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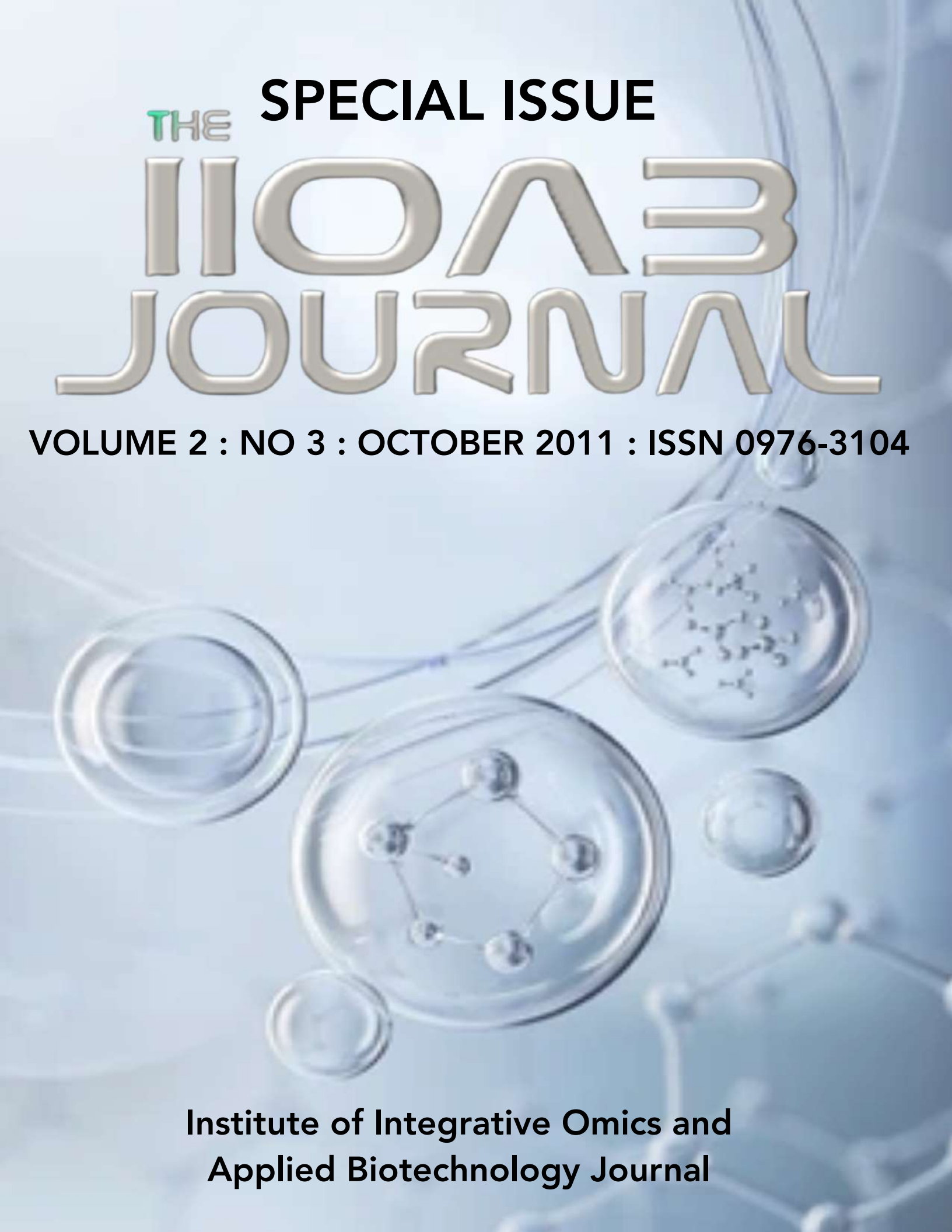

Institute of Integrative Omics and
Applied Biotechnology Journal

Dear Esteemed Readers, Authors, and Colleagues,

I hope this letter finds you in good health and high spirits. It is my distinct pleasure to address you as the Editor-in-Chief of Integrative Omics and Applied Biotechnology (IIOAB) Journal, a multidisciplinary scientific journal that has always placed a profound emphasis on nurturing the involvement of young scientists and championing the significance of an interdisciplinary approach.

At Integrative Omics and Applied Biotechnology (IIOAB) Journal, we firmly believe in the transformative power of science and innovation, and we recognize that it is the vigor and enthusiasm of young minds that often drive the most groundbreaking discoveries. We actively encourage students, early-career researchers, and scientists to submit their work and engage in meaningful discourse within the pages of our journal. We take pride in providing a platform for these emerging researchers to share their novel ideas and findings with the broader scientific community.

In today's rapidly evolving scientific landscape, it is increasingly evident that the challenges we face require a collaborative and interdisciplinary approach. The most complex problems demand a diverse set of perspectives and expertise. Integrative Omics and Applied Biotechnology (IIOAB) Journal has consistently promoted and celebrated this multidisciplinary ethos. We believe that by crossing traditional disciplinary boundaries, we can unlock new avenues for discovery, innovation, and progress. This philosophy has been at the heart of our journal's mission, and we remain dedicated to publishing research that exemplifies the power of interdisciplinary collaboration.

Our journal continues to serve as a hub for knowledge exchange, providing a platform for researchers from various fields to come together and share their insights, experiences, and research outcomes. The collaborative spirit within our community is truly inspiring, and I am immensely proud of the role that IIOAB journal plays in fostering such partnerships.

As we move forward, I encourage each and every one of you to continue supporting our mission. Whether you are a seasoned researcher, a young scientist embarking on your career, or a reader with a thirst for knowledge, your involvement in our journal is invaluable. By working together and embracing interdisciplinary perspectives, we can address the most pressing challenges facing humanity, from climate change and public health to technological advancements and social issues.

I would like to extend my gratitude to our authors, reviewers, editorial board members, and readers for their unwavering support. Your dedication is what makes IIOAB Journal the thriving scientific community it is today. Together, we will continue to explore the frontiers of knowledge and pioneer new approaches to solving the world's most complex problems.

Thank you for being a part of our journey, and for your commitment to advancing science through the pages of IIOAB Journal.

Yours sincerely,

Vasco Azevedo

Vasco Azevedo, Editor-in-Chief
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RESEARCH ARTICLE

BIOINFORMATICS AND BIOSYNTHESIS ANALYSIS OF CELLULOSE SYNTHASE OPERON IN *ZYMOMONAS MOBILIS* ZM4

Sheik Abdul Kader Sheik Asraf, K. Narayanan Rajnish, and Paramasamy Gunasekaran*
Department of Genetics, Centre for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai -625021, INDIA

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***Corresponding author:** Email: gunagenomics@gmail.com Tel: +91-452-2458478; Fax: +91-452-2459873

ABSTRACT

Biosynthesis of cellulose has been reported in many species of bacteria. The genes encoding cellulose biosynthetic enzymes of Z. mobilis have not been studied so far. Preliminary sequence analysis of the Z. mobilis ZM4 genome revealed the presence of a cellulose synthase operon comprised of Open Reading Frames (ORFs) ZMO01083 (bcsA), ZMO1084 (bcsB) and ZMO1085 (bcsC). The first gene of the operon bcsA encodes the cellulose synthase catalytic subunit BcsA. The second gene of the operon bcsB encodes the cellulose synthase subunit B (BcsB), which shows the presence of BcsB multi-domain and is inferred to bind c-di-GMP, the regulator of cellulose biosynthesis. The third gene of the operon bcsC encodes the cellulose synthase operon C domain protein (BcsC), which belongs to super family of teratrico peptide repeat (TPR) that are believed to mediate protein – protein interactions for the formation of cellulose. Multiple sequence alignment of the deduced amino acid sequences of BcsA and BcsC with other closely related homologs showed the presence of PVDPYE, HAKAGNLN, DCD motif and TPR motif, the characteristic motifs of bacterial cellulose synthases. Analysis of the nucleotide sequence of the ORF ZMO1085 and neighboring ORFs namely ZMO1083 and ZMO1084 indicated that all the ORFs are translationally linked and form an operon. Transcript analysis using Real-time PCR indicated the expression of the genes involved in cellulose synthase operon in Zymomonas mobilis ZM4. Z. mobilis colonies grown on RM-glucose containing Congo red displayed a characteristic bright red-brown colour. Z. mobilis colonies grown on RM-glucose medium supplemented with Calcofluor exhibited fluorescence. The arrangement of Calcofluor stained microfibrils can be seen in fluorescence microscopy which is an indicative for cellulose biosynthesis. AFM micrograph of the extracellular matrix of Z. mobilis shows a relatively dense matrix with bacterial cell residues. The presence of cellulose was confirmed by the Acetic-Nitric (Updegraff) Cellulose assay. The Bioinformatics and biosynthetic analysis confirm the biosynthesis of cellulose in Z. mobilis.

Key words: *biosynthesis, cellulose; open reading frame; operon; transcript; Zymomonas mobilis*

[I] INTRODUCTION

Cellulose biosynthesis is widespread in plants and microorganism, and cellulose production by bacteria is of special interest. Among the bacterial species, cellulose biosynthesis has been established in *Gluconobacter xylinus*, *Rhizobium leguminosarum*, *Sarcina ventriculi*, *Escherichia coli*, *Klebsiella pneumonia* and several species of cyanobacteria [1].

Cellulose production in bacteria is attributed to several reasons. In the case of *Rhizobium*, cellulose biosynthesis is required for adhesion and aggregation of bacteria at the root hair tip. Similarly, cellulose is involved in sequential attachment of *A. tumefaciens* to carrot tissue culture cells. In the case of pathogens such as *E. coli* and *Salmonella*, cellulose biosynthesis occurs concomitantly with the production of thin aggregative fimbriae (AGF), the second component of the extracellular matrix of a multicellular morphotype. While thin aggregate of fimbriae form rigid, but fragile interconnections between cells, cellulose connects the cells through elastic, but stable bonds. One of the multicellular morphotype is biofilm formation on abiotic surfaces where cells producing cellulose and thin aggregative fimbriae form distinct adherence patterns [2]. In *Zymomonas mobilis*, cellulose biosynthesis has been implicated in the formation of cellular aggregates or flocs that can be separated using centrifugation. But, the genes

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responsible for cellulose synthesis have not been studied. Similarly, biochemical estimation of cellulose has not been carried out so far.

Zymomonas mobilis, a gram negative, anaerobic, micro aerotolerant, ethanologenic bacterium uses Enter-Dourdoff (ED) pathway to metabolize glucose [3]. The complete genome of *Z. mobilis* ZM4 consists of a singular circular chromosome of 2,056,416 bp with an average G+C content of 46.33 % [4]. The predicted ORFs (open reading frames, 1,998) cover 87 % of the genome. Among them, 1,346 ORFs (67.4 %) could be assigned with putative functions, 258 ORFs (12.9 %) were putative coding sequences of unknown functions and the remaining 394 ORFs (19.7 %) showed no similarities to known genes. The preliminary sequence analysis of the *Z. mobilis* ZM4 genome shows the presence of a cellulose synthase operon comprised of Open Reading Frames (ORFs) ZMO01083, ZMO1084 and ZMO1085. However, there are no reports on the detailed analysis of this operon. In the present study, several bioinformatics tools were used in functional analysis of the genes of the cellulose synthase operon. The precision of these bioinformatics tools has enhanced over a period of time and has its own advantages. In order to make the most precise predictions, numerous methods were used to build up the functional properties of the cellulose synthase operon of *Z. mobilis* to the highest possible accuracy. We have also provided the experimental evidence for the cellulose production by *Z. mobilis*.

[II] MATERIALS AND METHODS

2.1. Bioinformatics analysis

The protein sequences of the cellulose synthase catalytic subunit (BcsA), cellulose synthase subunit B (BcsB) and cellulose synthase operon C domain protein (BcsC) was obtained from the *Z. mobilis* ZM4 genome [NC_006526]. The primary sequence was analysed using Protparam [5]. ProtParam was used to calculate biochemical, biophysical and physicochemical properties like molecular weight, theoretical isoelectric point, instability index, extinction coefficient, aliphatic index, grand average of hydropathicity (GRAVY), estimated half-life (*Escherichia coli*, *in vivo*, in hours), and total number of negatively and positively charged amino-acid residues. Homology and similarity searching of the protein sequences against several sequences was performed using BLASTP [6] against NR and PDB databases. Multiple sequence alignment and analysis were performed using ClustalW2. Signal peptide and cleavage site was predicted using iPSORT [7], PrediSi [8], PSORT [9], SignalP [10], and SOSUI signal [11]. iPSORT is a subcellular localization site predictor for N-terminal sorting signals. Predisi is a software tool for predicting signal peptide sequences in real time with a high accuracy and is based on a position weight matrix approach by a frequency correction that takes the amino acid bias present in proteins in consideration. PSORT analyzes the input sequence by applying the stored rules for various sequence features of known protein sorting signals and reports the possibility for the input protein to be localized at each candidate site with additional information. SignalP 3.0. incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models. SOSUIsignal predicts signal peptide of which three-domain (tripartite) structure is recognized by three modules of the software system.

2.2. Transcript analysis

The mid-growth phase cultures of *Z. mobilis* grown in RMG were withdrawn and the total RNA was isolated as described previously [12]. All the RNA samples were treated with DNase I (MBI Fermentas, Opelstrasse, Germany) to eliminate the genomic DNA contamination and purified before the PCR was performed. The RNA was quantified using Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA), and the integrity of RNA was analyzed on a formaldehyde agarose gel [13]. Later, RevertAid First Strand cDNA Synthesis kit (MBI Fermentas, Germany) was used for the synthesis of first strand cDNA from total RNA template using gene-specific primers **[Table-1]**.

Table: 1. List of primer pairs used for real time PCR

Primer Name	Sequence (5'-3')	bp	Tm(°C)
BcsAF	TGCCGTCGCCCATGA	15	68.2
BcsAR	ACGGAACGGAAAACGAACTG	20	66.0
BcsBF	GTTGCGTGAAAATGCGAATG	20	66.3
BcsBR	GGAAGATCGCCGGATCAA	18	66.2
BcsCF	GGTCACCGCAAATTATAGACCAAT	24	65.5
BcsCR	CATGACAGGACGACGTTCCA	20	67.3
AdhBF	CGCAGAAGCCACCATTCAG	19	66.9
AdhBR	GCTGGAATACCAATGGAAGCA	21	65.9

A negative control reaction was performed using *Taq* DNA polymerase with RNA as template. qPCR (quantitative PCR) primers were designed using PrimerExpress 3.0 software (Applied Biosystems, USA) and primers were ordered from Sigma Genosys, Bangalore. The levels of expression of ZMO01083, ZMO1084 and ZMO1085 transcripts in *Z. mobilis* ZM4 was determined by real-time PCR. *adhB* (alcohol dehydrogenase) gene was used as endogenous control using 100 ng of cDNA as template. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) kit in an Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, USA) according to manufacture's instruction. Power SYBR Green PCR Master Mix (25 µl) consisted of 2x SYBR Green (12.5 µl), 900 nM of each of forward and reverse gene-specific primers for the respective genes, 100 ng cDNA template. qPCR cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles each of 95 °C for 15 sec and 60 °C for 1 min. The resulting PCR products were examined to dissociation-curve analysis to confirm the presence of single amplicon obtained from the cDNA template. The qPCR was performed in duplicates. For each gene analyzed, Power SYBR Green PCR Master Mix without template controls were performed. After, qPCR was completed, the threshold cycle (C_t) was calculated using ABI 7500 SDS software version 1.3 (Applied Biosystems, USA). The transcript levels of target ZMO01083, ZMO1084 and ZMO1085 were normalized with respect to endogenous control, *adhB*. The resultant aliquots (25 µl) of each qPCR product were electrophoresed on 1.2 % agarose gel in 1 x TAE (40 mM Tris acetate, 1 mM EDTA, pH 8) buffer at constant voltage of 80 V/cm and stained with ethidium bromide (0.5 µg ml^{-1}). The size of each amplified qPCR product was double-checked by Bio-Rad Quantity One software to confirm the single amplicon.

2.3 Reagents, microorganisms, and culture conditions

The RNeasy mini kit (Qiagen, Hilden, Germany) was used for the

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isolation of total RNA. *Zymomonas mobilis* ZM4 (ATCC31821) was obtained from NRRL, Peoria, IL, USA. Power SYBR Green PCR Master Mix (Applied Biosystems, USA) was used for real-time PCR experiment. *Z. mobilis* ZM4 was grown in Rich Medium (glucose, 20 g l^{-1}; KH_2PO_4, 20 g l^{-1} and yeast extract 10 g l^{-1}) under static condition at 30 ºC for RNA isolation.

2.4. Cellulose assay and characterization of cellulose-related phenotype

Cellulose was estimated by Acetic-nitric (Updegraff) cellulose assay. To study the secretion of cellulose, *Z. mobilis* was grown for 72 h at 30 ºC on RMG agar plates (glucose, 20 g l^{-1}; KH_2PO_4, 20 g l^{-1} ; yeast extract 10 g l^{-1} and 1.6 % agar) supplemented with Congo red 40 µg.ml^{-1}. Calcofluor binding by *Z. mobilis* was observed on RMG agar plates (glucose, 20 g l^{-1}; KH_2PO_4, 20 g l^{-1}; yeast extract 10 g l^{-1} and 1.6 % agar) supplemented with Calcofluor 200 µg.ml^{-1}. The colonies fluorescence was observed under fluorescence microscope (Nikon eclipse Ti). The morphology and microstructure of the extracellular cellulosic material was evaluated by atomic force microscope (APE Research A 100).

[III] RESULTS AND DISCUSSION

3.1. Bioinformatics analysis of cellulose synthase operon

Analysis of *Z. mobilis* genome identified a putative operon comprising of the cellulose synthase catalytic subunit (BcsA), cellulose synthase subunit B (BcsB) and cellulose synthase operon C domain protein (BcsC). This arrangement is similar to the cellulose biosynthase operon in *Acetobacter xylinum* [14]. The domain analysis at the Conserved Domain Database **[Figure–1]** provided the following results. The *bcsA* encodes the cellulose synthase catalytic subunit BcsA, which belongs to super family of Glycosyl transferase A and PilZ. The PilZ domain is the binding protein for cyclic diguanylic acid (c-di-GMP), an allosteric activator of the cellulose synthase. The presence of this domain perhaps indicates that the BcsA protein could be regulated by cyclic-di- GMP [15]. BcsA shows the presence of PVDPYE, HAKAGNLN, DCD motifs, the characteristic motifs of bacterial cellulose synthase [2] and a presence of CelA multi-domain. The specific hit of BcsA is CESA_CelA_like family proteins. The BcsA protein is transmembrane protein and belongs to a family of progressive β-glycosyltransferases. The second gene of the operon *bcsB* encodes the cellulose synthase subunit B (BcsB), which belongs to super family of PRK11114 and shows the presence of BcsB multi-domain.

Like the BcsA protein, the BcsB protein also poses transmembrane domain at the C-terminus, and is inferred to bind c-di-GMP, the regulator of cellulose biosynthesis. The third gene of the operon *bcsC* encodes the cellulose synthase operon C domain protein (BcsC), which belongs to super family of teratrico peptide repeat (TPR) and Cellulose synthase operon protein C. BcsC shows the presence of TPR motif. TPR is a 34 amino acid repeated motif that is widespread among prokaryotes and eukaryotes [16]. In the case of cellulose biosynthesis, TPR repeat domains are believed to mediate protein – protein interactions for the formation of cellulose. The BcsC has transmembrane domains and the TPR repeat domain at the N-terminus.

The biochemical, biophysical and physicochemical properties of BcsA, BcsB and BcsC are listed in the **[Table-2]**.BLASTP analysis of the cellulose synthase catalytic subunit (BcsA), cellulose synthase subunit B (BcsB) and cellulose synthase operon C domain protein (BcsC) showed maximum identity to putative cellulose synthase of *Sphingobium japonicum* UT26S and cellulose synthase protein C precursor of *Sphingobium japonicum* UT26S respectively.

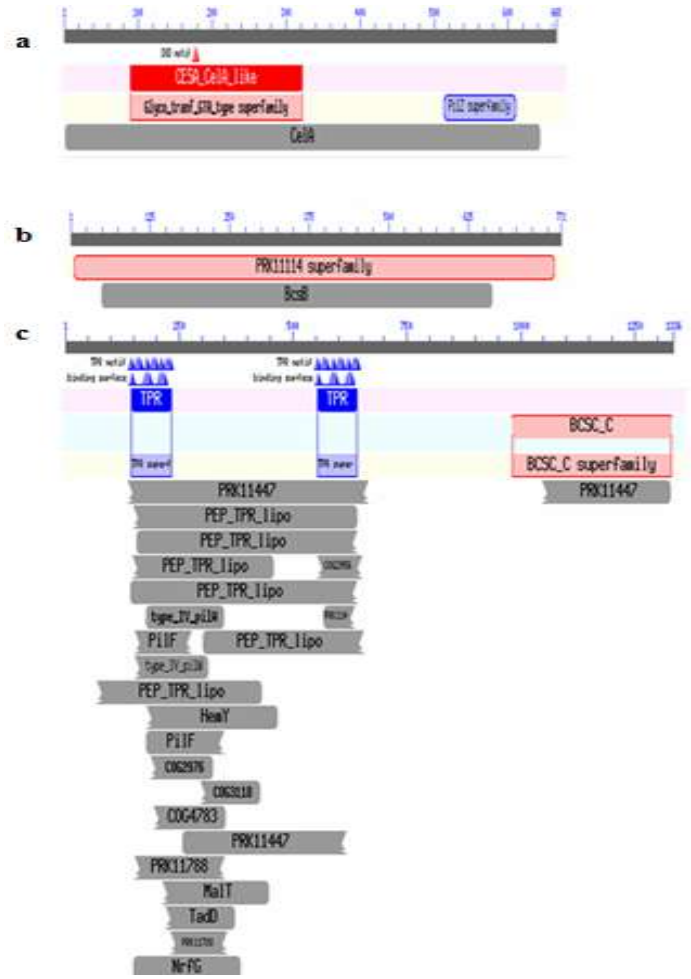

Fig: 1. Conserved Domain analysis of cellulose synthase operon. a) The first gene of the operon *bcs*A encodes the cellulose synthase catalytic subunit BcsA, which belongs to super family of Glycosyl transferase A and PilZ. BcsA shows the presence of DXD motif. The specific hit of BcsA is CESA_CelA_like family proteins. **b)** The second gene of the operon *bcs*B encodes the cellulose synthase subunit B (BcsB), which belongs to super family of PRK11114. There is a presence of BcsB multi-domain. **c)** The third gene of the operon

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*bcs*C encodes the cellulose synthase operon C domain protein (BcsC), which belongs to super family of TPR and Cellulose synthase operon protein C. BcsC shows the presence of TPR motif.

Table 2: Predicted properties of BcsA, BcsB and BcsC

Biochemical / biophysical / physicochemical properties	BcsA	BcsB	BcsC
Number of amino acids	665	771	1336
Molecular weight (in KDa)	75.38	84.62	147.07
Theoretical isoelectric point (pI)	8.49	6.54	6.83
Aliphatic index	101.08	95.5	73.17
Half-life (*E. coli*, *in vivo*, in hr)	>10	>10	>10
Gravy index	0.06	-0.101	-0.589
Extinction coefficient	94685	92945	142910
Total number of negatively charged residues (Asp + Glu)	65	79	146
Total number of positively charged residues (Arg + Lys)	69	75	142

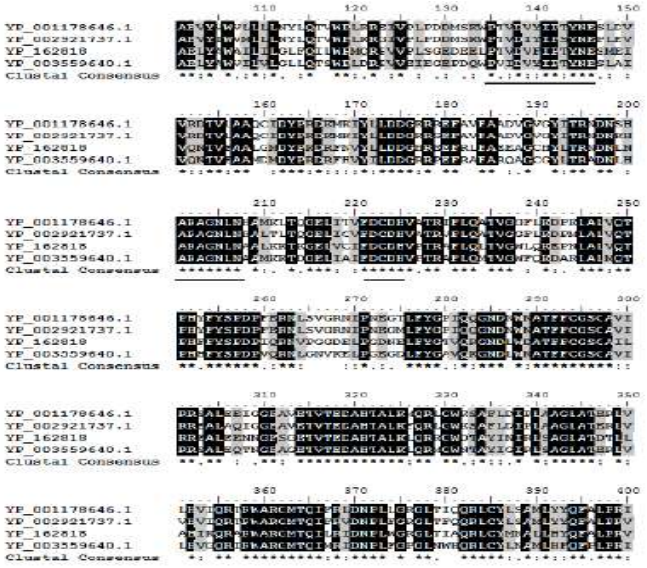

Fig: 2. Multiple sequence alignment of the deduced amino acid sequence of ZMO1083 with other bacterial cellulose synthases: The multiple sequence alignment was computed using the CLUSTAL W2 program. The amino acids that form the conserved motifs of bacterial cellulose synthases are PVDPYE (135-147), HAKAGNLN (200-208) and DCD (222-224) are underlined. The sequences compared with BcsA (ZMO1083, YP_162818.1), include the following: YP_003559640.1 (putative cellulose synthase, *Sphingobium japonicum* UT26S), YP_001178646.1 (cellulose synthase, *Enterobacter* sp. 638), and YP_002921737.1 (putative cellulose synthase, *Klebsiella pneumoniae* NTUH- K0244). Asterisks and dots indicate identical and similar amino acids respectively.

Multiple sequence alignment of the deduced amino acid sequences of BcsA and BcsC with other closely related homologs showed the presence PVDPYE, HAKAGNLN and DCD motifs **[Figure−2]** and TPR motifs **[Figure−3]** respectively. Predisi, SignalP 3.0., iPSORT, SOSUIsignal, PSORT predicted that BcsA has no signal peptide and it is an intracellular protein. BcsB and BcsC have been predicted to have signal peptide and are extracellular proteins.

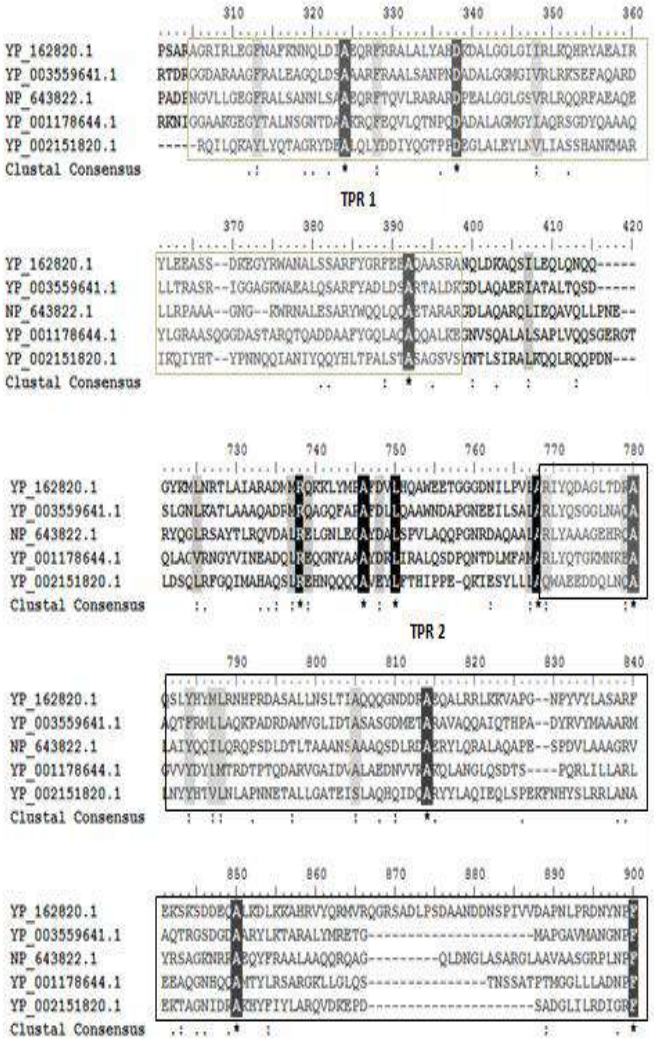

Fig: 3. Multiple sequence alignment of deduced amino acid sequence of *Z. mobilis* ZMO1085 with other bacterial cellulose synthase subunit C amino acid sequences: The multiple sequence alignment was computed using the ClustalW2 program. The amino acids from 305 -395 and 768-900 represent the TPR 1 and 2 repeats of BcsC. Sequence alignment revealed a putative consensus characteristic of the TPR family of proteins. The sequences compared with BcsC (YP_162820.1, *Z. mobilis*) include YP_003559641.1 (cellulose synthase protein precursor, *Sphingobium japonicum* UT26S), YP_00178644.1 (cellulose synthase domain containing protein, *Enterobacter* sp), NP_643822.1 (cellulose synthase subunit C, *Xanthomonas*

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axonopodis pv. Citri. Str.306) and YP_0021518201 (cellulose synthase protein, *Proteus mirabilis* H14320) respectively. Asterisks and dots indicate identical and similar amino acids respectively.

Genes	Shine-Dalgarno sequence	Start codon
eda	TAAAGC**AGGA**GTCTAAG	ATG
glf	GGCGGGA**GAGG**AATCGCC	ATG
zwf	TGTTTTA**AGGA**CGAGAAT	ATG
glk	TTTAGAAA**AGGA**ATATT	ATG
pgi	TCATTT**AGGAG**AGCGTT	ATG
gap	TAAGTT**AGGAG**AATAAA	ATG
pgk	GCCAAA**AGGAG**GATATA	ATG
adhB	GTAGGGT**GAAGG**TTATAGC	ATG
ZMO1085	AAA**AGGAT**GCTTCC	ATG
Z. mobilis *RBS consensus*	**AGGA**	

Fig: 4. Comparison of Ribosome binding site of the ORF ZMO1085 with *Z. mobilis* highly expressed ethanologenic genes. An examination of the nucleotide sequence upstream to ORF ZMO1085 indicated the presence of a putative Ribosome Binding Site AGGA. The putative RBS of the ORF ZMO1085 matched the RBS sequences of *eda, zwf, glk*.

Gene	Promoters		
	"-35"	intervening	"-10"
adh P1	AGCAGCCTTGCTC	ATCACCGCTGTCGCGAG	TAGAAAAT TCG
*adh*P2	GAACCCCTTGATC	TGATAAAACTGATAGAC	TATTGCTTT TGC
*gap*P1	AGCAGATTGGCTG	GGAAACGCTA----------TACTGGAAT AAT
*gap*P2	GGTATACTGGAAT	AAATGGTCTTCG------- TATTGATGT TTT
pdc	ATGCCTATAGCTA	AATCCGGAACGACACTT-- TAGAGGTTT CTG
Z.mobilis Consensus			
	A*****CTTG***		TA*TG*A*T
	- - -		- -
	G A G		A T
ZMO01081	**ATATTATTAA**	AGTTAGCCTTAAAAAGC	**TACATTCT TTT**

Fig: 5. Comparison of Promoter of the Cellulose synthase operon with *Z. mobilis* highly expressed ethanologenic genes. The putative promoter in the upstream region was predicted using the BPROM tool and aligned with the *Z. mobilis* promoter consensus sequence using BioEdit. The nucleotides in the predicted promoter regions that mismatched from the *Z. mobilis* promoter consensus are underlined.

An examination of the nucleotide sequence upstream to ORF ZMO1085 indicated the presence of a putative Ribosome Binding Site AGGA **[Figure–4]**. The putative RBS of the ORF ZMO1085 matched the RBS sequences of *eda*, *zwf*, *glk*. The intervening sequence between the RBS and the start codon consisted of 3 purines and 4 pyrimidines. Moreover, the start codon of the ORF ZMO1085 overlapped with the 3' end of the ORF ZMO1084. Such overlapping ORFs have been observed in the *gap* operon where the *eno* gene overlaps with the distal end of the *gap* gene. This result indicated that the ORFs ZMO1084 and ZMO1085 are linked and may form an operon.

The BPROM promoter prediction tool was used to identify the putative promoter sequences in the upstream region **[Figure–5]**. Analysis of the nucleotide sequence of the ORF ZMO1085 and neighboring ORFs namely ZMO1083 and ZMO1084 indicated that all the ORFs are translationally linked and form an operon. BPROM analysis identified a putative -10 sequence (TACATTTCT) and a -35 sequence (ATATTATTA). Comparison of the predicted promoters revealed considerable similarity to the *Z. mobilis* promoter consensus. In particular, the -10 region was well conserved and matched with the *Z. mobilis* promoter consensus sequence. In the case of the -35 region an adenine replaced the conserved guanine of the consensus sequence. Moreover the intervening sequence between the putative -10 and -35 promoter regions consisted of 18 nucleotides, which is considered as the optimal spacing for promoter activity. Similarly the promoter prediction indicated absence of promoter in the intervening region, nevertheless presence of a putative promoter was evident. Thus, the promoter of the ORF ZMO1084 drives the transcription of the ORF ZMO1085.

The gene neighborhood analysis performed using STRING server yielded the following results. The results indicated that strong association of ORF ZMO1085 (cellulose synthase operon C domain protein) with ORFs ZMO1084 (cellulose synthase subunit B; score 0.963), ZMO1083 (cellulose synthase catalytic subunit; score 0.958), ZMO1086 (endoglucanase; score 0.840). These results suggested that the association of the ORF ZMO01085 with ORFs ZMO1084, ZMO1083 and ZMO1086 is considered with high confidence. Moreover co expression data also showed evidence for interaction of the ORFs ZMO1084, ZMO1083 and ZMO1086 with the ORF ZMO1085. The results indicate that the interaction of ZMO1085 with the neighboring ORFs ZMO1084, ZMO1085 and ZMO1086 is highly possible.

3.2. Transcript analysis of the cellulose synthase operon

In this study, the transcript level of the ORFs ZMO1083, ZMO1084 and ZMO1085 was quantified with respect to the endogenous control gene, *adhB* **[Figure–6]**. The SDS v 1.3 software was used to calculate the C_t values and the average C_t values obtained are shown in **[Table–3]**. As seen in the **[Table–3]**, the C_t value of ORFs ZMO1083 and ZMO1084 was lower than the C_t value of the endogenous control gene (*adhB*). Since C_t is inversely proportional to logarithm of the copy number, low C_t values correspond to high copy numbers of the target sequence, and high C_t value correspond to low copy numbers. Thus, the level of the transcript of the ORFs ZMO1083 and ZMO1084 was found at higher level compared to the control *adhB*. The melt curve analysis of the amplicons

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indicated single peaks corresponding to the adhB, ZMO1083, ZMO1084 and ZMO1085 amplicons **[Figure–6b]**. Similarly, gel electrophoresis analysis of PCR products obtained as the result of qPCR indicated the presence of amplicons corresponding to 50 bp **[Figure–6c]**.

Table: 3. Relative quantification of the genes of cellulose synthase operon

Sample	Task	Average C_t
Alcohol dehydrogenase	endogenous control	13.05
ZMO1083	target	12.42
ZMO1084	target	12.11
ZMO1085	target	14.53

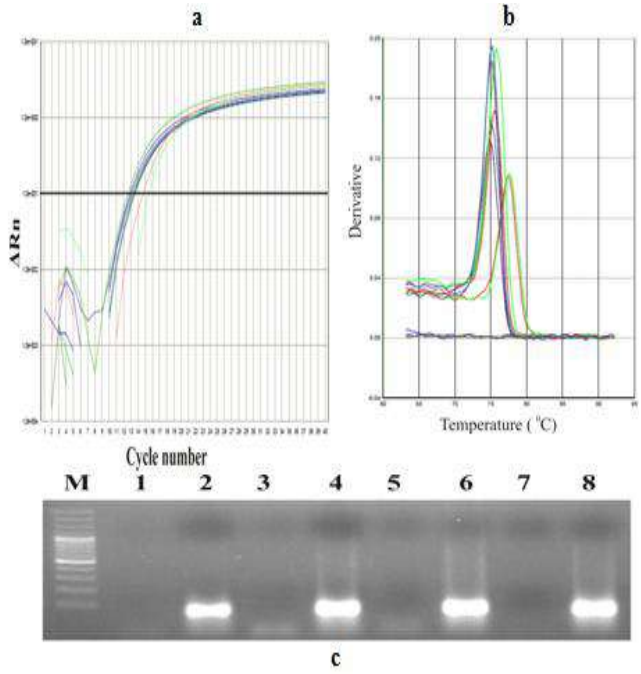

Fig: 6. a) qPCR amplification plot of ZMO01083, ZMO1084, ZMO1085 and *adhB*. The cDNA specific to ZMO01083, ZMO1084, ZMO1085 (target) and *adhB* (reference) were analyzed using relative quantification. The delta R_n values represent the difference of the fluorescent signal between the endogenous control gene (*adhB*) and the target (ZMO01083, ZMO1084 and ZMO1085). The grey line indicates the threshold set using SDS v 1.3 software **b) Melt curve analysis of ZMO01083, ZMO1084, ZMO1085 and *adhB* amplicons**. Melt curve analysis indicated the presence of four distinct peaks corresponding to the amplicons of *adhB* (Tm, 77.7°C) and ZMO01083 (74.9°C), ZMO01084 (77°C) and ZMO01085 (75.2°C). **c) Gel electrophoresis analysis of relative quantification assay amplicons.** The amplicons obtained from the relative quantification experiment were resolved on a 1.5 % agarose gel and stained with ethidium bromide. The lanes 1, 3, 5, 7 are the No Template Controls while the lanes 2, 4, 6, and 8 represent the amplicons of *adhB* and ZMO01083, ZMO01084 and ZMO01085 respectively.

3.3. Identification of Cellulose synthesis by Z. mobilis

Z. mobilis colonies grown on RM-glucose containing Congo red displayed a characteristic bright red-brown colour **[Figure–7a]**. This property was similar to that of *S. typhimurium*, *E. coli* and *C. violaceum*. *Z. mobilis* colonies grown on RM-glucose medium supplemented with Calcofluor exhibited fluorescence **[Figure–7b]**.

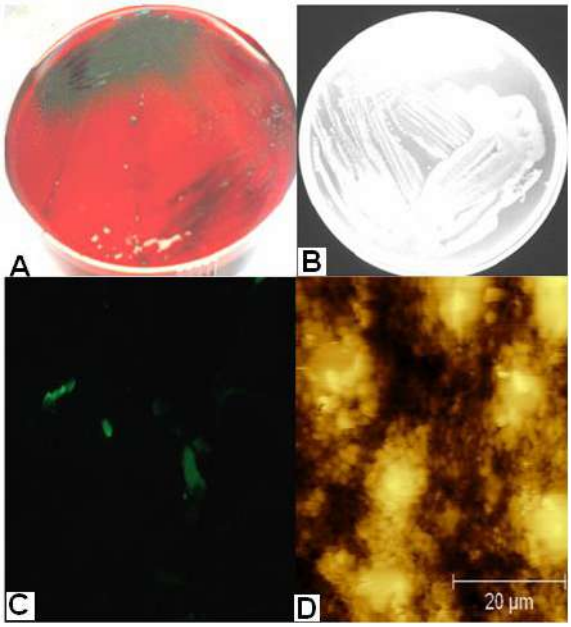

Fig: 7. a) Congo red assay. *Z. mobilis* colonies bound Congo red displayed a characteristic bright red-brown colour when grown on RM-glucose containing the dye after 72 h. **b) Calcofluor assay.** Fluorescence of *Z. mobilis* colonies that were grown on RM-glucose supplemented with calcofluor and visualized.**c) Fluorescence microscopy**. The arrangement of Calcofluor stained microfibrils can be seen in fluorescence microscopy. Calcofluor binding to microfibrils in the cell clumps is an indicative for cellulose biosynthesis. **d) Atomic force microscopy.** AFM micrograph of the extracellular matrix of *Z. mobilis* shows a relatively dense matrix with bacterial cell residues.

The arrangement of Calcofluor stained microfibrils can be seen in fluorescence microscopy **[Figure–7c]**. Calcofluor binding to microfibrils in the cell clumps is an indicative for cellulose biosynthesis. AFM micrograph **[Figure–7d]** of the extracellular matrix of *Z. mobilis* shows a relatively dense matrix with bacterial cell residues. The presence of cellulose was confirmed by the Acetic-Nitric (Updegraff) Cellulose assay.

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[IV] CONCLUSION

The Bioinformatics and biosynthetic analysis confirm the biosynthesis of cellulose in *Z. mobilis*.

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ABOUT AUTHORS

Prof. Paramasamy Gunasekaran *is Senior Professor & Head, Department of Genetics and Coordinator, Center for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University. His research interests include: Metagenomics and bioprospecting, Molecular biology and Genomics of Zymomonas mobilis and filamentous fungi, Bioprocess technology for enzymes and biofuel production: Recombinant strains, Enzyme engineering and enzyme technology, Bioremediation of industrial effluents, and Genetic diversity of Plant growth promoting rhizobacteria. He has more than 130 publications in the peer-reviewed national and international journals. He has written 8 books/manuals in the field of microbial genomics.*

Mr. K. Narayanan Rajnish *is a Senior Research Fellow in Department of Genetics, Center for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University. He has completed his Ph.D. thesis entitled "Studies on functional genomics of Zymomonas mobilis" His research interests include Molecular biology and Genomics of Zymomonas mobilis, Enzyme engineering and enzyme technology and Bioprocess technology for enzymes and biofuel production: Recombinant strains. He has published 1 Research article, 1 Book-chapter and has communicated 1 Research article (In Review) and 1 Book chapter (In Press).*

Mr. Sheik Abdul Kader Sheik Asraf *is a Junior Research Fellow in Department of Genetics, Center for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University. He is presently working upon the Ph.D. thesis entitled* "Functional genomics of selected carbohydrate hydrolases in *Zymomonas mobilis*". *His research interests include Molecular biology and Genomics of Zymomonas mobilis, Enzyme engineering and enzyme technology and Bioprocess technology for enzymes and biofuel production: Recombinant strains. He has communicated 2 Book chapters (In Press) and 1 Research article (In Review).*

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RESEARCH ARTICLE

REMOVAL OF HEXAVALENT CHROMIUM USING ACTIVATED COCONUT SHELL AND ACTIVATED COCONUT COIR AS LOW COST ADSORBENT

Asha Gupta*, Reena Yadav, and Parmila Devi
Department of Environmental Science & Engineering,Guru Jambheshwar University of Science & Technology, Hisar-125001, INDIA

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***Corresponding author:** Email: gupta06amit@gmail.com Tel: +91-9416372247; Fax: +91-1662-276240

ABSTRACT

Studies were conducted to assess the comparative efficiency of activated coconut shell and activated coconut coir in removing toxic hexavalent chromium from its synthetic solution and tannery industrial effluent in batch mode. The parameters studied include- pH, adsorbent dose, contact time & initial metal ion concentration and the applicability of adsorption isotherms. Activated coconut shell exhibited more adsorption potential as compared to Activated coconut coir and maximum removal exhibited by ACS was 88% and 83.0 % for 0.3 mm and 1.0 mm in case of synthetic solution & 76%(0.3mm) and 66%(1.0mm) in case of tannery effluent at optimized conditions (pH =2, dose=1.0g/100ml, contact time=60 minutes & initial metal ion concentration=10 ppm). In general ACS was observed to be better adsorbent than ACC while adsorbents of particle size 0.3 mm have greater efficiency than 1.0 mm. On application of adsorption kinetics, Adsorption of Cr (VI) by ACS and ACC followed the pseudo first order and pseudo second order model. The results showed that the activated coconut coir is an efficient adsorbent for hexavalent chromium removal.

Key Words: *dose; removal efficiency; effluent; activated carbon; sorption*

[I] INTRODUCTION

Heavy metal contamination of industrial effluents is one of the significant environmental problems due to their toxic nature and accumulation throughout the food chain as non-biodegradable pollutants [1]. Tannery is the one of the oldest and fastest growing industry in India. There are about 2161 tanneries in India; however sustenance of tanneries is becoming increasingly difficult because of alarming level of environmental pollution caused by various tannery operations and practices. The main pollutants of concern in tanneries are BOD/COD, suspended solids and heavy metals. Heavy metals like mercury, lead, cadmium, copper, chromium and nickel are extremely toxic even at minute quantities [2]. Chromium is more abundant in earth's crust and is widely used in electroplating, leather tanning, metal finishing & chromate preparation. It exists in two stable oxidation states Cr (III) & Cr (VI). Cr (VI) is of particular concern as because of its high toxicity, it may cause many adverse effects on human health such as epigastric pain, hemorrhage, severe diarrhea, vomiting, nausea, dermatitis by skin contact, ulcer, lung cancer and tissue necrosis. Thus it becomes essential to remove Cr (VI) from industrial waste water before discharging it into water body or on to land.

Widespread concern over the cumulative toxicity and environmental impact of heavy metals has led to extensive research into developing effective alternative technologies for the removal of these potentially damaging substances from effluent and industrial wastewater [3]. Conventional technology for the removal of metal ions from aqueous solution includes chemical precipitation, ion exchange, chemical oxidation/reduction, reverse osmosis, electrodialysis, ultra filtration, etc. which have their own inherent limitations such as less efficiency, sensitive operating condition, production of secondary sludge and further the disposal is costly affair [4]. The search for alternate and innovative treatment techniques has focused attention on the use of biological materials for heavy metals removal & has gained important credibility during recent years because of the good performance and low cost of these materials [5]. So the efforts are being directed towards the use of natural low cost adsorbents for removal of heavy metals. Use of natural materials which are available in large quantities [6] or certain waste products from industries or agriculture may have potential as inexpensive

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adsorbents. Recently some of these low cost adsorbent (natural or processed) have been tested as adsorbents for heavy metal removal [7-10]. Conversion of this waste to a useful adsorbent contributes not only for the treatment of heavy metals contaminated environment but also to minimize the solid wastes. These research activities indicated promising results but further efforts are still required in order to maximize metal removal efficiency and minimize preparation costs.

Coconut shell and coconut coir are agricultural based waste materials and these materials have the potential to sequester metals from solutions. Abundant availability, high biosorption capacity, cost-effectiveness and renewability are the important factors making these materials as economical alternatives for water treatment and waste remediation [11]. The activated carbon prepared from coconut shell and coconut coir is highly porous, amorphous solid consisting of microcrystallites with a graphite lattice. They are non-polar and cheap. Keeping this in view, in the present study the potential of agro-waste-Activated coconut shell and Activated coconut coir was investigated for removal of hexavalent chromium from its synthetic solution and industrial effluent.

[II] MATERIALS AND METHODS

2.1. Preparation of adsorbents

Coconut shell and coir was collected from local market of Hisar, Haryana. Then properly washed with water, sun dried and finally kept in muffle furnace for 60 minutes at 270 °C temperature for carbonization. Then they were washed with distilled water to make the pH near neutral and kept the material in oven at 120°C for 18 hours. Dried material was ground and sieved through standard sieve to obtain particles of size 0.3mm and 1.0 mm. These adsorbents were treated as ACS and ACC.

2.2. Preparation of Cr (VI) solution

A stock solution of Cr (VI) of concentration 1000 mg/l was prepared using an accurately weighed quantity of the $K_2Cr_2O_7$ in double-distilled water. Experimental solutions of the desired concentrations were obtained by successive dilutions. pH values of metal solutions were adjusted using 1 N NaOH and HNO_3.

2.3. Batch adsorption experiments

Batch adsorption experiments were conducted in triplicates to evaluate the effects of adsorbent dose, contact time, pH and initial metal ion concentration on removal of Cr (VI) ions by ACS and ACC(0.3 mm and 1.0 mm). All the adsorption experiments were conducted in 250 ml round bottomed flask on rotatory shaker at 150 rpm. After desired contact period, flasks were removed and allowed to stand for two minutes. The solution was filtered through What man filter paper 41 and filtrate was analyzed for Cr (VI) concentration spectrophotometrically at wavelength 540 nm using the complexing agent 1,5-diphenyl-carbazide in acid medium according to the standard methods [12]. Finally all the adsorbents were applied on the tannery industrial effluent under the optimized conditions (Obtained from experiment on synthetic solution) for removal of Cr (VI).

2.4. Adsorption isotherms and kinetics

The adsorption data obtained for the Cr (VI) was analyzed using Langmuir and Freundlich isotherm and the kinetics for adsorptions of Cr (VI) on activated coconut shell and coir were studied for their utilization in the treatment of industrial effluents.
Langmuir's isotherm model is valid for monolayer adsorption onto a surface containing a finite number of identical sites, which is represented as equation:

$$\frac{Ce}{qe} = \frac{1}{Qob} + \frac{Ce}{Qo}$$

Where qe is the amount adsorbed at equilibrium (mg/g); Ce is the equilibrium concentration (mg/l); Qo and b are the Langmuir constants.

The Freundlich equation [13] proposes an empirical model that is based on the sorption on heterogenous surface and has the form:
Log x/m = log k + 1/nlog Ce
Where k (mg/g) and n are Freundlich isotherms constants; Ce is the equilibrium concentration (mg/l); x/m is the amount adsorbed (mg/g) and m is the adsorbent dose (g/l).

The kinetics for adsorption of Cr (VI) on coconut shell and coir were studied for their utilization in the treatment of industrial effluents. The first order rate constant for adsorption of Cr (VI) has been studied with the help of Lagergran's equation.

$$\text{Log } (qe-q) = \log q - Kt/2.303$$

Where qe =The amount of metal adsorbed at equilibrium (mg/g)
q = Amount of metal adsorbed at time t (mg/g)
k = rate constant of adsorption (per minute)
t = Time (minute)
Pseudo-second order rate equation

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

Where,

k_2 = Second order rate constant for adsorption (g mg^{-1} min^{-1})

q_e = Amount of metal adsorption at equilibrium (mg g^{-1})

q_t = Amount of metal adsorption in time t (min^{-1})

In order to obtain the rate constant graph was plotted between t/q_t vs t.

Line obtained after plotting log ($q_e - q_t$) vs t and t/qt vs t shows degree of fitness of metal sorption to first and second order rate kinetic models respectively. Straight line indicates best fitness of experimental data to corresponding models. This is based on the assumption that the adsorption capacity for metal on the adsorbent is proportional to the number of active sites occupied on the sorbent and metal uptake is due to chemisorption [14].

[III] RESULTS AND DISCUSSION

3.1. Effect of Adsorbent dose

Adsorbent dose had a very profound effect on Cr (VI) removal. Adsorption experiments were carried out at varying adsorbent dose (0.2-1.2gm/100ml), while other parameters like contact time (1 hr.), pH (2.0) and initial metal ion concentration (10 mg/l), were kept constant. The removal of Cr (VI) by ACS (0.3 mm & 1.0 mm) and ACC (0.3 mm & 1.0 mm) as a function of adsorbent

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dose is shown in **[Figure–1]**. As evident from the results increase in Cr (VI) removal was observed with increase in adsorbent dose and ACS showed greater removal than ACC. Both the adsorbents showed maximum removal at 0.3 mm particle size as compared to 1.0 mm. The reason may be the higher porosity of 0.3 mm size as compared to 1.0 mm due to which it provides greater adsorption sites to the adsorbate molecules. It was observed from

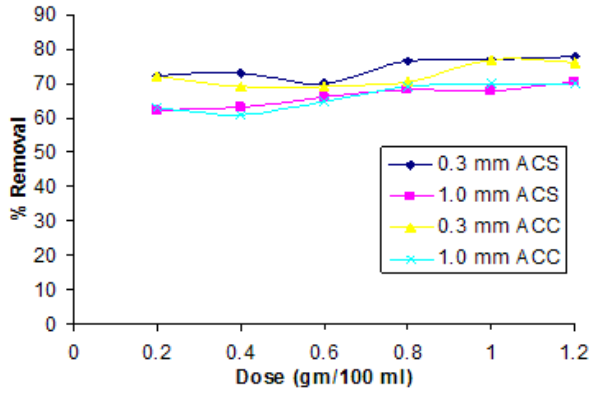

Fig: 1. Effect of adsorbent dose on Cr (VI) removal using activated coconut shell and activated coconut coir

the results that the percentage removal of Cr (VI) increases with increase in adsorbent dose up to some extent, thereafter with further increase in adsorbent dose; there was no appreciable increase in percentage removal. The optimum dose for removal of Cr (VI) was found to be 0.8 gm/100 ml for ACS with 76.5%(0.3mm) and 68.1%(1.0 mm) removal respectively, while for ACC it was 1.0 gm/100 ml with 76.8%and 70.1% for 0.3 mm and 1.0 mm respectively. The phenomenon of increase in percentage removal of Cr (VI) with increase in adsorbent dose up to certain level and beyond that more or less constant removal may be explained as with increase in adsorbent dose, more and more binding sites becomes available for the complexation of Cr (VI) ions and this increased the rate of adsorption. However very slow increase in removal beyond an optimum dose may be attributed to attainment of equilibrium between adsorbate and adsorbent at the existing operating conditions [15].Higher adsorbent dose cause screening effect of dense outer layer of cells, blocking the binding sites from metal ions, resulting in lower metal removal per unit adsorbent [16].

3.2. Effect of contact time

The effect of contact time on Cr (VI) removal was investigated at optimized adsorbent dose by varying the contact time (20-80 min.), while other parameters were kept constant **[Figure–2]**. Increase in percentage removal was observed with increase in contact time for all type of adsorbents.

Optimum time observed was 60 min. for all adsorbents at which % removal of Cr (VI) was 77.8%(0.3 mm) and 76.4%(1.0mm) for ACS while in case of ACC ,% removal was 72.3% and 66.3% for 0.3 mm and 1.0 mm respectively. There was no appreciable increase in percent Cr (VI) removal after these optimum times. As shown in Figure–2, the adsorption process took place in two stages. The first stage was rapid, where about 60% adsorption was completed within first 20 min. The second stage represented a slower progressive adsorption. The rapid initial biosorption may be attributed to the accumulation of metals on to the surface of adsorbent, due to its large surface area. With the progressive occupation of these sites, process became slower in the second stage. Moreover the initially deposited metal ions penetrate to the interior of the biosorbent through intra-particle diffusion which was slower process. This is in accordance with the observations of other similar studies [17]. The adsorption process attained equilibrium in 1 hr. Based on the results of kinetics experiments, a time of 1 hr was considered to be adequate for remaining experimentations. It may be explained as initially adsorbent showed the fast adsorption which gets slowed down later on, because initially large number of vacant surface site may be available for adsorption and after some time the remaining vacant surface sites may be exhausted due to repulsive forces between the solute molecules of solid and bulk phase [18].

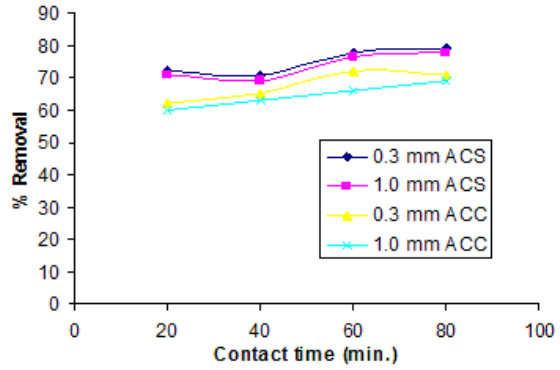

Fig: 2. Effect of contact time on Cr (VI) removal using activated coconut shell and activated coconut coir

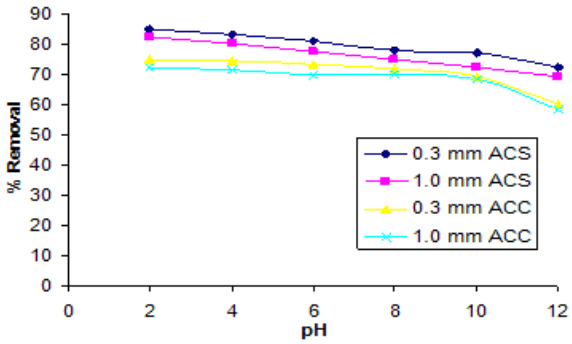

Fig: 3. Effect of pH on Cr (VI) removal using activated coconut shell and activated coconut coir

3.3. Effect of pH on Cr (VI) removal

The effect of pH on Cr (VI) removal by Activated coconut shell (ACS) and Activated coconut coir (ACC) was investigated at

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optimized adsorbent dose and contact time by varying pH from 2.0-12.0 **[Figure–3]**. Progressive decrease in Cr (VI) adsorption was observed with increase in pH from 2 to 12 and maximum adsorption was observed at pH 2. ACS showed maximum removal i.e. 85 % and 82 % (0.3 mm and 0.1 mm) while ACC showed 75% and 72% (0.3 mm and 1.0 mm) respectively at pH 2. The effect of pH on adsorption of Cr (VI) onto the adsorbent can be interpreted on the basis of the structure of the sorbent and the speciation of chromium. Chromium solution contains a larger number of $Cr_2O_2^{7-}$ ions and a smaller number of $HCrO^{-4}$ ions in the regions of lower pH and only CrO_2^{-4} ions above pH 8.0. In the pH range 3 to 6, the equilibrium shifts to dichromate according to the overall equilibrium, $2CrO_2^{-4} + 2H+ = Cr_2O_2^{7-} + H_2O$. A major fraction of negative sites are occupied by H^+ ions via electrostatic attraction in the regions of lower pH and these positively charged sites of the adsorbent are occupied by $Cr_2O_2^{7-}$ ions [19]. Hence the maximum chromium removal was observed at lower pH i.e. 2. Higher removal of chromium at low pH may also be due to reduction of chromium (VI) to chromium (III) [20, 21], which was then adsorbed by the adsorbent.

3.4. Effect of initial metal ion concentration

Effect of initial Cr (VI) ion concentration on its removal was carried out at optimized adsorbent dose, contact time and pH by varying the metal ion concentration from 10-50 ppm **[Figure–4]** Adsorption of Cr (VI) was found to decrease with increase in metal ion concentration from 10-50 ppm. This is due to increase in number of metal ions competing for available binding sites and due to lack of binding sites for complexation at higher metal ion concentration. At lower concentration almost all the metal ions could interact with binding sites facilitating maximum adsorption [22] at 10 ppm concentration i.e. 88% and 83 % for ACS (0.3 mm and 1.0 mm) while 79% and 75% for ACC (0.3 mm and 1.0 mm). Maximum Cr (VI) removal was observed at 10 ppm concentration using low cost adsorbents [23]. At higher concentration more chromium ions are left unadsorbed in the solution due to saturation of adsorption sites [24].

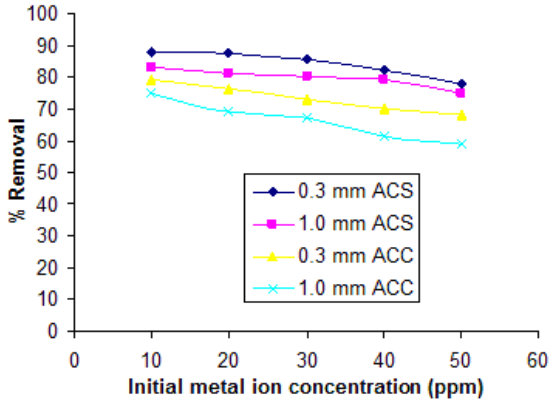

Fig. 4: Effect of initial Cr (VI) ion concentration on removal using activated coconut shell and activated coconut coir

3.5. Industrial feasibility of adsorbent

The industrial feasibility of ACS and ACC was studied for the removal of hexavalent chromium from tannery effluent at optimized conditions obtained from experiments on synthetic solution in batch mode. The adsorption efficiency of the adsorbent was reduced from 85 % to 76% and 80.2% to 66% in case of ACS for 0.3 mm and 1.0 mm respectively. Similarly, in case of ACC, adsorption efficiency decreased from 75% to 67% for 0.3 mm and 72 % to 59 % for 1.0 mm adsorbent particle size. This reduction in percent removal may be due to presence of co-metal ions present in industrial effluents [25].

3.6. Adsorption isotherms and kinetics

The Langmuir and Freundlich adsorption models were used for mathematical description of adsorption of Cr (VI) ions and isotherms constants were determined to find out the adsorption capacity of adsorbent. As shown in **Table–1 and –2**, the value of correlation coefficients $R^2 \geq 0.8$ 0 indicated that the adsorption data are well fitted in Langmuir and Freundlich models. The values of n more than 1 for all the adsorbents indicated that significant adsorption takes place at low concentration of the adsorbent.

Table: 1. sotherm model constant and correlation coefficient for adsorption of hexavalent chromium

	Langmuir constant (mg/g)			Freundlich Constant (mg/g)		
Adsorbents	**Qo (mg/gm)**	**B**	**R^2**	**K**	**n**	**R^2**
ACS (0.3 mm)	2.337	0.761	0.9256	14.09	6.067	0.8962
ACS (1.0 mm)	5.319	0.1332	0.3622	25.61	3.577	0.4958
ACC (0.3 mm)	3.409	0.387	0.690	194.5	4.466	0.6955
ACC (1.0 mm)	7.849	0.0778	0.1508	11.24	2.33	0.216

Table: 2. Kinetics model constant and correlation coefficient for adsorption of hexavalent chromium

	Pseudo first order		Pseudo second order		
Adsorbents	**K_d**	**R^2**	**qe**	**K_2**	**R^2**
ACS (0.3 mm)	0.0495	0.8291	10.384	0.2239	0.9961
ACS (1.0 mm)	0.0479	0.945	10.2459	0.04488	0.9951
ACC (0.3 mm)	0.0439	0.9717	7.5988	0.0855	0.9966
ACC (1.0 mm)	**0.0231**	**0.9737**	**7.2727**	**0.9705**	**0.9986**

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The pseudo-first order and second order kinetics models were successfully employed for explaining the kinetics data of adsorption process. Straight line obtained after plotting Log ($q_e - q_t$) vs t and t/qt vs t shows degree of fitness of metal sorption to first and second order rate kinetics model. This is based on the assumption that the adsorption capacity for metal on the adsorbent is proportional to the number of active sites occupied on the sorbent and metal uptake is due to chemisorption [14]. The values of constants K_d and R^2 were calculated from the plots. The pseudo-first order equation was able to describe the adsorption of Cr (VI) onto both forms of adsorbents as evidenced from the coefficient of determination values (R^2> 0.90).

[V] CONCLUSION

The adsorption of hexavalent chromium was observed to pH, adsorbent dose, contact time and initial metal ion concentration dependent .ACS can remove more Cr (VI) as compared to ACC from its synthetic solution and tannery effluent at optimized conditions. Both activated coconut shell and Activated coconut coir were found suitable adsorbents for the removal of Cr (VI) from its synthetic solution and tannery effluent and may also have wide applicability. The adsorption efficiency of both forms of adsorbents of particles size 0.3mm for Cr (VI) is also well evident from the coefficient of determination values (R2> 0.90). The coconut shell and coconut coir are abundantly available as an agro waste which makes this technology industrially feasible and economically cheaper.

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RESEARCH ARTICLE

ALUMINA AND CHITOSAN TYPE BIO-ADDITIVE MODIFIED ELECTRONIC INDUSTRY WASTE SLUDGE FOR HEAVY METAL STABILIZATION BY MICROWAVE HEATING

Rajabathar Jothiramalingam[1] and Shang Lien Lo[2]
[1] *School of distance education, University Sains Malaysia, MALAYSIA*
[2] *Graduate Institute of Environmental engineering, National Taiwan University, Taipei, TAIWAN*

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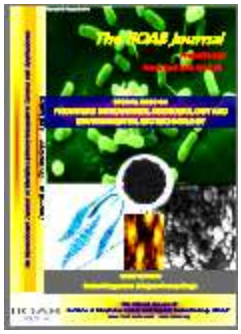
Corresponding author: Email: rjothi@usm.my or rjothiram@gmail.com Tel: +60124249642; Fax: +6046575657

ABSTRACT



Chitosan type biopolymer is used by several researchers recently for biosorption application and dye removal in aqueous solutions. In this study, Alumina and chitosan type naturally available low cost material is used as an alternate bio-additive to stabilize the leaching toxic heavy metal ions in industrial sludge. Cement solidification is used as the common method to stabilize the industrial waste water sludge at Taiwan. However, this method has the disadvantage of an increase in waste volume. The effects of additive dosage amount, power of microwave irradiation and reaction time are studied in the present work. Heavy metal leaching capacity is determined by using standard TCLP (Toxicity Characteristic Leaching Procedure) test and elemental content in the leachate is analyzed by ICP (Inductively Coupled Plasma). Alumina and chitosan is effectively stabilizing the copper ions and control the leaching of metal ion in the presence of microwave irradiation. The optimized additive mixing in industrial sludge control the copper ions and other heavy metal ion leaching <5 mgL^{-1} upon microwave heating process in nitrogen atmosphere instead of passing air. The complete stabilization achieved for chromium and cadmium (not detected after TCLP test) compared to other heavy metal ions leaching. Finally, heavy metal ion stabilized sludge is tested for dye discoloration efficiency. Chitosan modified sludge shows the complete discoloration for the dye compounds such as methylene blue and acid red 114.

Keywords: *chitosan, microwave heating process; sludge stabilization; TCLP*

[I] INTRODUCTION

Chitosan type naturally available low cost biomolecule such as synthetic biopolymer is used by several researchers for industrial wasterwater treatment and dye removal reaction in aqueous solutions [1-3]. Chitosan is derived by deacetylation of the naturally occurring abundant polysaccharide in the world after cellulose and its show the outstanding binding behavior for pollutants removal such as dye and heavy metal ions [4]. Electronic industrial wastewater sludge with copper and other heavy metal ion content produces in a large amount of hazardous waste at Taiwan from industrial sources. Even though most of the copper ions in sludge are reclaimed by the acid-extraction process with sulfuric acid, the concentration of copper ions in the residue after the toxicity characteristic leaching procedure (TCLP) test may exceed 15 mg L^{-1}. Hence, acid-extracted industrial sludge still needs to be stabilized for heavy metal ion leaching problem. The common method for stabilizing the hazardous waste at Taiwan is cement solidification process. However, this method has the disadvantage of an increase in production of waste volume [4-7]. Hsiau and Lo [8] reported the heavy metal extractability and leacheability characteristics of chemically fixed sewage sludge and the copper and other heavy metal ions are shown the higher affinity with organics except zinc [8]. Chen et al. (2007) recently reported the various modified procedure for sludge stabilization and solidification process with the assistance of hybrid microwave irradiation heating process followed by conventional heating process [9].

Compared with other conventional thermal treatment technologies, the microwave technique with the characteristics of polar oscillation and effect of dielectric losses offers the

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advantage of selective, uniform and rapid heating [10]. The effect of microwave irradiation and cooling gas treatment in microwave heating process to stabilize the industrial sludge was studied in detail.[11] Sodium sulfide and sodium phosphate type chemical additive are used to stabilize the heavy metal ion for industrial sludge by adopting different methodology with the assistance of microwave heating as well as conventional heating effects in the past years [12-13]. Heavy metal stabilization by spinel structure formation on industrial waste sludge by suitable metal precursor and heating conditions are currently growing field of interest [14].

In order to understand the effect of additive property of alumina and chitosan to find out the optimized dosage for heavy metal stabilization in industrial electronic sludge in presence of microwave irradiation, experiments regarding the required dosage of additive, required reaction time and reaction conditions. Further, in order to reduce the use of cooling gas, several modified hybrid microwave processes were performed. The stabilization efficiency of additive modified industrial sludge is evaluated by analyzing the filtrate of the sample by US-EPA TCLP test and ICP analysis for determine the heavy metal concentration in the leachate.

[II] MATERIALS AND METHODS

2.1 Basic nature of the sludge

Sludge used for the experiments was the residue of electronics industry sludge that had gone through an acid-extraction process with sulfuric acid and from which most of the copper ions had been reclaimed. The acid-extracted sludge was dried at 105 °C until all moisture was removed. The particle size of the sludge was between 100 and 400 mesh after being crushed by a grinder. Powdery sludge was stored in a tightly closed 20 L bucket. Commercial chitosan was purchased from a chemical company in Taipei, Taiwan, and used without further purification.

2.2 Methodology and instrumentation

The potential maximum power of the industrial microwave oven employed in this study was 1600 W. A simplified diagram of the microwave oven is shown elsewhere.9 The valve on the top of the oven is to let gas out. The valve on the right is for addition of gas and was connected to a float flow meter to control the flow rate of gas. The valve on the left was connected to a vacuum pump that can provide near-vacuum conditions in the oven. A container filled with the prepared sample was placed in the center of a flat tray at the bottom of the oven. The The stabilization efficiency of additive-modified industrial sludge was evaluated by analyzing the filtrate of the sample using the U.S. Environmental Protection Agency TCLP test, and inductively coupled plasma (ICP) analysis was used to determine the heavy metal ion concentration in the leachate. Various amounts of chitosan (1.0-6.0 g) were mixed with 40 g of industrial sludge and 50 mL of deionized (DI) water for each experiment. After samples had undergone the abovementioned experimental treatments, 5.0 g of each sample was subjected to the TCLP test. The required amount of 1.0 M acetic acid solution with a pH value of 2.88 (0.05 is added with a suitable amount of sludge. The ratio of liquid to solid was 20: 1, and the rotation frequency was 30 rpm. After an extraction time of 18 h, the leachate was filtered, and the filtrate pH was adjusted to below 2. The filtrate was immediately analyzed with an inductively coupled plasma (ICP) analyzer (Jobin Yvon JY24) to prevent the formation of colloid and sediment.

[III] RESULTS AND DISCUSSION

3.1. Hybrid microwave process on a- and γ-alumina additive modified sludge

The heavy metal leaching capacity after the TCLP test for α and γ-alumina modified industrial sludge is shown in **Supplementary Table–1**. Both the microwave heating as well as conventional furnace heating effect was studied. The microwave heating time is fixed at 9 min at 600 W, after completion of microwave heating process the samples were heated further by conventional oven at two different temperatures separately such as 800 ^{o}C for one set of sample and another set of samples heated directly at 1000 ^{o}C for 6 h. The X-ray diffraction pattern of alumina modified sludge materials are studied by our group recently and the same were reported elsewhere [15]. The intensity of the peak is slightly decreased for α-alumina modified sludge samples compared to all other sample. The α-alumina modified sludge, heated at 800 ^{o}C shows the increased leaching concentration for copper ion compared to 1000 ^{o}C heat treated sample. Hence, the above point concluded that the increase in heating temperature by conventional method after the microwave heating, impact the heavy metal leaching capacity in industrial sludge.

In case of γ-alumina modified industrial sludge heated separately at 800 and 1000 ^{o}C are shown in **Supplementary Table–1**, efficient heavy metal stabilization was observed and no zinc leaching was observed. The increasing in dosage amount above 1.0 g of γ-alumina addition causing the overloading effect and results in leaching of heavy metal ion after TCLP test. The barium manganate modified sludge was mixed with 1.5 g of α and γ-alumina separately and heated in conventional heating furnace at two different temperatures **[Supplementary Table–1]**. The TCLP test of modified sludge samples are showing complete stabilization for copper and other heavy metal ions upon hybrid microwave process. The overall observation concludes that the appropriate amount of γ-alumina (1.0 g) addition with industrial sludge in presence of microwave and conventional heating process shown the efficient stabilization for industrial sludge. The γ-alumina modified industrial sludge shown the effective result for copper ion stabilization in industrial sludge in terms of less reaction time and decrease in waste volume compared to aluminum powder usage in industrial sludge treatment. The textural property and surface area of the γ-alumina is the main reason for the effective stabilization of copper and other heavy metal ion in industrial sludge compared to alumina powder mixing.

3.2. Effect of bioactive, dosage amount for electronic industry waste sludge on copper stabilization and dye decoloration activity

Effects of bioadditive, dosage amount and microwave power for copper stabilization in industrial sludge by microwave heating. **Supplementary Figure–1a** shows the copper leaching amount

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(mg L^{-1}) against different dosage of chitosan addition and **Supplementary Figure–1b** shows the effect of microwave heating time against copper leaching for modified industrial sludge at two different microwave power such as 600 W and 800 W. The increasing amount of chitosan addition decreases the copper leaching at optimized additive addition such as dosage amount, microwave power and reaction time. The lowest leaching concentration of about 2.52 mg L^{-1} was observed at 4.0 g of chitosan modified sludge treated at 800 W for 9 min and increasing the dosage amount of chitosan above 4.0 g results the copper leaching occurred **[Supplementary Figure–1a]** due to overloading of additive mixing in sludge and chitosan particle aggregation occurred. The higher microwave power like 800 W shows the effective control of copper leaching compared to low power microwave heating **[Supplementary Figure–1]**. The lowest copper leaching concentration of 4.09 mg L^{-1} is observed for 800 W microwave treatment at 6 min **[Supplementary Figure–1b]**. Hence, chitosan type bioadditive could control the copper leaching in industrial sludge by adopting optimized reaction condition such as 800 W power microwave heating at 6 to 9 min reaction time. Increasing the reaction time such as above 12 min at high power (800 W) microwave heating could cause the leaching problem.

Supplementary Table–2 shows heavy metal leaching concentration for various metal ions after the TCLP test and chitosan is completely stabilize the chromium and cadmium at 1.0 - 6.0 g of chitosan addition in sludge. Other major heavy metal ions like Zn, Cr, Cd and Ni are also shown the effective control for heavy metal leaching from sludge. The dosage amount between 4.0 g to 6.0 g of chitosan with sludge after the microwave heating shows the effective control over heavy metal ions leaching.

The modified sludge (microwave method treated heavy metal ion stabilized in chitosan mixed industrial sludge) is tested for possible sorption application related with hazardous dye discoloration experiment. Dye removal and discoloration process in the presence of biosorbent is the important topic related with wastewater treatment released from textile and chemical industries. Hence, the chitosan modified industrial sludge could act as a low cost biosorbent for dye removal as well as discoloration of the toxic dye compounds. In present study, methylene blue and acid red 114 are tested for dye discoloration activity. **Supplementary Figure–IIb** shows the filtrated solution of aqueous dye solution of mehtylene blue and acid red dye discoloration activity after 30 min of sorption experiment, increasing the sorbent weight causes the complete dye discoloration at very short time duration, due to the presence of chitosan particle in the bulk solid sludge. Chitosan itself is a popular sorbent for dye removal in wastewater treatment experiments. Hence chitosan modified sludge is tested for dye discoloration and it shows the better activity **[Supplementary Figure–IIa and IIb- No.2-5]** compared to untreated sludge **[Supplementary Figure–IIa and IIb- No.1]**. The maximum absorption wavelength for methylene blue and acid red 114 are measured at 665 nm and 522 nm, respectively. In the sorption experiment every 10 min dye solution was removed and continued the process upto 30 min and adsorbed dye solution filtered followed by analysis of the filtrate by spectrophotometer. Initially the dye adsorption efficiency is more and discoloration is very faster at the initial 10 min period and equilibration attained with in 30 min. Dye discoloration is more efficient in presence of chitosan modified materials compared to raw industrial sludge and **Supplementary Figure–IIa and IIb** shows the comparison of various amounts of sorbents in presence of dye solution.

3.3 Impact of heavy metal binding mechanism with chitosan modified bulk sold sludge

Chitosan is a basic polymer with functional groups like amino and hydroxyl groups present in its structure are playing a key role in Lewis acid base type reaction in aqueous medium. Chitosan, being a base, forms salts with acids and originates polyelectrolyte with a solubility that is a function of the nature of the anion involved and deacetylation degree. Ngah and Musa [16] reported that the adsorption of organic matter over chitosan takes place in the amino groups forming coordinated bonds, but to our knowledge just a few studies have been conducted, to understand the roll of NOM in the removal and selectivity of metal ions (such as cadmium, copper, and lead) by this biosorbent [16]. For instance, Yan and Bai [20] studied the adsorption capacity of lead onto chitosan hydrogels beads and reported that amine groups in chitosan were found to play the major role in the adsorption of lead ions or humic acids (HA), and when these two were simultaneously adsorbed their removal was significantly lower [17]. In our present work chitosan is mixed with sludge in aqueous medium and results in the possible chitosan transformation and binding reaction with heavy meat ions in the bulk sludge *via* co-ordination bond formation might occurred. The pH of the medium is playing the important role in formation of heavy metal ions binding on chitosan molecules. Several functional groups present in the chitosan molecules is involve in the possible Lewis type acid base reaction in aqueous medium with modified sludge [18]. After the microwave treatment by uniform heating effect facilitate the chitosan-heavy metal ion binding for stabilization process in the bulk sludge.

[V] CONCLUSION

The chitosan type bioadditive has been tested and successfully adopted for copper and other heavy metal ion stabilization in industrial sludge for the first time and the present methodology is not reported elsewhere. The methodology for mixing chitosan with industrial waste sludge has been developed to control the leaching of copper and other heavy metal ions. The chitosan addition dosage of 4.0 g per 40 g of solid sludge in microwave heating for 12 min at 800 W had shown the best stabilization effect for heavy metal ions. The microwave treatment at 600 W is not effective at shorter time duration (3 min) with less amount of chitosan, in the case of 800 W microwave heating treatment at longer time (15 min) duration with less amount (1.0 g) chitosan

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shows the effective stabilization for heavy metal ion in waste sludge. Hence the present study concludes that the chitosan type bio-additive mixed industrial sludge stabilize the copper and heavy metal ions effectively as well as used as an possible dye sorbent applications.

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ABOUT AUTHORS

Dr. R. Jothiramalingam, *M.Sc. Ph.D., UNIDO Fellow, is expertise in the field bio-chemical technology and catalysis for biofuel and biobased product development. Presently working as a visiting scientist at school of distance education, University Sains Malaysia, Penang, Malaysia. He earned his PhD (2005) from Department of chemistry, Indian Institute of Technology, Madras. In 2009 he received research fellow award from ICS-UNIDO, Italy.*

Prof. Lo, *is Distinguished professor at Graduate school of environmental engineering and Director of Pollution prevention and control technology at National Taiwan University, Taipei. His research area includes water quality treatment, solid waste management and nanoscience for chemical and biotech applications.*

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SUPPLEMENTARY FILES

Supplementary Table: 1. **Heavy metal leaching concentration of microwave heat treated alumina modified industrial sludge after TCLP test**

Sludge (S)-additive Composite	pH	Zn[a]	Cr[a]	Cd[a]	Pb[a]	Ni[a]	Mn[a]	Cu[a]
At 800 ° C								
S-α-alumina (0.5 g)	5.04	4.6	0.9	1.3	6.2	5.1	2.8	25.1
S-α-alumina (1.0 g)	5.60	0.9	0.6	1.3	5.8	6.0	3.9	1.8
S-α-alumina (1.5 g)	4.63	4.6	0.7	1.3	6.7	3.4	1.9	60.6
S-γ-alumina (0.5 g)	7.43	-	0.9	1.2	7.0	3.9	3.6	0.7
S-γ-alumina (1.0 g)	7.15	-	0.7	1.3	6.3	3.1	4.0	0.2
S-γ-alumina (1.5 g)	5.32	-	0.6	1.2	5.6	3.9	3.6	0.5
At 1000 ° C S-α-alumina (0.5 g)	4.32	0.2	0.7	1.2	5.7	2.9	2.3	10.0
S-α-alumina (1.0 g)	4.37	-	0.7	1.2	5.6	2.3	1.7	5.7
S-α-Alumina (1.5 g)	4.40	-	0.6	1.2	5.6	2.3	1.7	5.3
S-γ-alumina (0.5 g)	5.70	-	0.6	1.2	5.7	2.9	4.1	5.1
S-γ-alumina (1.0 g)	5.68	-	0.7	1.3	5.8	2.9	4.3	6.2
S-γ-alumina (1.5 g)]	4.59		0.9	1.2	5.4	2.8	2.9	11.0

[a] all the metal ion concentration in mg L^{-1}; detection limit error ±0.05

Supplementary Table: 2. **Effect of chitosan dosage on copper and other heavy metal leaching control in industrial sludge**

Additive Amount (g)	Zn	Cr	Cd	Pb	Ni	Mn	Cu
1.0	29.3	0.31	0.57	3.2	6.9	7.3	27.64
2.0	28.3	0.24	0.54	2.5	6.2	6.1	23.11
3.0	28.4	0.27	0.99	2.9	6.1	6.7	18.05
4.0	27.5	0.30	0.51	3.1	3.3	4.3	2.52
5.0	0.65	-	-	0.89	2.7	2.5	6.90
6.0	1.28	-	-	1.52	2.8	2.2	10.90

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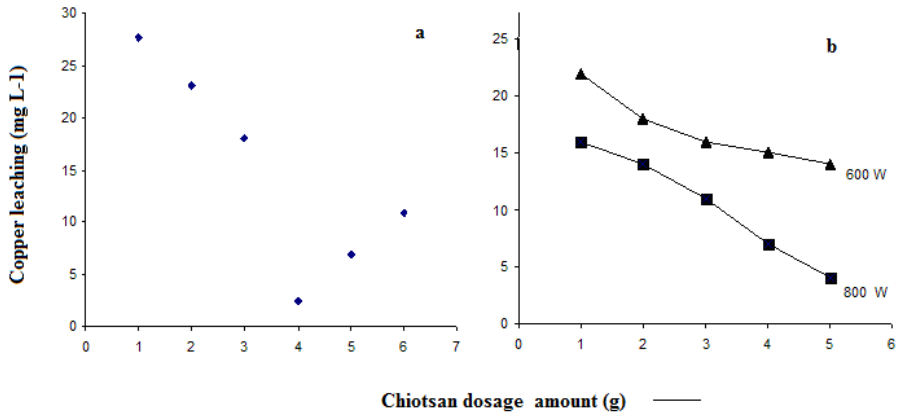
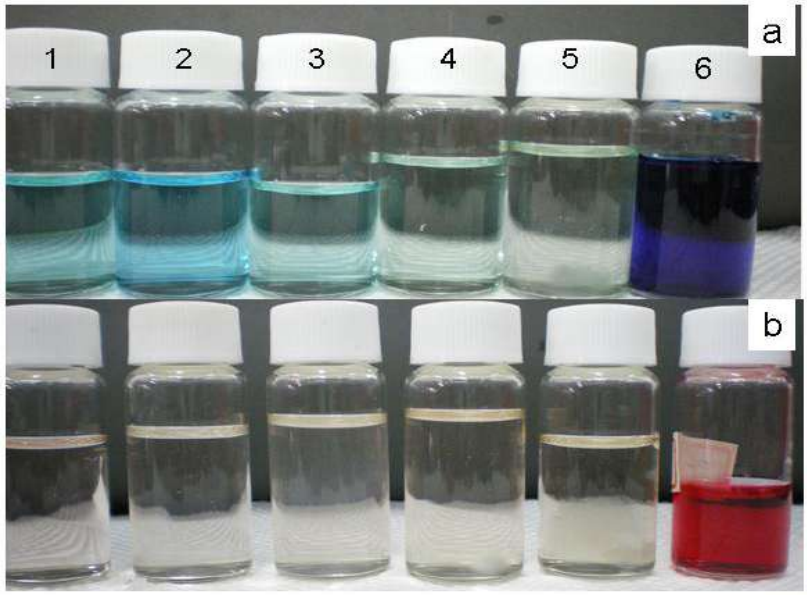


Supplementary Figure (Ia) Effect of chitosan dosage amount (1 -6 g) for copper leaching control in industrial sludge **(Ib)** Effect of microwave heating power on copper leaching control.



Supplementary Figure (IIa) No. 1- 1.0 g of unmodified sludge; No. (2 – 5) - 1.0 g – 4.0 g of modified sludge ; No, 6- methylene blue solution (100 mg L^{-1}) **(IIb)** No. 1- 1.0 g of unmodified sludge ; No. 2- 5-1.0 g – 4.0 g of modified sludge ; 6-Acid red 114 (100 mg L^{-1})

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RESEARCH ARTICLE

KERATINASE ACTIVITY OF DERMATOPHYTES AND YEAST SPECIES FOR POULTRY WASTE AND WASTE WATER TREATMENT

Richa Sharma[1*] and Meenakshi Sharma[2]
[1]*Dept. of Biotechnology and Allied Sciences, Jayoti Vidyapeeth Women's University, Jaipur 303007 Rajasthan, INDIA*
[2]*Dept. of Botany, University of Rajasthan, Jaipur 302004 Rajasthan, INDIA*

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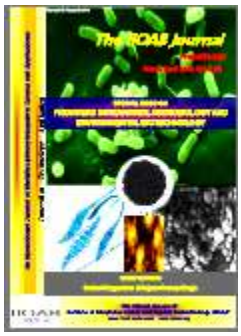
[*]**Corresponding author:** Email: richa.phd.15@gmail.com

ABSTRACT



Dermatophyte and yeast species have been screened for their degradative ability towards various keratin substrates. Application of soluble preparation of keratin (KS) of chicken feathers enables a preliminary evaluation of the growth of the fungi and screening of fungal isolates that possesed keratinolytic activity and keratinase enzyme. Five dermatophytes i.e. Trichophyton rubrum, Trichophyton mentagrophytes, Microsporum gypseum, Microsporum canis and Chysosporium tropicum and two yeasts i.e. Candida albicans and Malassezia furfur were used in this study for keratinase activity. Out of five dermatophytes species and two yeast species studied, all tested fungal species showed a keratinase activity except one species i.e. M.canis. From findings, the results provide a scientific validation for the use of these microorganisms (dermatophytes and yeast species) for keratinase enzyme activity for the treatment of poultry waste and sewage waste water treatment.

Key words: *keratinase; enzyme; keratin; sewage; dermatophytes; yeasts*

[I] INTRODUCTION

Feather is composed of over 90% protein, the main component being keratin, a fibrous and insoluble protein highly cross-linked with disulphide and other bonds. The feather can be hydrolysed by keratinase which is a proteolytic enzyme specific to keratins [1]. The keratinase enzyme is a potential enzyme for removing hair and feather in the poultry industry [2]. This enzyme has been produced by fungi, including the species of Aspergillus, Onygena, Absidia and Rhizomucor. Some species of dermatophytes, including *Trichophyton mentagrophytes*, *T. rubrum*, *T.gallinae*, *Microsporum canis* and *M.gypseum* [3]. Keratinophilic fungi was also screened for their degradative ability towards various keratin substrates like hair, nails, feathers etc [4]. Suntornsuk and Suntornsuk reported growth and efficient utilization of feather by Bacillus sp. FK 46 with release of 0.9 unit/ml of keratinase [5]. Doratomyces microsporus also produce keratinase enzyme and degrade skin epidermis in vitro under different experimental conditions [6]. Hydrolysis of feathers by microorganisms possessing keratinolytic activity represents an attractive alternatives method for improving the nutritional value of feather meal, compared to currently used physiochemical methods [7]. Most of the fungi exhibited variable efficiency in producing extracellular keratinase when grown in plates with chicken feathers as the sole carbon and nitrogen source [8]. In the present study, keratinase activity of dermatophytes and yeast species were investigated with the aim to use the keratinase enzyme for various biotechnological applications like removal of hair and feathers in leather and poultry industries, in sewage system for cleaning obstructions during waste water treatment and digestion of abundant waste generated from poultry processing industries.

[II] MATERIALS AND METHODS

Keratinase activity or degradation of keratin substrate (chicken feathers) by keratinolytic fungi was studied by agar plate method [3].

2.1. Keratin media

The keratinolytic properties of the fungi were examined on a solid mineral medium according to Wawrzkiewicz et al. (1991) [3]. The media

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was supplemented with the keratin substrate as the sole sources of carbon and nitrogen. Chicken feathers or a soluble preparation of keratin protein of chicken feathers described as KS (Soluble keratin) [3].

2.2. Preparation of keratin substrate

Preparation of soluble keratin protein is constituted from white chicken feathers where 10 gms of native chicken feathers where 10 gm native chicken feathers is dissolved with 500ml of DMSO (Dimethylsulfoxide) by heating at 100C temperature for 2 hours. Solution is precipitated for soluble protein (keratin) by using 2 volume of cold acetone for 1 volume of protein and then caseous precipitate of keratin protein was suspended in 0.1 M of phosphate buffer. Afterwards, soluble keratin protein was added in the solid media at a concentration of 0.06%. Solid media were inoculated with micropipette, injecting 1ml of standard suspension into centre of the petriplate and petriplates were incubated at 37ºC at an optimum pH 7.4 for keratinase activity. Control plates were prepared containing above medium without keratin substrate. Formation of precipitate zones around the colonies indicate keratinase enzyme production and zone was examined and measured.

[III] RESULTS

Keratinase activity of dermatophytes and yeasts were studied under in vitro laboratory conditions on the solid mineral medium incorporated with soluble preparation of keratin protein (KS). Keratin substrate used for in vitro degradation was chicken feathers. Five dermatophytes i.e. *T. rubrum*, T. mentagrophytes, M. gypseum, M. canis and C. tropicum and two yeasts i.e. C. albicans and M. furfur were used in our study for keratinase activity. All fungal isolates used in our study, grew on the solid mineral medium containing 0.06% of keratin protein (KS) with 14 days of incubation. Out of five dermatophytes species and two yeast species studied, all tested fungal species showed a keratinase activity except one species i.e. *M. canis* **[Table– 1 and Figure–1]**.

Table: 1. Keratinase activity of fungal isolates on solid mineral medium

Fungal species	Incubation		
	3	10	14
C. albicans	12*/40**	12*/40**	12*/40**
M. furfur	10*/40**	13*/47**	13*/47**
T. rubrum	-	20*/0**	20*/45**
T. mentagrophyts	20*/0**	20*/26**	22*/49**
M. gypseum	-	20*/34**	47*/65**
M. canis	-	-	20/0
C. tropicum	10*/20**	15*/25**	40*/60**

*Indicates fungal colony diameter; ** Indicates diameter of the zone of keratin solubilization; - Indicates no growth of the fungus; All numbers represent the average of triplicates; Control showed growth of selected test organisms but not cleared zone around fungal colonies

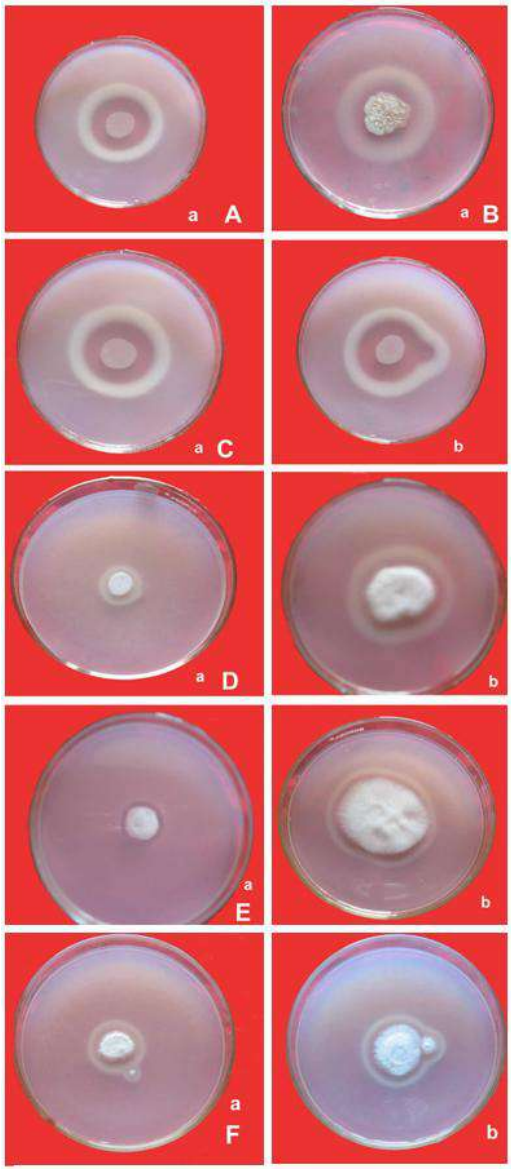

Fig: 1.keratinase activity of fungi. A. *C. albicans:* (a) at third, tenth and fourteenth day of incubation. **B.** *T. rubrum*: (a) at fourteenth day of incubation. **C.** *M. furfur*: (a) at third day of incubation, (b) at tenth and fourteenth day of incubation. **D.** *T. mentagrophytes*: (a) at tenth day of incubation, (b) at fourteenth day of incubation. **E.** *M. gypseum*: (a) at tenth day of incubation, (b) at fourteenth day of incubation. **F.** *C. tropicum*: (a) at third day of incubation, (b) at tenth and fourteenth day of incubation.

It was found that, the application of soluble preparation of keratin (KS) of chicken feathers enables a preliminary evaluation of the growth of the fungi and screening of fungal isolates that possesed keratinolytic activity and keratinase enzyme. C. albicans also showed a good keratinase activity. On third day of incubation colonies of *C. albicans* (12 mm in diameter) were surrounded by broad zone of (40 mm) of

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degraded keratin. Within an extended period of incubation (10 and 14 days), no change in the zone diameter was observed. M. furfur also showed good keratinase activity when compared with C. albicans. Colonies (about 10 mm in diameter) was surrounded by broad zone of (40 mm) of degraded keratin within three days of incubation. At extended incubation period (10 and 14 days) clear zone of 47 mm was found. *T. rubrum* showed keratinase activity on a solid mineral medium within incubation of 14 days. No growth of *T. rubrum* was observed on medium at third day of incubation. *T. rubrum* showed 20 mm diameter of fungal colony and no degradation on tenth day but on fourteenth day of incubation, *T. rubrum* colony (20 mm in diameter) was surrounded by a broad zone (45 mm in diameter) of degraded keratin, thus indicating an active secretion of keratin decomposing enzymes to the medium. T. mentagrophytes showed colony diameter of 20 mm at third day of incubation but clear zone of keratin degradation was not reported within 3 days but after on tenth day of incubation, colonies of (20 mm in diameter) surrounded by clear zone of (26 mm) diameter of degraded keratin was observed and on fourteenth day of incubation large broad zone (about 49mm in diameter) of keratin degradation was found. Formation of clear zone around fungal colonies was reported on both tenth and fourteenth days of incubation. Zone of keratin degradation of fourteenth day of incubation was broader and larger than tenth day of incubation period. Growth of M. gypseum was not observed on third day of incubation. At tenth day of incubation, both growth and clear zone of degradation was observed. Colony diameter of 20 mm surrounded by narrow clear zone of 34 mm was found and within fourteenth day of incubation, large broad zone of 65 mm was reported when compared with tenth day of incubation (34 mm in diameter). M. canis showed no growth on solid mineral medium at third day and tenth day of incubation. On fourteenth day of incubation, growth of 20 mm in diameter was reported but clear zone of keratin degradation was not observed around fungal colony. Thus indicating an absence of active secretion of keratin decomposing enzymes into the medium. The excellent keratinolytic activity was observed in C. tropicum. Within 3 days, this fungi grew on solid medium and also showed clear zone of 20 mm. Further increase in incubation period of 10 days, showed a higher zone of degradation (25 mm in diameter) than third day of incubation. On fourteen days of incubation, highest zone of keratin degradation of about 60 mm in diameter was reported. In the present study, increased keratinase enzyme activity for C.albicans, M.furfur, T.rubrum, T.mentagrophytes, M.gypseum and C.tropicum was found at optimum temperature i.e. 37±2°C and pH 7.4.

[IV] DISCUSSION

In this study, degradation of keratin substrate (chicken feathers) by dermatophytes and yeasts species were studied. It was found that, M. gypseum (65 mm in diameter) showed highest keratinase activity followed by C. tropicum (60 mm), T. mentagrophytes (49 mm), M.furfur (47 mm), *T. rubrum* (45 mm) and C. albicans (40 mm). In our study, all the tested fungal isolates showed a diameter of clear zone of keratin degradation within range of 40-65 mm and possessed good keratinase activity except one species, M. canis which showed growth on solid mineral medium but clear zone of keratin degradation was not found by further increase of incubation period from 14 days to 21 days. This reflected the fact that keratin is the main substrate for dermatophytes and therefore these fungi are called keratinophilic.

The examined species of dermatophytes and non dermatophytes (yeasts) were both keratinase procedures and they are apparently capable of damaging the keratinized structure of the skin as previously reported [3]. The high keratinase activity of *M. gypseum* in comparison with other related fungi explain their ability to invade chicken feathers and keratin degradation. This was in agreement with work of earlier workers [9,10] which revealed that *M.gypseum* showed highest keratinase activity among examined dermatophytes and non-dermatophytes.

The present experiments indicate a possibility of appearing modified keratin of chicken feathers / KS (Soluble keratin) as a very useful model for a preliminary estimation of keratinolytic activity of dermatophytes. This keratin, introduced as a source of carbon and nitrogen to the mineral agar medium, allows a quick selection of active strains. Native keratin contained in hairs or feathers did not constitute such a universal source of C and N for dermatophytes as the preparation of KS employed in our experiments. The few strains degrading keratin of guinea pig hair included strains of T.verrucosum and T. mentagrophytes of a wide infections spectrum [3]. In our studies, T. mentagrophytes also produced high keratinase activity (49 mm).

These results are also similar to Muhsin et al. [10] which revealed that the three tested varieties of T. mentagrophytes showed high keratinase activity. Sharma (2009) also reported that maximum keratinase (2.57± 0.028 unit/ml) was released from T.mentagrophytes when 35ºC temperature was provided [11]. In case of M. canis, keratinase activity was not detected. These result are in agreement with Wawrzkiewiez et al. [3] where M. canis was found negative for keratinase activity but disagree with the results of Muhsin et al [10] who reported (15 mm) zone of precipitation around fungal colony of *M. canis* [4]. Chrysosporium tropicum was also found in this study superior for keratinase production, forming zone of 60 mm after 14 days of incubation at 37°C. These results was in agreement with El-Naghy et al. (1998) [12] who reported that the Chrysosporium georgiae possessed high keratinase activity and completely degraded the added keratin after 9 days of incubation.

Moreira et al. (2007) [13] investigated, degradation of keratinous materials by the plant pathogenic fungus Myrothecium verrucaria using poultry feathers as the only substrate. According to da Gippo et al. (2009) [14], the association of two residues poultry feather powder (PFP) plus cassava bagasse could be an excellent option as a cheap culture

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medium for the production of keratinase in submerged and solid state cultures. New feather degrading filamentous fungi was studied by Rodrigues Marcondes et al. (2008) [15]. The study found that the highest keratinolytic activity were produced by Alternaria tenuissima after 4-6 days of cultivation in submerged conditions followed by *Acremonium hyalinulum, Curvularia branchyspora, Beauveria bassiana*. The results of this work contribute to showed that keratinolytic activity was relatively widespread among common filamentous fungi and may have an important role in feather decomposition. According to Gupta and Ramnani (2006), microbial keratinases have become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptide "keratin" recalcitrant to the commonly known proteolytic enzymes trypsin, pepsin and papain [16]. These enzyme are largely produced in the presence of keratinous substances in the form of hair, feather, wool, nail, horn etc. during their degradation. Raju et al. (2007) [17], in their studies, clearly indicates the presence of enzyme keratinases in the dermatophyte Microsporum gypseum and found that maximum biomass and keratinase activity was observed at pH 8 and at 35°C. Muhsin et al. (1997) [10] also found in their studies that *T. rubrum* and C. albicans also showed good keratinase activity which was coincides with the results of our present work. *T. rubrum* and C. albicans produced clear zone of 45 mm and 40 mm in our studies. Peyton and Weary (1968) found that M. furfur capable of degrading keratin. work agree with their studies. M.furfur showed positive keratinase activity but disagree with Muhsin et al. who reported negative keratinase activity in M. furfur. Difference in the properties of keratinases in particular strains of dermatophytes have been noted by Takiuchi et al[18].

[V] CONCLUSION

From the results, it is evident that dermatophytes and yeast species were capable of producing keratinase enzyme. Finally this study suggests that microorganisms (dermatophytes and yeast species) can be used to degrade keratin instead of using physiochemical methods for the removal of wastes of poultry industries and for cleaning obstructions in sewage waste water treatment.

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RESEARCH ARTICLE

DEINKING OF NOTE BOOK PAPERS AND EFFUENT DEGRADATION USING ASPERGILLUS FUMIGATUS

Parkavi and Priya R Iyer*
Department of Biotechnology, Women`s Christian College, Chennai-600006, INDIA

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***Corresponding author:** Email: brajuraj@yahoo.com

ABSTRACT

Aspergillus fumigatus was isolated from soil sample was efficient in degradation of ink , paper stained with water soluble fountain pen ink and also could be deinked by boiling for 5 minutes. The effluent obtained by deinking process could be effectively treated with Aspergillus fumigatus. The quality of the deinked paper is acceptable under paper recycling norms.

Key words: *deinking, Aspergillus fumigatus, paper quality*

[I] INTRODUCTION

Writing is a fundamental need of education and schools insist on the use of fountain pens to improvise the handwriting of young children. Thus in every academic year papers stained with fountain pen inks are being thrown out and recycling of these papers stained with fountain pen inks becomes essential. Paper recycling is the process of recovering waste paper and remaking it into new paper products [1]. Today the strong market in ink-reservoir writing instruments is still dominated by the ball-ended pen, now with several ink options, but the fountain pen will not lie down, it is still highly valued in our electronic communication culture [2]. The paper has two main levels. The first level stores the ink that is 'floating' on the paper. This is wet ink that can be dragged across the page. The second layer is the ink that has dried – that is, that has been absorbed by the page and is no longer able to flow [3]. Carbon black now replaces spinel black, rutile black and iron black in nearly all black inks. In fact the ink industry is the second largest consumer of carbon black [4]. The protocol for the enzymatic deinking of laser printed waste papers on a laboratory scale using cellulase and hemicellulase of *Aspergillus niger* was developed as an effective method for paper recycling [5].

[II] MATERIALS AND METHODS

Approximately one kilogram of marine soil sample was collected from Chennai beach (east coast) and Trivandrum beach (west coast). This soil sample was spread neatly on separate trays. Another tray filled with soil from staining tray was also taken [6].

2.1. Isolation of bacteria and fungi capable of deinking

The method was followed as described in Zollner et al [7]. Three pairs of paper of size 3x3 was cut and dipped in ink. These papers were dried. One pair of the paper was buried in Chennai beach soil sample, other in Trivandrum beach sample and the third pair of paper was buried in the staining tray. This set up was left undisturbed for 7 days. On the 7th day 5 nutrient agar plates and 5 nutrient agar plates were prepared. The pair of papers buried in soil sample was carefully taken out using a sterile forceps and was used to inoculate one nutrient agar plate and one potato dextrose agar plate.This completes inoculation of one set of plates with papers from Chennai soil sample, other set with Trivandrum soil sample, the third with staining tray paper sample and the fourth set of plates was inoculated with 1g of soil sample collected directly from the staining tray. The fifth set is marked as control. These plates are incubated at 37°C for 24 hours.

2.2. Enrichment Culture

Minimal media was prepared and autoclaved. After cooling, ink mix was added at a concentration of 0.1ml/ml to the minimal media. The

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organisms from the nutrient agar plates and Czapek-dox agar plates were inoculated separately. It is incubated at 37^{0}C for 7 days. Lactophenol cotton blue mounting was used to identify the presence of characteristic mycelia and fruiting structures.

2.3. Cellulose assay and characterization of cellulose-related phenotype

Minimal salt broth was prepared [Glucose (0.1g), Dipotassium phosphate (0.7g), Monopotassium phosphate (-0.2g), Sodium citrate (-0.05g), Magnesium sulphate (-0.01g), Ammonium sulphate (-0.15g), Agar (-2.5g), Distilled Water (-100ml)], poured in 10 conical flasks (250ml) and sterilized by autoclaving. These flasks were arranged in two sets (each set containing 5 conical flasks).four conical flasks were marked as blue, black, red and green, the fifth was marked as control. 0.5ml of the respective ink was added to each of the flask separately, one set was inoculated with *Aspergillus fumigatus* and the other with *Penicillium* spp. These flasks were incubated at the room temperature and observed for color reduction. The color reduction was measured by visible spectrophotometer.

2.4. Degradation of gel ink

Minimal salt broth was prepared, poured in 6 conical flasks (250ml) and sterilized by autoclaving. These flasks were arranged in two sets (each set containing 3 conical flasks).two conical flasks were marked as, blue gel and black gel. The fifth was marked as control.0.5ml of the respective ink was added to each of the flask separately. One set was inoculated with *Aspergillus fumigatus* and the other with *Penicillium* spp These flasks were incubated at the room temperature and observed for color reduction.

2.5. Deinking of papers directly using Aspergillus fumigatus

Trial 1: A paper dipped in ink was dried and placed on a sterile Petriplates. Spores of A*spergillus fumigatus* was collected and dispersed over the paper directly. The plates are incubated at 37ºC. **Trial 2:** A paper dipped in ink was placed on a sterile Petriplate and around 1 ml of minimal media was added to wet the paper. To this wet paper spores of *Aspergillus fumigatus* were added and incubated. **Trial 3:** A paper dipped in ink was placed on a sterile petriplate and was soaked in excess of minimal media. Spores of *Aspergillus fumigatus* was collected and dispersed over the medium. These plates are incubated at 37ºC. **Trial 4:** Sterile Minimal agar plates were prepared. A paper dipped in ink was placed over the medium. The organism was spread evenly on the plate using sterile peptone swabs and incubated at 37ºC.

2.6. Deinking of fountain pen inks

A mixture of ink was prepared by mixing equal volume of all the four colors (blue, black, red and green) of fountain pen ink. Using a dry cotton swab the ink mix was spread evenly on the small standard A4 paper (TNPL copier).The paper was dried, immersed in the beaker containing water, which was heated for 5 minutes.

2.7. Degradation of gel pen inks

A mixture of ink was prepared by mixing equal volume of gel blue ink and gel black ink. Using a dry cotton swab the ink mix was spread evenly on the small standard A4 paper (TNPL copier).the paper was dried and was immersed in the beaker containing water. It was heated for 5 minutes [8, 9].

2.8. Degradation of effluent from deinking trial

The quantity of effluent was measured as described by Scott and Ollis 1995 [10]. The minimal media components were added to the effluent based on the amount of effluent. It was sterilized by autoclaving. The fungal culture was then inoculated using a sterile inoculation loop. It was incubated at 37ºC and observed for color reduction. The color reduction was measured by visible spectrophotometer [11]. **Brightness:** Whiteness Meter was used to measure the brightness, yellowness and shade of the deinked paper. This whiteness meter is mainly used to measure the whiteness of paper, paperboard, paper pulps, pulps of chemical fiber, cotton, chemical fiber, textile, plastic, starch, salt, white cement, porcelain clay and talcum powder, etc. **Porosity:** Paper is a highly porous material and contains as much as 70 % air. Porosity is a critical factor in Printing Papers, Laminating Paper, Cigarette Paper and Bag Paper. The POROLOG MICRO 5000 is an instrument which is widely used to detect changes in porosity on the moving paper web [12]. **Smoothness**: Smoothness tester determines the smoothness and porosity of paper and board, based on rotameter airflow principle. It is provided with three imported variable area flow meters (Rotameter). **Caliper:** Caliper used to measure the thickness of a sheet of paper expressed in thousandth of an inch.

[III] RESULTS AND DISCUSSION

3.1. Isolation

Czepek-dox agar plates were inoculated with paper from staining tray paper and staining tray soil showed white and green color fungal colonies **[Figures–1 to 4]**. Nutrient agar plates showed white colonies **[Figure–2]**.

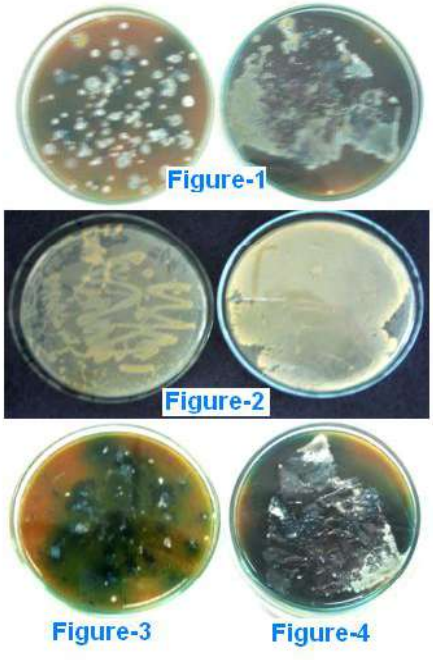

Fig: 1. Fungal colonies. Fig: 2. Bacterial colonies. Fig: 3. Growth of fungus. Fig: 4. Growth of fungus.

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3.2. Enrichment culturing

The isolated organisms were cultured on medium enriched with medium containing ink of which only the two fungal species showed considerable reduction in color [13].

3.3. Identification

Lactophenol cotton blue preparation of a slide culture showed a typical flask- shaped vesicles and characteristic rows of conidia on fruiting bodies and was found to be *Aspergillus fumigatus* **[Figure–5]**. Lactophenol cotton blue preparation of a slide culture showed dense brush-like spore-bearing structures with simple conidiophores terminated by clusters of flask-shaped phialides, was found to be *Penicillium* spp **[Figure–6]**.

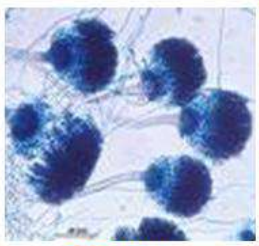
Figure-5

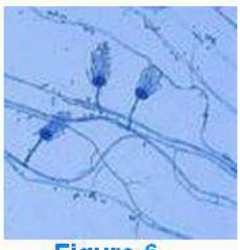
Figure-6

Fig: 5. *Aspergillus fumigatus.* Fig: 6. *Penicillium spp.*

3.4. Visible spectrophotometeric analysis

The OD value taken for every four days for a period of 16 days was recorded **[Tables–1 to 4]**. *Aspergillus fumigatus* was found to be effective in degrading ink than *Penicillium* spp.

Table: 1. Blue ink degradation

Number of days	0	4	8	12	16
A. fumigatus	0.026	0.013	0.009	0.007	0.004
Penicillium sp	0.026	0.022	0.019	0.007	0.006

Table: 2. Black ink degradation

Number of days	0	4	8	12	16
A. fumigatus	0.091	0.065	0.051	0.042	0.03
Penicillium sp	0.091	0.091	0.088	0.076	0.062

Table: 3. Red ink degradation

Number of days	0	4	8	12	16
A. fumigatus	0.861	0.57	0.535	0.162	0.14
Penicillium sp	0.861	0.654	0.579	0.448	0.441

Table: 4. Green ink degradation

Number of days	0	4	8	12	16
A. fumigatus	0.048	0.044	0.037	0.032	0.023
Penicillium sp	0.048	0.048	0.046	0.04	0.035

3.5. Deinking of papers directly using Aspergillus fumigatus

All the four trials carried out to remove ink from the paper directly was found to be failure since the organisms stain the paper with its metabolic product after four days of incubation [14]

3.6. Deiniking of papers by boiling

The fountain pen inks are easily separated form the paper by boiling for 5 minutes as it does not fuse with the paper tightly but the gel pen inks fuse with the paper and cannot be removed from the paper by boiling.

3.7. Degradation of ink from effluent

Degradation of effluent from deinking trials using *Aspergillus fumigatus* for 16 days was carried out the initial and the final concentration of the effluent was measured in visible spectrophotometer and was ploted on a standard graph for ink mix dilution [15]**[Table–5]**.

Table: 5. Effect of degradation

Effluent	OD at 620 nm
Before treatment	1.79
After treatment	0.414

The effect of this deinking process can be calculated by calculating the loss of brightness

$$\text{Loss of brightness} = \frac{\text{Brightness of base paper - Brightness of the deinked paper x 100}}{\text{Brightness of base paper}}$$

$$= \frac{79.9\text{-}68.9 \times 100}{79.9}$$

$$= 0.1376 \times 100$$

$$= 13.76$$

Therefore the loss of brightness percentage is 13.76. According to Neal et al. treatment of news paper by Agglomerate Floatation using kerosene oil showed average the loss of brightness of 14.79 which is an acceptable standard for recycling of grade one papers [16].

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Table: 6. Quality of deinked Paper

Property	Base paper	Deink paper
Brightness [%]	79.9	68.9
Yellowness [%]	-5.5	-3.1
L* [%]	89.2	85.0
a*[%]	0.9	0.8
b*[%]	-0.3	-1.7
Smoothness [ml/inch]	Top-320 Bottom-220	Top-800 Bottom-600
Porosity [ml/inch]	100	130
Caliper [µ]	103	114

Since the porosity of the deinked paper is high compared to the base paper, it cannot be reused as writing material but can be used to make boards, boxes and tissue papers [17] **[Table –6]**.

Pulp recovery: The pulp weight was 0.271 before and after the treatment. There is no change in the pulp weight. Hence the recovery of pulp is 100%. According to Tradecom International Ltd, Mumbai, Recycled paper recovery in India is 22% of total consumption in comparison to 55-60% recovery in developed countries. In chemical deinking of newspaper waste, the maximum recovery is 60% [18]. In enzymatic deinking process 90-98% recovery is possible but it is an expensive process [19]. Hence this method of deinking using *Aspergillus fumigatus* is efficient as well as economical.

[IV] CONCLUSION

Aspergillus fumigatus isolated from soil sample is efficient in degradation of ink. Paper stained with water soluble fountain pen ink can be deinked by boiling for 5 minutes. The effluent from deinking process can be effectively treated with *Aspergillus fumigatus*. The quality of the deinked paper is acceptable under paper recycling norms.

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RESEARCH ARTICLE

DEGRADATION OF PROCION RED MX-5B REACTIVE DYE COUPLING A PHOTO FENTON SYSTEM

K. Swaminathan, K. Pachade, and S. Sandhya*
National Environmental Engineering Research Institute, CSIR-Complex, Chennai, INDIA

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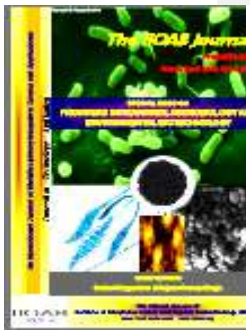
***Corresponding author:** Email: sswami_in@yahoo.com_ Tel: +91–44 22541964; Fax: +91–44 22541964

ABSTRACT



The oxidative degradation of Procion Red MX-5B by solar Fenton's has been investigated using sunlight as irradiation source in presence of hydrogen peroxide and Fe2+. Maximum decolorization (99%) and COD removal (97%) for MX-5B was obtained at H2O2 dose of 16.6 mM and at 30 min irradiation time. Fe2+ dose of 0.36 mM was optimum for Procion Red MX-5B degradation. The solar degradation mechanism occurs in three steps in which initially the more active bonds were hydroxylated. The N=N and the C-S bonds of sulphonate group form organic acids, SO42+ and NH4+. At the same time, the aromatic acids produced initially were further oxidized to aliphatic acids. MX-5B undergoes oxidation leading to non toxic and biodegradable ultimate breakdown products, such as, oxalic acid and acetic acid. Solar Fenton process not only oxidizes but also helps in decolorization and nearly mineralization of the dyes at low dose of reactants.

Key words: *Solar Fenton's degradation; solar energy; Procion Red MX-5B; H2O2; decolorization; product of degradation*

[I] INTRODUCTION

Wastewaters generated from textile and dyeing industries are highly colored, rich in organic and inorganic materials and saline. With the presence of complicated color causing compounds, decolorization and degradation of these wastes is a difficult and challenging task. Hence, it is essential to remove/destroy the refractory/ recalcitrant pollutants before its discharge into the surrounding water bodies. In recent years, new technologies such as Advanced Oxidation Processes (AOP) show great potential for the treatment of wastewater containing toxic organic compounds. The AOPs involve generation of hydroxyl radicals (HO), which non selectively attack the organic compounds faster than the commonly known oxidizing agents. The radicals can be produced on site, in a reactor where the radicals can contact the organics in the wastewater.

Fenton's process, one of the oldest advanced oxidation processes that use a mixture of Fe2+ and H2O2 as the oxidizing agent, has been proven as a powerful oxidant of organic compounds and has attracted interest recent years in wastewater treatment. Hydrogen peroxide reacts with ferrous ions in water and generates free hydroxyl radicals, which have high oxidation potential and can oxidize wide range of organic compounds [1].

Fe2+ + H2O2 □.OH+ Fe3+ +OH

Studies have shown that textile and commercial dyes can be successfully treated by Fenton's oxidation [2,3,4]. Fenton's process has been applied for the pretreatment of organic compounds to reduce toxicity and improve biodegradability [5].

In recent years, many studies have shown that the oxidizing power of the Fenton type system can be enhanced greatly under UV light irradiation and can be used for the degradation and mineralization of toxic organic contaminants present in wastewater [6, 7]. The efficiency of degradation of organic compounds was considerably improved due to the continuous regeneration of Fe (II) via photo reduction of Fe (III) by Fenton's reagent combined with UV-visible irradiation [8].

Solar energy was successfully used for the photo catalytic degradation of dyes [9,10]. Bandara et al.[11] reported that the degradation and decolorization of Orange II could be achieved

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in less time in the presence of natural sunlight via Fenton type reaction. Solar assisted Fenton's process was efficient to decolorize and mineralize reactive dyes used in the textile dyeing industries [1,12]. The objective of the work is to study the efficiency of solar Fenton's process for the degradation of MX-5B in the presence of natural sunlight

[II] MATERIALS AND METHODS

2.1. Reagents

FeSO4 • 7H2O (MERCK, purity 96%) was prepared at a predetermined concentration of 10 g l-1. H2O2 (30%) and other chemicals used in the experiment were of analytical grade. Commercial grade dyes Procion Red MX-5B obtained from Color Chem (India) Ltd. were used without any purification. A known concentration of the dye was prepared in deionized water and used for all studies. **Supplementary figure–1** gives the structure of dyes Procion Red MX-5B.

2.2. Experimental procedure

Solar experiments were carried out on sunny days of April to June 2004 between 11 AM and 2 PM. A known volume dye solution was taken in an open borosilicate glass tray of 500ml capacity, to which known volume of FeSO4 was added. The addition of H2O2 to the reacting solution marks the beginning of the reaction. The solution was mixed with the magnetic stirrer and exposed to sunlight. Solar light intensity was measured for every 1 hour and the average light intensity over the duration of each experiment was calculated. The intensity was 825x100 Lux. The experiments were carried out at pH 3.0. Samples were collected at regular time interval and analyzed immediately.

2.3. Analysis

COD and pH were measured as per Standard Methods [13]. The UV–VIS spectra of the samples were recorded from 200 to 800 nm using spectrophotometer (Shimadzu Model UV 160A). Color removal was measured for each dye at the wavelength in the visible range, where maximum absorbance was obtained. Residual hydrogen peroxide was determined by potassium iodide titration method [14]. Accordingly, correction was made in the COD determination for residual H2O2 [15].

Low molecular weight organic acids formed during AOP processes were analyzed by ion chromatography. Ion chromatography employed to analyze organic acids was equipped with an Anion Ion 12 (AS-4) column (Dionex) and operated in suppressed conductivity detection mode. The filtered eluent used was 3.3 mM Na2CO3/1.0 mM NaHCO3 at a flow rate of 0.7 ml/min.

Fourier Transform Infrared (FTIR) spectroscopy was carried out using a Perkin Elmer Brucker Vector 22 FTIR instrument for identification of functional groups. The samples were prepared for FTIR analysis by drying at 900C and mixing the powdered samples with dried KBr. The resulting powder was then pressed to produce a pellet for analysis. IR data were collected over the wave number range of 700 – 4000 cm-1.

[III] RESULTS AND DISCUSSION

3.1. Effect of hydrogen peroxide

Decolorization and degradation of Procion Red MX-5B for different H2O2 dose from 8.83 mM to 33.2 mM at constant iron dose (0.36 mM) are presented in **[Supplementary figure–2]** and **Supplementary figure–3**. Maximum decolorization (99%) and COD removal (97%) for MX-5B was obtained at H2O2 dose of 16.6 mM and at 30 min irradiation time. The enhancement in the removal rate is due to increase in hydroxyl radical production by solar light. Light intensity determines the amount of photons absorbed by the catalyst. With increase in the solar power, Fenton's generate more photons and produce more hydroxyl radicals, which help in improving the degradation efficiency. But at high dosage of H2O2 the decrease in decolorization is due to the hydroxyl radical scavenging effect. Costa et al. [12] reported complete decolorization of Bright Blue Ramazol and Red Proceon H-E713 in the mixture on exposure to solar and ultraviolet radiation. Hung et al. [15] observed that increase in H2O2 concentration from 3.75 mM to 42.8 mM resulted in 90% color removal of azo dye Acid Black 1 by the UV/H2O2 process. Murganandham et al. [16] also reported that solar Fenton process was found to be more effective during the decolorization of Reactive Yellow 14.

3.2. Effect of ferrous dosage

Effect of iron dose on decolorization and COD removal for Procion Red MX-5B is presented in **Supplementary figure–4 and Supplementary figure–5**. H2O2 dose was fixed at 16.6 mM and ferrous sulphate dose as Fe2+ was varied from 0.18 mM to 0.72 mM. Optimum ferrous iron dose observed was 0.36 mM with 92.4% decolorization and 89.4% COD removal at 30 min irradiation time. Solar light has the largest fraction of photons with the energy needed to drive photoreaction. The photo generated ferrous ion participates in Fenton's reaction generating additional .OH radicals thereby accelerating the oxidation process compared to Fenton's process. Kavitha and Palanivelu [17] reported that catalytic nature of iron helps in enhancing the reaction occurring at lower concentration of iron in solar Fenton's process. Selvam et al. [7] reported 70% decolorization and 51.3% degradation of Reactive Orange 4 by solar light at lower iron dose.

3.3. Decolorization and degradation by solar Fenton's process

The decolorization of MX-5B by solar Fenton's process at optimum conditions is shown in **[Supplementary figure–6]**. Color removal was very fast as the solution was decolorized in less than 5 min in solar Fenton's process the color completely changed from dark brown to colorless. The UV-visible spectral changes of Procion Red MX-5B at different irradiation time are presented in **[Supplementary figure–6]**. The absorption band at 538 nm decreased in 3 min irradiation and the solution turned from dark red to colorless. The faster decolorization of dye is due to the initial electrophilic cleavage in chromophoric azo (-N=N-) bonds attached to the naphthalene ring. The absorption

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band corresponding to MX-5B at 538 nm completely disappeared indicating degradation and disappearance of the conjugated structure. Chacon et al. [18] observed that the intensity of absorption at 430 nm decreased as the irradiation time increased during solar photo catalytic degradation of Acid Orange 24. Similar results are reported by Murganandham et al. [17] Light plays two different roles that lead to an improvement of the reaction yield. It drives solar-Fenton's reaction, producing extra hydroxyl radicals and the recovery of Fe (II), which helps in improving the degradation and decolorization efficiency. The use of sunlight and Fenton's reagent may become an effective way of eliminating color and organic load. Selvam et al. [7] reported that Reactive Orange 4 was almost decolorized and degraded in 30 min by solar Fenton's process.

Torrades et al. [19] stated that Procion Red H-E7B, and Red Cibacron FN-R could be efficiently decolorized by solar photo-Fenton's process. Chacon et al. [18] reported that photo-Fenton's process by sunlight was effective for the degradation and mineralization of Acid Orange 24. The results obtained demonstrate that use of solar Fenton's process is more effective in decolorization and degradation of both compounds as compared to Fenton's process alone.

To obtain detailed information on the reaction during solar Fenton's process the resulting oxidation products were determined. Conductivity increased from 1490 µS cm-1 to 2300 µS cm-1 for MX-5B **[Supplementary figure–7]**. A rapid increase in the conductivity indicates the formation of acidic by-products during the degradation of MX-5B by solar Fenton process. Release of inorganic ions at different irradiation time is presented **Supplementary figure–8**. Sulphate concentration increased from 124 mg L-1 to 286 mg L-1 for MX-5B. During oxidation process, sulphonic group may be substituted by .OH at the initial stage resulting in release of sulphate in the solution. The formation of chloride from the mineralization of organic chlorine linked to the triazine ring. Nam et al. obtained similar results during the study on azo dye oxidation by the FeIII–EDTA–H2O2 system. MX-5B contains three sources of nitrogen including an azo group, a triazine ring and a nitrogen atom linking naphthalene ring to the triazine ring. It was found that primary aromatic amine would disappear with the decolorization of MX-5B [20]. Ammonia was detected in traces from the oxidation of primary aromatic amine. Nitrate concentration increased from 60 mg l-1 to 110 mg L-1 for MX-5B. Chloride concentration increased from 83 mg L-1 to 105 mg L-1 from 5 min to 30 min of reaction time for MX-5B. The degradation leads to conversion of organic carbon to inorganic ions such as nitrate and sulphate and chloride. Houas et al. [21] reported complete mineralization of carbon, nitrogen, sulfur, and heteroatom of Methylene Blue (MB) by TiO2/UV process into CO2, NH4+, NO3− and SO42−. Stylidi et al. [22] reported that during solar induced photocatalytic degradation of azo dyes in aqueous TiO2 suspensions generates sulphate and nitrate as oxidation products.

3.4. Ion chromatography analysis

The data on organic acids formed during solar Fenton's process is presented in **Supplementary figure–9**. For MX-5B, oxalate formed during initial stages of reaction was oxidized at 30 min irradiation time whereas acetate was remaining at the end of reaction. It was observed that nearly complete oxidation of acids was achieved in solar Fenton's process. The lower values of acetate and oxalate obtained in solar Fenton process indicates that these compounds are oxidized under the influence of solar light leading to higher mineralization efficiency. Aromatic and aliphatic acids were identified during photo catalytic degradation of Acid Orange 7 before complete mineralization to water and CO2 [7, 23]. Herrmann et al. observed formic and lactic acids as intermediates on solar photo catalytic irradiation of Amaranth indicating fast and easy naphthalene ring rupture [9]. Kavitha and Palnaivelu [17] reported that carboxylic acids like acetic and oxalic acids were formed as the end products during the degradation of phenol by solar Fenton processes. Mahamoodi et al. [23] detected format, acetate, and oxalate as aliphatic intermediates during the photo catalytic degradation of Reactive dyes RB8 and RB 220. They have reported that the amount of inorganic ions reached the maximum when solution color completely disappears.

3.5. Degradation kinetics by solar Fenton's process

The pseudo first order kinetics of MX-5B during solar Fenton's process is presented in the following reaction.

$$-dcA/dt = K'd\,[CA]\,C.[OH]$$

At constant hydroxyl radical concentration C.OH should be constant hence, the above equation becomes

$$-dc / dt = K[CA]$$

K is the pseudo first order rate constant at time, and CA is the concentration. COD removal was used to study the degradation kinetics for MX-5B at optimum reaction conditions Fe2+/ H2O2 and irradiation time. The plot of -ln(CODt/COD0) vs. reaction time is presented in **Supplementary figure–10**. The data fitted linearly with the coefficient values of 0.96 for MX-5B. The rate constant for MX-5B was 0.1214 min-1. Shu and Chang [24] reported pseudo first order rate constant on decolorization of azo dyes by UV /H2O2 process. Feng et al. [25] observed pseudo first order reaction on discoloration of dye by solar photolysis of ferri-oxalate.

3.6. Biodegradability assays

The data presented in **Supplementary figure–11** show that after solar Fenton's process the ratio of BOD5/COD was increased. The ratio before treatment was 0.13 for MX-5B. After treatment the ratio increased to 0.45 for MX-5B indicating an effective increase of biodegradability. Torrades et al. [19]

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reported that solar Photo Fenton has proven beneficial in increasing BOD5/COD ratio of Procion Red H-E7B and Red Cibacron FN-R. The increase of BOD5/COD value 0.4 is considered as the quantitative index for complete biodegradability of organic matter [26].

3.7. FTIR analysis

FTIR spectra of MX-5B before and after Fenton's treatment are presented in **Supplementary figure–12**. Majority of peaks observed before Fenton's process disappeared during the reaction. The bands observed in the raw MX-5B in the range at 1370m-1 to 1042 cm-1 disappeared after 15 min of reaction time. The new peaks formed at 1709 cm-1 (oxalate), 1403 cm-1 (formate) and 1062 cm-1 (carboxylic acid) disappeared after 30 min indicating the decrease in the aromaticity. The sample at this stage showed the presence of bands at 1666 cm-1 ,1147 cm-1 and 803 cm-1 may be due to stretching vibrations of O-H bond, apparently became strong. A new peak at 1403 cm-1 may be formed due to interaction between the C-O stretching and O-H bending in a carboxylic group from acetic acid. Galindo et al. [26] reported that bands corresponding to oxalate (1709 cm-1 and 1690 cm-1) and acetate (1416 cm-1) were observed during photo degradation of Acid Blue74. Formation of carboxylic acid was reported by others [22, 23]. Based on the IR data it can be concluded that the naphthalene ring in the dye is oxidized, and broken down to carboxylic acid, which is in accordance with oxalate and acetate identified by ion chromatography. Zhao et al. [27] reported formation of carboxylic acid as acetic acid by IR spectroscopy indicating the breaking of -N=N- during photo degradation of an azo dye, Mordant Yellow 10 (MY10), under UV irradiation. By comparing IR data of MX-5B, is was observed that some of the carboxylic acids were formed in the early stages of MX-5B of solar irradiation and were oxidized completely at 30 min of reaction time. Similar results are reported by Hu et al. [28] during photo degradation of Procion Red dye.

[IV] CONCLUSION

The decolorization and degradation of MX-5B was studied using solar Fenton's process. The optimum dose of H2O2 for MX-5B was 16.6 mM . 0.36 mM Fe dose of was optimum for MX-5B . For MX-5B almost 99% decolorization 97% COD removal was achieved by solar Fenton's process in 30 min of irradiation time.

Study of FTIR and IR results shows that, solar degradation mechanism follows in three steps in which initially the more active bonds were hydroxylated. The N=N bond linked to the benzene ring or the naphthalene ring, and the C-S bond of sulphonate group form organic acids, SO42+ and NH4+. The triazine ring further oxidized to form NO3- and Cl-. At the same time, the aromatic acids produced initially were further oxidized and led to the cleavage of the aromatic ring opening to form aliphatic acids. MX-5B undergoes oxidation leading to non toxic and biodegradable ultimate breakdown products, such as, oxalic acid and acetic acid. Solar Fenton process at reduced Fe2+ and H2O2 concentration not only oxidizes but also helps in decolorization and nearly mineralization of the dyes at low dose of reactants. By solar Fenton's process better biodegradability of MX-5B could be achieved. The study shows that solar Fenton process can be prove an effective advanced oxidation process

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SUPPLEMENTARY FIGURES
(Not verified by Journal. Authors are responsible for any error)

Fig. 1. Structure of Procion MX-5B

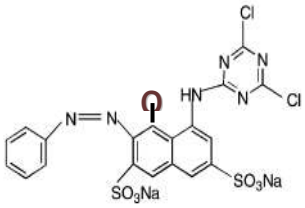

Fig. 2. Effect of H_2O_2 dose on decolorization of MX5B by solar Fenton's process

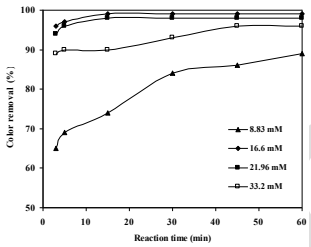

Fig.3. Effect of H_2O_2 dose on COD removal of MX5B by solar Fenton process

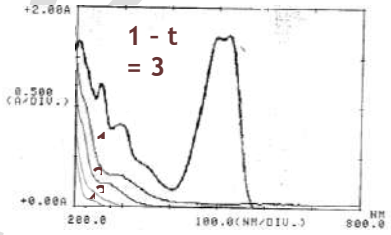

Fig: 4. Effect of Fe^{2+} dose on decolorization of MX-5B by solar Fenton's process

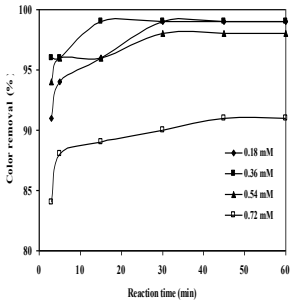

Fig. 5. Effect of Fe^{2+} dose on COD removal of MX-5B by solar Fenton's process

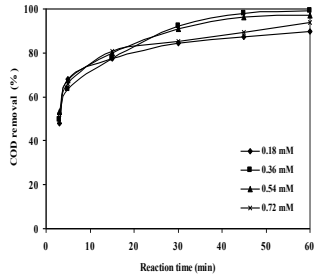

Fig. 6. UV –Visible spectra of MX-5B during solar Fenton process with different irradiation time

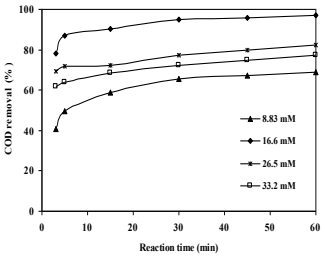

Fig: 7. pH and conductivity of MX5B during solar Fenton process

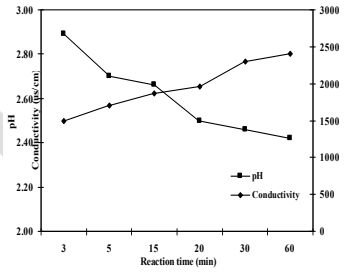

Fig: 8. Release of inorganic anions of MX5B by solar Fenton Process

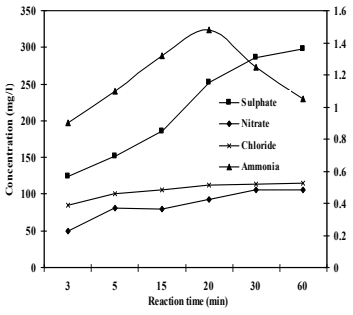

Fig: 9. Formation of acetate and oxalate of MX5B by solar Fenton's process

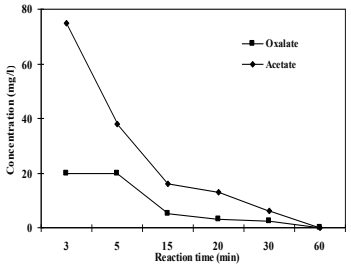

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Fig. 10. Degradation kinetics of MX-5B by solar Fenton's process

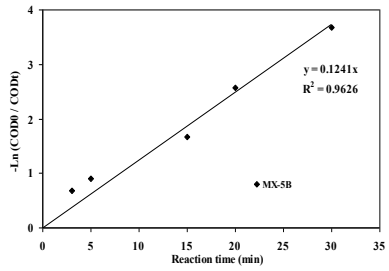

Fig. 11. BOD_5/COD ratio by solar Fenton's process

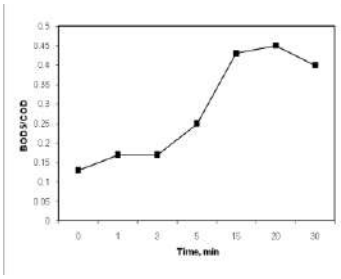

Fig. 12.FTIR spectra of MX-5B during solar Fenton process with different time. a – before treatment, b, c, after treatment. (15min, 30 min)

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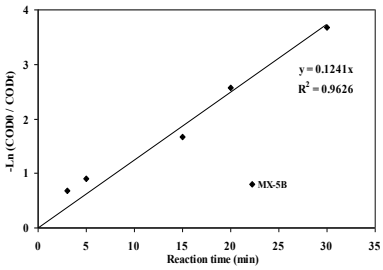

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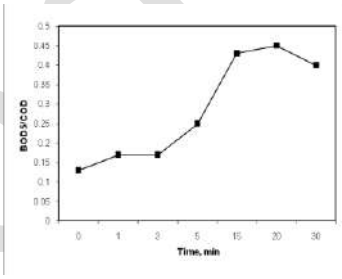

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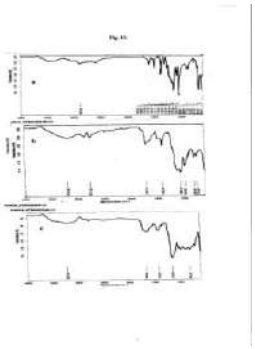

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COMMENTARY

INNOVATIVE APPROACHES FOR PROMOTING ENVIRONMENTAL EDUCATION IN INDIA

Swati Gupta[1*], Surendra Kumar Yadav[2], and Shweta Saini[3]
[1]*Anand College of Education, Agra (UP), INDIA*
[2] *School of Studies in Environment Management, Vikram University, Ujjain (MP), INDIA*
[3] *Anand College of Education, Agra (UP), INDIA*

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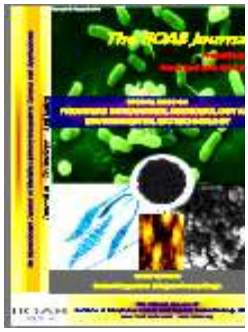
[*]**Corresponding author:** Email: swati11986@yahoo.com Tel: +91–9027932042, Fax: +91–7417072772

ABSTRACT



Environmental education system needs intervention, communication and education through short or long term courses to enhance skills and capacity building to improve higher education system. Environmental management issues in higher education by providing information, running events, developing cases and guidance materials, and other means like identifying and disseminating best practice is a good tool for evaluation. Developing the capacity of staff with environment-related responsibilities to achieve positive environmental change within their institutions through action workshops and other means is important. Teachers do have a relatively broad conceptualization of environmental education (EE) but that the perceived link between EE and school improvement is not strong. Environmental education can be improved through various means of communication and media tools. For establishing a sustainable society that realizes sound economic development with reduced environmental loads while maintaining a healthy and productive environment, providing a basic principle on environmental conservation activities, encouragement of willingness for environmental conservation and environmental education , clarifying the responsibilities of citizens and private bodies etc., the State, local governments, establish a basic policy and other necessary matters to encourage their willingness for environmental conservation and promote environmental education, thereby contributing to ensure healthy and cultured living for both the present and future generations of the nation. There is a need to develop sensitivity in population. Some innovative approaches are- Use of Various Media and Technology, Promote partnership and cooperation among non-government organizations, promote Research and exchange visits, Progression towards greener curricula etc. we all (NGOs, schools, and other community groups) must all work together to ensure the future success of environmental education campaigns ranging from the global to the local level.

Key words: *Environmental education; higher education; exchange visits; greener curricula; capacity building*

[I] INTRODUCTION

Human populations have significant impact on the natural environment. As the global population continues to rise, humans place more and more pressure on a finite number of resources and degrading environment [1]. However, In India, The National Conservation Strategy and Policy Statement has emphasized the importance of Environmental Education (EE) and there are some efforts which emphasized environmental education in India. A Supreme Court of India order has required the University Grants Commission to prescribe courses on the environment in higher education system. The Prayavaran Vahini Scheme is underway to create environmental awareness. In-service and pre-service training are provided to teachers and civil servants on different aspects of environment. The ENVIS (Environmental Information System) has been established to collect, retrieve and disseminate environmental information in the country. A National Environmental Awareness Campaign (NEAC) has been initiated for public awareness and some 3500 eco-clubs are actively run across the country for Grades 6 to 10 [2]. But, they are not sufficient because there are so many problems which hinder the work.

Recent research on the impacts of the environmental education program [3] suggests that an EE-based curriculum, properly implemented, may indeed positively impact student

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achievement. A positive relationship between EE and school improvement (i.e., improvements in teaching and student learning) has been shown in other studies, including a nationwide study conducted by the State Education and Environment Round table [4]. Two separate open-ended conceptual content cognitive map (3CM) tasks [5] were used to assess each participant's conceptualization of "environmental education" and "school education system improvement." The 3CM method is a means of assessing mental models – that is, how an individual or group thinks about, or conceptualizes, a particular topic on environment. Data obtained through a 3CM task provide information on the factors an individual perceives to be relevant to a topic and the relationships among these factors. The technique is particularly effective in measuring people's understanding of abstract issues and hence is suitable for the investigation of teachers' conceptualizations of EE and "school improvement."

Therefore, important parameter/ factors before researchers and academic community are causes which prevent promotion of environmental education and the way of transit environmental education in best way to recipient. EE is a learning process that increases people's knowledge and awareness about the environment and associated challenges, develops the necessary skills and expertise to address the challenges, and fosters attitudes, motivations, and commitments to make informed decisions and take responsible action [6]. The causes prevalent in environmental education are -

1.1. Formulation of national policy

Except few countries such as the Philippines, Australia and Thailand, no country has formulated a national policy on (EE). No coherent plan provides a link from the kindergarten to university levels. As a result, EE receives no priority action, no allocation of resources, no budget and no support, and thus is marginalized from the national mainstream. Because of this, even those countries that have initiated EE programs show inconsistencies and discontinuities in implementing EE programs and activities. There is no evidence of serious efforts being made towards building institutional capacity in EE.

1.2. Unbiased approach towards physical science

Before some time, environmental topics were taught only in physical science and geography classes. After that, however, the focus has been gradually moving towards social science, liberal arts and the humanities. Yet explicit incorporation of environmental themes is still biased towards physical science courses. Nevertheless, because human activity is the primary factor responsible for the deterioration and destruction of the environment, social science aspects should be given the same level of attention as the sound management of environmental resources. For example, water pollution is the result of human actions. Therefore, in order to prevent it, it is not only necessary to understand its physical basis, but also to promote human awareness of the problem and encourage compliance with environmental laws. This can only be done through the integration of environmental themes into areas of education other than physical science.

1.3. Purposeful of whole-of-government commitment

Although environmental themes have been integrated into the formal education system, most of the EE initiatives come first from the sectoral ministries such as Environment, Fisheries, Agriculture, Forestry or Natural Resources, and not from the Ministries of Education. Their efforts are mostly related to specific issues and geared towards changing knowledge, attitudes and skills. They are not broad and comprehensive in terms of achieving sustainability. It is not possible to get the necessary full commitment from the government towards environmental education activities unless it is addressed in totality.

1.4. Improvement in institutional coordination

All the countries report a lack of coordination amongst responsible agencies in the region. Because of this, the agencies either duplicate activities or compete for resources. When the situation degrades further, mutual mud-slinging becomes a common phenomenon resulting in no action or delayed action. Several ministries adopt individual policies and procedures to pursue their own mandates without any collective action or vision. Usually, there is no consultation among these groups and if there is any agreement, it is loose, vague and morally non-binding.

1.5. Adequate manpower

There is a notable shortage of trained manpower, especially of environmental educators and facilitators, to teach integrated courses such as environmental studies, man and environment and nature science. No major efforts have been initiated to promote teachers' competency and capability. Conventional teaching methods, such as lecture methods, are applied to teach dynamic courses such as these. This reduces the quality of the education because there are no opportunities for students to observe directly the environment, or to be exposed to real-life situations.

1.6. Rigid curricula and teaching methods

Existing curricula are book-based and examination-oriented. Further, the curricula are not oriented toward nourishing a sustainable society. Because classroom instruction is geared towards examinations, students prepare to appear for their final examinations and achieve high scores rather than develop actual

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skills and competencies in the subject matter. Despite the fact that environmental concerns are integrated into the curricula, they are neither vertically integrated nor horizontally coordinated. There are no vertical links between educational activities in one level with other levels, nor are educational activities within the same level horizontally coordinated with other course activities. Activities are duplicated, and teachers are often unaware of what other teachers are doing in other subjects. Students do not learn about the environment in critical ways and fail to see the interconnections that contribute to the overall complexity of the environment. Curricula are centrally controlled, and their development process is quite bureaucratic in nature. Furthermore, existing courses are tightly arranged and do not allow additional subjects to be incorporated. The unavailability, inaccessibility and irrelevancy of textbooks, instructional materials, manuals and guides have further aggravated the problems of effective curricular structure and processes. The pedagogy is mostly the "chalk-and-talk" method, and learning is based on the rote method and spoon-feeding. Because of this, students are encouraged to memorize rather than examine the problems critically.

1.7. Adequate physical facilities

In many countries, especially in rural areas, school buildings are dilapidated and do not have even minimal facilities such as furniture, classrooms, laboratories, libraries, resources, tools and equipment. Due to space limitations in some areas, several classes are being run in shifts. For example, in mountainous areas of Nepal and India, more than two classes share the same classroom. In Cambodia, the number of students is as high as 100 to 150 in a single class [2].

1.8. Conceptual ambiguity in issues

The concept of EE means many things to many people. In some countries, it is taken as another academic course without any relevance to, or bearing on, real-life situations, while in other countries, it is still in its infancy. Some believe that environmental education is a new perspective towards education and focuses more on values. There still exists confusion over its concepts and, therefore, its approach. There are different perceptions about the meaning and objectives of EE [7].

1.9. Availability of data and information

There is a great dearth of data and information on the problems of EE. Even when data and information are available, they are not necessarily accessible. The data and information should be designed so that they are both usable by and easily accessible to the general public. In many countries these days, data are stored in computer files [2].

The innovative technologies in the field of environmental education are limited. The environmental damage already inflicted due to alarming on-going population explosion, rapid movement towards urbanization and industrialization, increasing needs of energy and fast scientific and technological advancement cannot be reversed unless there is collective thinking, will and effort. These call for public awareness and participation for bringing about an attitudinal change and finally restricting further damage to the environment [8]. There is a need to develop sensitivity in population. Because, most of us have knowledge and awareness but, we are not sensitive. At present time Environmental education could be seen as just another of a number of pressures on already over-crowded teacher education programmes [9]. So, there is a need to develop a more extensive and effective environmental education strategy to better prepare the public to understand and take action regarding current and future environmental issues. We have to make our children to realize that they are part of the problem, and therefore they have to be part of the solution [10].

[II] DISCUSSION

Application and use of various media and technology to meet the objectives of EE in any particular situation is major concern. The importance of using latest technological developments to leapfrog and achieve a wide reach is recognized [11]. In traditional rites and media (e.g. ceremonies, folklore), use of traditional media is a very effective way to transfer messages especially in rural areas. Traditional ceremonies often have links to environmental issues, and take into account the history of the area and the perceptions and relationships of the people to the environment. Stories may encompass a wide range of topics. The tales or legends told by elders can serve as a means of transferring feelings of respect and appreciation for animals, forests and other wildlife. For effective community-level communication strategies, an integrated and planned use of both folk and mass media is necessary for achieving optimum impact and for desired feedback. In drama and puppet shows humor, sound, color and human figures provide entertainment and so attract large crowds, especially in rural areas. Questions, discussions, problems and solutions about the environment can be dramatized and production is relatively inexpensive. Simulations/Role Playing technique is useful for finding out new issues and gaining other people's perspectives to similar situations. Exhibition is show of artifacts and pictures, with simple explanations, will enable viewers to learn at their own pace. They allow use of realistic, three-dimensional models that facilitate understanding through use of sight, sound, and touch. This technique can be used over a period of time and thus cover a wider population.

Environmental /comic magazines can present various environmental topics through a variety of methods, e.g. games, cross-word puzzles, activities, stories, cartoons, and so are very effective teaching tools. A special teacher's page can assist the teacher to use the material in existing subjects in the curriculum.

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In addition, these magazines supplement the limited reading materials for children in places, such as schools, rural libraries and literacy centers. Newsletters circulation technique has the advantage of being able to provide information about current events that have environmental implications. A bulletin board that is regularly updated with newsletters, posters and leaflets and placed in a strategic area in school, village, training college, and the like, is an attraction and a good source of information for the general population. Posters are cost effective, and opportunities for development and use of posters depicting national priorities exist. Visual Art workshop to develop environmental posters is also effective tool. This technique could be used in many different ways and even to produce a "wall news- paper" on specific issues.

Games are always popular and through the production of games related to environmental issues, it is possible to discuss, improve knowledge and better understand the relationship between the use of natural resources and sustainable development. The flannel board helps the participants to build the statement of their environment (past and present). This technique is also useful as a teaching approach in schools. It has the advantage of being easy to replicate and update, and it is relatively inexpensive as it can be constructed with local materials.

Radio, a powerful medium, reaches a large number of people with relatively few inputs. Use of different techniques, e.g. quizzes, competitions, talks, music, radio drama, interviews, jingles, folk stories in different types of programs catering for different age and/or interest groups, can be effective in raising awareness. Series of high quality slide presentations, arranged in a carefully planned sequence, is an effective teaching medium. Learners' understanding is enhanced by the combination of sound and sight stimuli, yet production cost is relatively low compared to films. Videos/Films/Television techniques combine picture, sound, color and motion, and are thus the closest medium to reality. Environmental issues, development processes, technologies can be captured and shown to the learners at a convenient time and place. They also combine entertainment and education and so have wide appeal. Project technique in the formal education system is excellent for the integration of several subjects and activities around an environmental theme or issue. The best projects are those where the participants actually engage in an activity to protect and/or rehabilitate the environment. Demonstration of a proposed activity, located within easy access of the target population, is an effective technique. This technique can be supplemented by involving key people such as chiefs, political leaders, prominent farmers, businessmen, opinion leaders, and others.

Research carried out by participants, where they can actually see the impact of an intervention, will go a long way towards helping change behavior. Field Trip technique is a popular one. Participants look forward to a 'trip', and having new experiences. Field trips are widely used to motivate wildlife school clubs in Malawi [2]. Exchange Visits are planned to look at what other people have done. They are useful for sharing successful interventions, and allow the participants to compare their achievements with those of others from other areas. Field trips for farmers are also useful in helping them adopt successful farming practices which they see for themselves and then want to replicate [7]. Incorporation of EE into all forms of education that is formal, non-formal and informal education. Teaching materials have been developed locally and disseminated. Also, a variety of innovative methods of teaching and learning are being practiced. Progression towards greener curricula that is cross-curriculum approaches have been adopted to integrate environmental themes into curricula. Some countries have begun to "green" their curricula by incorporating environmental concerns and have emphasized the use of local resources in teaching and learning processes. This involves the integration of environmental principles, problems and solutions into other disciplines. Both the natural environment and the man-made environment are involved. Creation of new initiatives through innovative works can be done include such as the designation of model schools and honor schools, the development of optional courses, establishment of Teacher's Centers for Excellence and awards, Supreme Court orders to include EE in universities and collaboration between ministries and state universities for education, training and research are also some reforms in EE system. Other initiatives include the creation of a green bank, an eco-polis center (a place for environmental information, education and hands-on activities in the community), a green press (collecting and publishing news related to environment), and eco-clubs, eco-farming and eco-harvesting In Indonesia, green banking programs have been initiated to provide insurance against environmental degradation.

Perception of EE as a new approach to education is seen as an integrated approach to education. While some countries see it as values education (concerning respect for nature and life, stewardship over natural resources, simple living, personal responsibility and gratitude for the lavish gifts of nature), others think that it provides a new perspective on education (concerning education in, about and for the environment). All these suggest that environmental education should not be an independent subject in its own right. Rather, it is a holistic approach to education that takes into consideration the environment that surrounds and affects people. Development of composite courses at the primary level such as Environmental Studies, The Environment Around Us, The World Around Us, Environmental Science, Man and the Environment, Nature Science and Life Experiences have been adopted at the primary level, and environmental themes are either integrated into existing subjects or are developed as compulsory courses at the secondary level. Movement of focus from physical science to social science courses is needed. The trend shows that there has been a shift from incorporating environmental matters only in

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physical science courses towards including environmental matters in social science, liberal arts and humanities courses as well. In addition to physical science courses, environmental concerns can now be found in courses such as in Moral Education, Hygiene, Religion and Civic Education. Nevertheless, environmental themes have been dominant in the physical science courses only. However, environmental issues do not exist solely within physical contexts.

Some countries have placed more emphasis on formal education because they envision that children will help educate their parents and can more easily influence their parents' actions. In turn, these parents will have a greater impact on environmental resources. In order to make environmental education successful, all types of education, both formal and non-formal, should be utilized. Establishment of successful eco-business activities- such as the green bank, eco-labeling, eco-consumerism, environmental advocacy and green press are becoming popular. These activities have been successful in enhancing environmental education in the region. Various innovative ways are undertaken to provide opportunities for students to acquire knowledge, attitudes and skills in school as well as out of school. The opportunities include eco-clubs, green clubs, nature clubs, camp and outdoor education, intra-mural competition, project work, street theatre, internships, mock congresses and junior eco-clubs. These activities provide students with out-of-classroom opportunities to relate their knowledge to practice; obtain direct, first-hand experiences with the local environment and apply what they have learned in the classroom to real-life situations. The integration of theory into practice has had a great impact on the environmental activities of society. The children in schools should be taught the role of trees, wild life etc. [11].

Professional development attempts have been made to improve EE in the regions. These attempts include holding pre-service, in-service, on-the-job and professional programs and forming of environmental educator associations as forums for environmental educators to share and exchange their knowledge, expertise and experiences. Likewise, funds for conducting research, scholarship grants for professional development and networks for education have helped foster environmental education in the region [2]. Preparation of the Teachers for EE through innovative strategies because teachers normally teach the way they were taught. Study done by [12] investigated the effects of value analysis, value clarification and action learning on the environmental knowledge, attitudes and problem solving skills of pre-service teachers in some Nigeria Colleges of Education. The study found out that value education strategies were more effective in promoting subjects' cognitive and affective achievement in environmental education than conventional lecture method [12]. Promoting the use of electronic media in the classroom and the inclusion of courses on their use in pre- and in-service teacher education programmes. It will also illustrate, through a case-study approach, how teachers can actively use such media in the promotion of environmental education [13]. Research done by [14] recognize the potential of the Web to enhance local, national and international co-operation, and to facilitate a better understanding of geographical and environmental issues at the grass-root level. Web-based learning can also help to increase and deepen the pupils' cultural understanding.

Environmental sites, through exploration of the surrounding environment, sites of special environmental significance, e.g. market place, waste dump, eroded land, forest, pond, farm, enterprise, can be identified and used for EE. Reorient the pedagogical approach: There is an urgency to reorient out existing teaching methods from 'chalk to talk' and lecture methods to problem-solving methods, from activity and issue-based approach to field work and case studies, from didactic to advise-based approach, and from rote learning to attitudes and competence development and learning through participation and educational training. Unless we care for the environment and use these materials carefully, some materials will get depleted gradually and get exhausted one fine day [15]. Networking and partnerships for strengthening of mechanisms for flexible, cross-sectoral, multi stakeholder and inter-governmental cooperation amongst relevant organizations, institutions and civil society to enhance the shared development and wise use of environmental education resources and programmes is encouraged [16]. Networks of the schools and their communities were strengthened through their interactive involvement in the learning process [8]. Participatory Monitoring and Assessment by engaging the learners in some aspect of environmental monitoring and/or assessment, e.g., measuring rain, quality of their own water supplies, amount of wood available, is a useful strategy for teaching about interactions and demonstrating man's impact on the natural resource base [4]. Use Youth Strength in Planning, Policy-Making, and Decision Making for EE through Youth Network, and Action Research, etc. are also an effective tool for reformation of EE. [17].

[III] CONCLUSION

In the light of country specific situations, more support for education, training and public awareness activities related to environment and development could be provided, in appropriate cases, through measures such as giving higher priority to those sectors in budget allocations, protecting them from structural cutting requirements; shifting allocations within existing education budgets in favor of primary education, with focus on environment and development; promoting conditions where a larger share of the cost is borne by local communities, with rich communities assisting poorer ones; obtaining additional funds from private donors concentrating on the poorest countries, and those with rates of literacy below 40%; encouraging debt for education swaps; lifting restrictions on private schooling and increasing the flow of funds from and to non-governmental

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organizations, including small-scale grass-roots organizations; promoting the effective use of existing facilities, for example, multiple school shifts, fuller development of open universities and other long-distance teaching; facilitating low-cost or no-cost use of mass media for the purposes of education; encouraging twinning of universities in developed and developing countries. There is still a considerable lack of awareness of the interrelated nature of all human activities and the environment, due to inaccurate or insufficient information. Developing countries in particular lack relevant technologies and expertise. There is a need to increase public sensitivity to environment and development problems and involvement in their solutions and foster a sense of personal environmental responsibility and greater motivation and commitment towards sustainable development.

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