VI) and other forms) respectively are fixed as maximum limits in the drinking water (Gupta and Rastogi 2009).

Physico-chemical methods such as reverse osmosis, precipitation, electrodialysis and ion exchange are used for effective removal of chromium. But these are unreliable and are not cost effective (Camargo et al. 2003). Hence, biological processes for remediation of chromium are gaining importance as an alternative method. Chromium remediation through biological methods was very effective and eco-friendly for cleaning the heavy metal contaminants in the environment (Camargo et al. 2003; Megharaj et al. 2003). Biosorption of heavy metals using microorganisms such as algae, fungi and bacteria have greater selectivity and high metal loading capacity for the transition of heavy metals (Rajasimman and Murugaiyyyan 2010). Algae biomass is considered as a pollution indicator and for filtering the toxic heavy metals (Cossich and Tavares 2002). Many studies have demonstrated that significant amount of heavy metals were up taken, reduce and accumulate by the algae present in aqueous solution (Radway et al. 2001; Tien et al. 2005; Khattar et al. 2007). Additionally Cr(VI) and (III) are biosorped by marine macroalgae such as *Sargassum* (Chojnacka et al. 2005), *Ecklonia* sp. (Kratochvil et al. 1998), micro algae like *Spirulina platensis* (Donmez and Aksu 2002), *Chlorella miniata* (Gupta et al. 2001), *Spirulina* sp. (Li et al. 2006), *Dunaliella* sp. (Han et al. 2006) and fresh water algae *Spirogyra* sp. (O'Neal et al. 1985). The objective of the present study is to optimize the growth of algae in various media and to determine their chromium removal capacity. Further, SEM-EDAX analysis was performed to determine the changes in the algal morphological structure after the treatment.

2 Materials and Methods

2.1 Collection of Algae

Algae were collected from different locations like Kalasalingam University campus, Thirunelveli, Chennai, Kanniyakumari, Karaikudi and Thondi. The samples were collected from both marine and fresh water in these different locations. The algal samples were collected in 50 mL tubes and maintained in ice box while transferring to laboratory.

2.2 Various Algae Medium Preparation and Optimization

Eight different types of media were selected and prepared for the growth of algae. They were Blue green medium, blue green medium 11, ASN111, Bristol's, Prochlorococus, Jaworski's, ATCC and SES Medium. Each of the entire medium was prepared for 1 L and adjusted to pH 7.4 using 0.1 M Sodium hydroxide. Algae were washed with distilled water and filtered using Whatman No. 1 filter paper. The filtered algae were inoculated into the entire medium. Inoculated cultures were incubated under sunlight for two weeks and pH 7.5.

Composition of Blue green medium: $NaNO_3$, 15; K_2HPO_4, 4; $MgSO_4$, 7.5; $CaCl_2$, 3.6; $C_6H_8O_7$, 0.6; $NH_4Fe(SO_4)_2$, 0.6; EDTA, 0.l; Na_2CO_3, 2; H_3BO_3, 2.86; $MnCl_2{\cdot}4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.222 and $CuSO_4$, 0.079 mg/L, **Blue green 11 medium:** $C_{10}H_{12}N_2O_8Na_2Mg$, 0.3; $C_6H_8FeNO_7$, 0.6; $C_6H_{10}O_8$, 0.6; $CaCl_2{\cdot}2H_2O$, 3.6; $MgSO_4$, 7.5; K_2HPO_4, 3.05; H_3PO_4, 2.86; $MnCl_2$, 1.81; $ZnSO_4$, 0.222; $CuSO_4$, 0.079; $COCl_2$, 0.050 and Na_2MoO_4, 0.391 mg/L, **ASN111 medium:** NaCl, 2.5; $MgSO_4{\cdot}7H_2O$, 0.35; $MgCl_2{\cdot}6H_2O$, 0.2; $NaNO_3$, 0.075; $K_2HPO_4{\cdot}3H_2O$, 0.075; $CaCl_2{\cdot}2H_2O$, 0.050; KCl, 0.050; Na_2CO_3, 0.002; Citric acid, 3; $C_6H_8FeNO_7$, 3; Mg EDTA, 0.5 mg/L; Vitamin B12, 10 µg and trace metals 0.1 mL. Trace metal composition such as H_3BO_3, 0.286; $MnCl_2{\cdot}4H_2O$, 0.181; $ZnSO_4{\cdot}7H_2O$, 0.022; $Na_2MoO_4{\cdot}2H_2O$, 0.0039; $CuSO_4{\cdot}5H_2O$, 0.0079; $Co(NO_3)_2{\cdot}6H_2O$, 0.0494 mg/L, **Bristol's medium:** $NaNO_3$, 10; $CaCl_2.2H_2O$, 1; $MgSO_4.7H_2O$, 3; K_2HPO_4, 3; KH_2PO_4, 3 and NaCl 1 g/L. Each of the chemical was prepared as stock solution and dissolved in 400 mL of distilled water. 100 mL of each stock solution was taken and the solution was made up to 1000 mL using distilled water. One drop of 1% of ferric chloride solution was added to 40 mL of Pringshein's soil water extract and autoclaved. **Prochlorococus medium:** Na_2HPO_2, 1 mL; NH_4Cl, 1 mL; $C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$, 0.43 g/L; $Na_2SeO_3{\cdot}5H_2O$, 1 g/L; $ZnSO_4$, 2.3 g/L; $(SO_4)_3{\cdot}6H_2O$, 2.63 g/L; $COCl_2$, 1.19 g/L; $MnCl_2$, 17.8; $Cl_2{\cdot}6H_2O$, 0.316 g/L and Oligotrophic seawater, 1 mL. The glassware's were washed with acid and rinsed using oligotrophic sea water before use. **Jaworski's medium:** $CaH_8N_2O_{10}$, 10; H_2KO_4P, 6.2; $MgSO_4$, 25; $NaHCO_3$, 7.95; $C_{10}H_{13}FeN_2O_8$, 1.12; H_3BO_3, 1.24; $MnCl_2{\cdot}4H_2O$, 0.69; $C_{10}H_{16}N_2O_3S$, 0.02; $NaNO_3$, 40; $(NH_4)_6Mo_7O_{24}$, 18 g/L. Add 1 mL of each stock solution (1–9) to 1 L of distilled water and autoclave at 121 °C for 15 min. **ATCC medium:** $C_2H_3NaO_2$, 0.01; Yeast extract, 0.01; Nutrient broth, 0.01; Na_2S, 0.5 g/L, **SES medium:** Soil extract 0.01, K_3PO_4 0.02, $MgSO_4$ 0.02 g/L.

2.3 Stock Solution Optimization of BG11 Medium

Four different types of stock solutions were prepared based on composition given in the BG11 medium and it was named as stock solution A ($(NH_4)_5Fe(C_6H_4O_7)_2$, 0.6 mg/L, $MnCl_2$, 1.81 mg/L), stock solution B ($C_6H_8O_7{\cdot}H_2O$, 0.6 mg/L, $ZnSO_4$,

0.22 mg/L), stock solution C ($CaCl_2H_4O_2$, 3.6 mg/L, $CuSO_4$, 0.079 mg/L) and stock solution D ($COCl_2$, 0.05 mg/L). For the preparation of suitable medium for algae growth, one of these stock solutions was skipped and another three stock solutions were added together and named as medium 1. Likewise, stock solution A was skipped and stock solution B, C and D were added. Like B was skipped and A, C, D were added. Then C was skipped and A, B, D were added. Finally D was skipped and A, B and C were added. Based on the absorbance readings of the algae growth in these four types of medium the suitable one was chosen for further experiments.

2.4 Cr (VI) Stock Solution Preparation and Calibration Plot

2.82 g of $K_2Cr_2O_7$ was dissolved in 1000 mL deionised water and kept in a shaker at 150 rpm for 15 min to completely dissolve. Cr (VI) stock solution was stored at 4 °C for further experiments. This stock solution was taken and used for making up various concentrations (i.e., 10–100 ppm). These concentrations of Cr (VI) were mixed with 1, 5-diphenylcarbazide (DPC) and the values measured with spectrophotometer at 540 nm. Absorbance versus concentration of chromium was plotted in a graph and the OD values were calculated.

2.5 Chromium Degradation by Using DPC Method (Mohammad Ilias et al. 2011)

Hexavalent chromium was determined using UV spectrophotometer (Genesys 5, USA) by 1, 5-diphenylcarbazide (DPC) (Nacalai Tesque, Inc., Japan) method. The DPC reagent was prepared by adding 24 mL of 85% H_3PO_4 to 56 mL of distilled water. This solution was mixed with 0.076 g of DPC dissolved in 20 mL of 95% ethanol. The reagent was stored in a dark place at 4 °C (store in a brown bottle). Cr (VI) was assayed by the addition of 0.5 mL of the DPC reagent with 4.5 mL of chromium samples. Samples were mixed gently and kept at room temperature for 20 min. The absorbance was measured at 540 nm.

2.6 Treatment Under Various Chromium Concentrations

Prepared chromium was taken and added into the isolated algae cultures. Chromium was added into different media based on the various concentrations like 100, 200, 300, 400 and 500 mg/L. This analysis was done to check the growth and chromium reduction at different ppm concentrations by algae.

2.7 Analysis of Chromium Reduction by AAS

Atomic absorption spectroscopy (AAS) is used to determine the chemical elements (analyte) in a sample by absorbing the optical radiation (light) with the help of free atoms in the gaseous state.

2.8 Scanning Electron Microscope (SEM) with EDX of Structural Analysis of Before and After Treated Algae

The surface morphology of algae is observed by Scanning Electron Microscope (SEM) (20 kV). After drying, the samples (0.71–1.0 mm) were covered with thin layer of gold (10 nm) using sputter coater under a vacuum of 1.33×10^{-6} mBar. EDX confirms the presence of chromium in the algal sample after treatment.

2.9 FT-IR Analysis Before and After Chromium Treated Algae

The pretreated and treated algae samples were analyzed using Fourier Transform Infrared (FT-IR) Spectroscopy. Samples were mixed with KBr at a ratio of 1:100. The 1 K spectrum was collected using a Perkin-Elmer spectrophotometer within the range 400–4000 cm^{-1}.

3 Results and Discussions

Three algal strains were isolated from Kalasalingam University campus, Thirunelveli, Chennai, Kanniyakumari, Karaikudi and Thondi and evaluated as potential sources of biomass and biosorption. The isolated colonies were categorized based on the morphological appearance of the culture and the microscopic cellular appearance such as green microalgae and cyanobacteria. The isolated strains of microalgae ranged from unicellular to filamentous in form.

3.1 Effect of Algae on Various Medium

The optimization of algae growth was performed by growing in eight different types of media viz. Blue green medium (BGM), blue green medium (BGM) 11, ASN111, Bristol's, Prochlorococus, Jaworski's, ATCC and SES Medium. When compared with other media, BG11 shows good result for all the three algal types (Fig. 1).

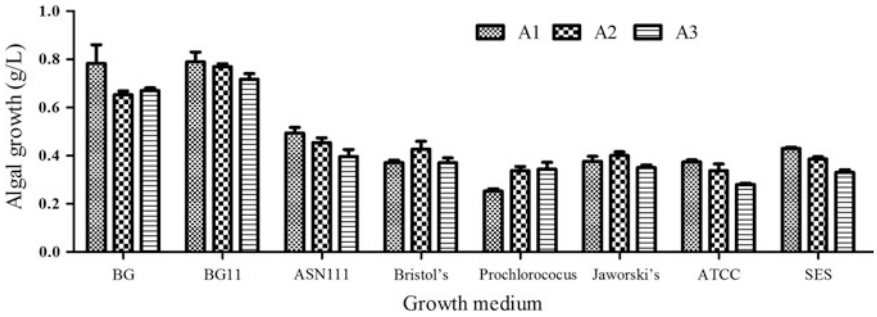

Fig. 1 Optimization of algae growth in various growth medium

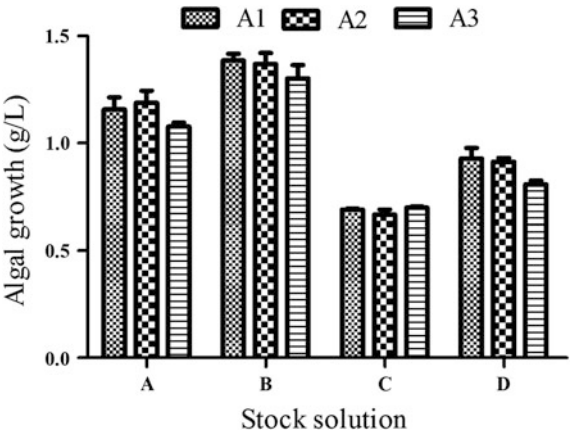

Fig. 2 Optimization of algal growth in BG11 medium with various micro elements (A1, A2 and A3)

This indicates that BGM 11 provides best nutrients for algal growth. The obtained optical density were 0.79 *Unidentified cyanobacteria* (A1), 0.77 *Chlorella* sp. (A2) and 0.71 g/L *Neochloris* sp. (A3) respectively at the end of 25 days of process. While the growth of algae was minimum in Prochlorococus medium followed by ATCC, Jaworski's, SES, Bristol's, ASN111 and BGM. Parameswari et al. (2009) cultured blue green algae and reported that BG-11 broth is best suitable for the mass multiplication of algal isolates.

3.2 Stock Solution Optimization of BG11 Medium

The optimization of BG11 medium was performed by growing the algae in different types of stock solution (A, B, C and D). When compared with stock solutions A, C and D, stock solution B shows increased level of algal growth with the g/L being 1.385 for *Unidentified cyanobacteria* (A1), 1.3697 for *Chlorella* sp. (A2) and 1.302 for *Neochloris* sp. (A3) (Fig. 2). This proves that stock solution B acts as a suitable

medium for the growth of algae. Additionally the growth of algae was minimum in A, followed by stock solution D and stock solution C.

3.3 Biosorption of Chromium by Three Different Algae

The ability of chromium removal by the algae *Unidentified cyanobacteria*, *Chlorella* sp. and *Neochloris* sp. was also investigated using Atomic Absorption Spectroscopy. Higher level of chromium absorption was noticed in *Unidentified cyanobacteria* (A1) 75.63% followed by *Neochloris* sp. (A3) 72.5% and *Chlorella* sp. (A2) 44.37% at the end of 25 days of the process (Fig. 3). High percentage of chromium removal by *cyanobacteria* is substantiated by the study of Shukla et al. (2012).

The growth of all the BGA, *Unidentified cyanobacteria* (A1) *Chlorella* sp. (A2) and *Neochloris* sp. (A3) in various chromium concentrations (100, 200, 300, 400 and 500 mg/L) were analyzed. The results show that *Unidentified cyanobacteria* (A1) was found to grow at a chromium concentration of 400 mg/L followed by *Neochloris* sp. (A3 300 mg/L) and *Chlorella* sp. (A2 200 mg/L) (Table 1).

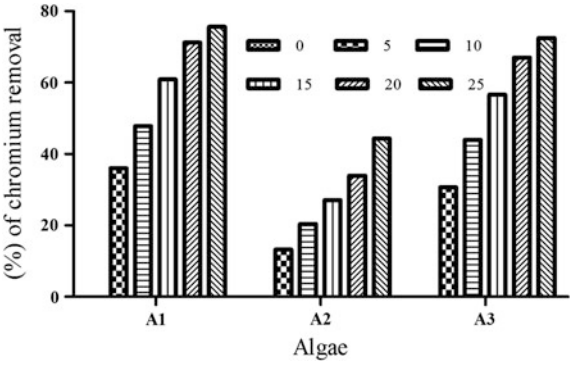

Fig. 3 Percentage of chromium removal in algae (A1, A2 and A3)

Table 1 Growth of *unidentified Cynobacteria* (A1), *Chlorella* sp. (A2) and *Neochloris* sp. (A3) treated with various concentrations of chromium

Concentration (mg/L)	100	200	300	400	500
A1	+++	+++	++	+	–
A2	++	++	–	–	–
A3	+++	++	++	–	–

3.4 SEM and EDX Analysis of Chromium Removal by Algae

The chromium absorbed by the algal samples was determined by SEM. During the initial stage of growth an aggregation of algal cells was noticed. After the absorption there was disaggregation of cells and the cell surface became shorter with roughness due to the absorption of chromium (Fig. 4a, b (A1); c, d (A2); e, f (A3)). Further the presence of chromium was confirmed by EDX (Fig. 4g, h). During initial growth there is an absence of chromium peak, which was clearly visible after the absorption of chromium peak. This is in corroboration with an earlier study demonstrating the reduction of chromium using the EDX spectra (Shukla et al. 2012).

3.5 Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

In the present investigation, the hexavalent chromium was found to undergo reduction via complexation with carboxyl or amide or hydroxyl moieties of components present in the algae. FT-IR study of the algae biomass A1 shows that the band observed at 3448 cm^{-1} result refer to absorbency of –OH and COO– groups. The intensity of peak gets diluted after 25 days of experimental process. It is suggested that –OH and COO groups could contribute to the binding of Cr^{6+}. Similarly in A2 and A3 also the intensity peak observed at 3579 and 3471 cm^{-1} get reduced due to the accumulation of chromium by the algal sample. Similar results were reported by Thillai et al. (2011) that the biosorption frequencies of chromium obtained in 3448 cm^{-1} in *Graciliria edulis*. Further the reduction in the intensity of peak at 1411 (A1); 1385 (A2); 1381 cm^{-1} (A3) indicates the complexation of carboxalate functional group by coordination with chromium metal (Fig. 5). The last changes in the peak range at 894 (A1); 833 (A2); 840 cm^{-1} (A3) could be attributed to the presence of chromium (Haxo 1960). Similar results were also reported by Shukla et al. (2012).

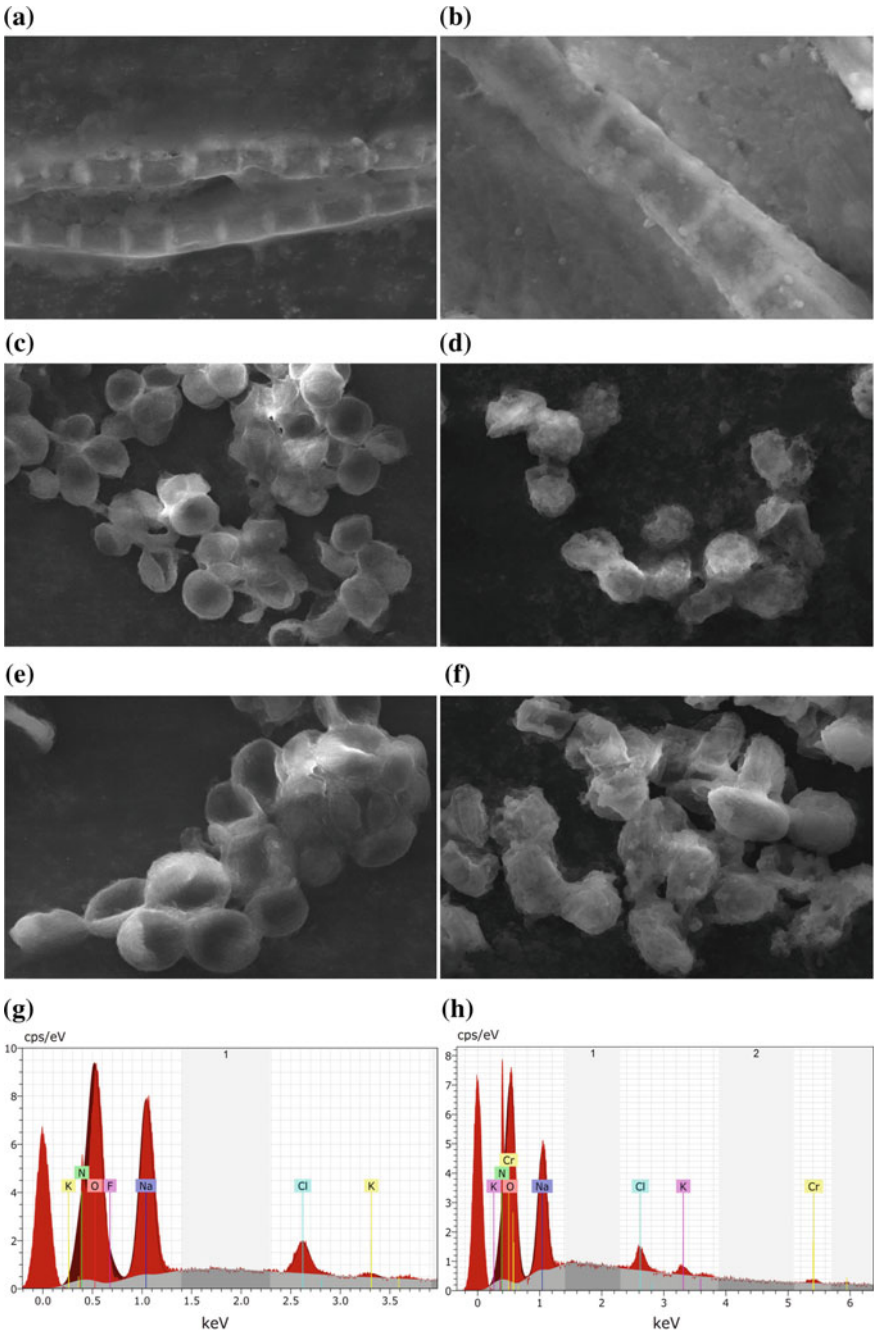

Fig. 4 SEX-EDX analysis for control (without Cr) and test (with Cr) algae A1 (**a** and **b**), A2 (**c** and **d**), A3 (**e** and **f**) and Cr conformation with control and test (**g** and **f**)

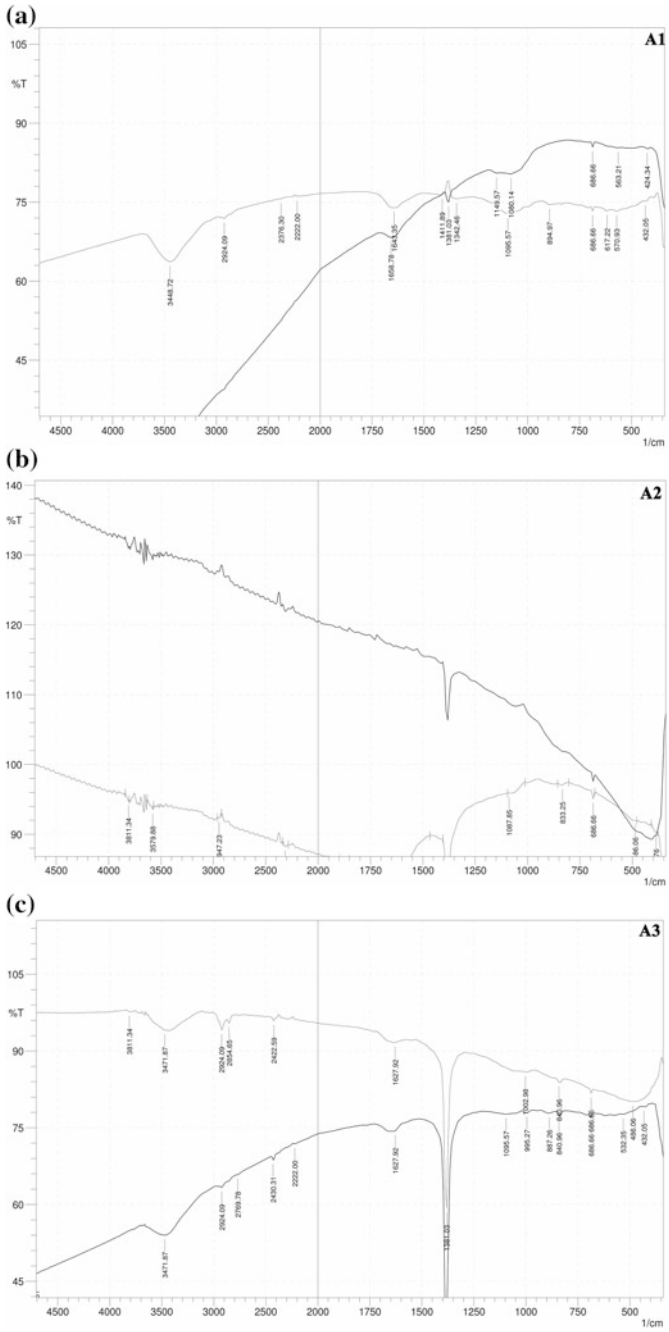

Fig. 5 FT-IR analysis for chromium adsorption by algae (A1, A2 and A3)

4 Conclusion

The conditions for the growth of algae were optimized and BG11 medium with stock solution B was found to be the suitable medium for algae cultivation. The unidentified cyanobateria exhibited a higher chromium removal capacity (75.63%) followed by *Chlorella* sp. (44.37%) and *Neochloris* sp. (72.5%). The chromium biosorption was further conformed by AAS, SEM and FT-IR which substantiate the chromium biosorption by the algae.

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