

Diversity and decolorization potential of fungi isolated from the coral reef regions off Kavaratti, India.

Kaliyan Barathikannan¹, Kesava Priyan Ramasamy², Cathrine Sumathi Manohar* & Ram Murti Meena

CSIR-National Institute of Oceanography, Dona Paula, Goa. 403 004. India.

¹Present address: Department of Plant Biology and Biotechnology, Loyola College, Chennai, India.

²Present address: Dept of Bioscience and Biotechnology, University of Camerino, Italy.

[E.Mail: *cathrine@nio.org]

Received 13 June 2014; revised 11 November 2016

Forty-six marine-derived fungi were isolated from sea water, sand, sea grass and coral mucus collected from the coral reef regions off Kavaratti, Lakshadweep Islands, Arabian Sea. Of these, six cultures were screened for their extra-cellular enzymes and decolorization potential by qualitative plate assay method. A basidiomycete isolate F-38 was identified as the most potential fungus as it exhibited maximum cellulase, xylanase, laccase and decolorization activity on plate assay. It has maximum similarity with *Pseudoglarobasidium acaciicola* based on the BLASTn analysis of its ITS rDNA gene sequence. The isolate was able to decolorize Poly-R at three different concentrations; 40, 80 and 120 mg L⁻¹ up to 82%, 35% and 30% respectively. Major lignin degrading enzymes such as laccase, MnP and LiP activity was determined in the culture filtrate. The culture could also grow on corncob, utilizing it as a nutrient source and effectively decolorized Poly-R (40 mg L⁻¹) and the laccase activity increased a thousand fold.

[**Keywords:** decolorization, coral reef, fungi, marine, corncob, poly-R]

Introduction

Fungi are known to be effective against a large number of recalcitrant materials and can effectively aid in the bioremediation and treatment of industrial wastes¹. Fungal taxa belonging to phylum Basidiomycota, are known to produce some of the most efficient lignin-degrading enzymes which are widely used. The lignin degrading basidiomycete fungi are commonly called as white-rot fungi because they can produce extra-cellular enzymes that can degrade the complex lignin and the residual wood is left behind as a white powdery mass². Lignin is found in the cell walls of the plants and trees that give them their rigidity and strength. It is a complex polymer of p-hydroxycinnamyl alcohols and the monomeric units are linked through stable, non-hydrolysable carbon-carbon bonds or inert ether bonds. The resulting polymer is an irregular structure with complex three-dimensional network. The lignolytic enzymes synthesized by white-rot fungi have the inherent property to breakdown such complex polymers. Potential fungal cultures and their enzymes have been used for biotechnological applications for

the bioremediation and biodegradation of a range of xenobiotics including textile dyes³.

Dyes are synthetic aromatic compounds used to colorise products of many different industries such as textile industries, paper and pulp mills, tannaries etc. Textile effluents are a serious threat to the environment because of the chemical and physical nature of the coloured compounds or dyes which are present in it. Considering both the volume and the effluent composition the textile industry waste water is rated as the most polluting among all industrial sectors⁴. Hence, there is always the need to isolate³ and improve the activity of potential strains⁵ for efficient treatment and bioremediation of the toxic effluents released from the industries.

In this study we have isolated fungi from the pristine coral reef habitats and screened some selected cultures for their decolorization efficiency. The most efficient culture F-38, a non-sporulating basidiomycete isolate was studied in detail for their enzyme activity and decolorization potential using a model compound Poly-R. The high costs associated with bioremediation process still hinder their use on a large scale. Application

of crude enzyme extracts of potential cultures grown on agro wastes is one of the best options to reduce the cost involved. The enzyme production by the microorganisms is also largely dependent on the growth condition and the nutrient source⁶. The agro wastes such as corncobs, wheat and paddy straw are an important source of the essential nutrients and utilizing these cost effective alternatives augments the value of the bioremediation process^{7,8}. Here, the potential of corncob, an agricultural waste product was also studied as a nutrient source and the enzyme activity of the culture grown on corncobs was also determined.

Materials and Methods

Sampling and isolation of fungi

Fungi were isolated from sea water, sand and coral mucus collected from the coral reef regions off Kavaratti, Lakshadweep Islands in the Arabian Sea. Coral sand and water samples were collected by divers from a depth of approximately 4-5 m in clean, sterile sampling tubes. Mucus samples were collected from the branching coral *Acropora hyacinthus* using sterile syringes. The sampling tubes and syringes were immediately stored at 4°C and brought to lab under aseptic conditions. They were processed immediately within 24 hours for isolation of fungi.

Fungi were isolated from the coral sand using particle plating technique⁹ on petri plates with microbiological growth media such as Malt extract agar, Corn meal agar or Czapek Dox agar. All the media (HiMedia Pvt. Ltd., India) were used at 1/5 strength to discourage the growth of fast growing fungi. They were prepared in seawater and fortified with 10% antibiotic solution (Hi-Media Pvt. Ltd., India) to inhibit bacterial growth. Fungal colonies were isolated from water samples, by filtering 100 ml of it on sterile Durapore filters (0.45 µm, Millipore (India) Pvt. Ltd. India). The filter papers were placed on the culture plates with solidified media and incubated. 200 µL of the coral mucus was spread plated on the different agar media for isolation of fungi from coral mucus. These plates were incubated for 15 – 20 days at room temperature (~30 °C). Each of the fungal morphotype was designated with a unique number and catalogued as a separate isolate. They were routinely sub-cultured and checked for purity by light microscopy. The relative abundance of the isolates was calculated in percentage by dividing the number of isolations of a particular species by the total number of isolations obtained and multiplied by 100.

Qualitative screening of fungi

Preliminary screening of the isolates for their lignin-degrading enzyme activity was carried out on Boyd and Kholmeyer (BK) agar medium using model compounds such as Poly R-478 (Poly-R), guaiacol, carboxy methyl cellulose (CMC) and birch wood xylan. Decolorization of Poly-R from pink to yellow or colourless indicated the presence of lignin peroxidase and/or manganese peroxidase. The production of an intense brown colour in guaiacol supplemented media plates indicated the presence of laccase activity¹⁰. Cellulolytic and xylanolytic activity was determined based on the utilization of CMC or xylan in the media plates. When the colony reached an optimal growth phase, the plates were stained with Congo red and the medium did not take up the colour in the absence of CMC or xylan¹¹.

Identification of fungi

The fungal isolates used in this study were identified based on the internal transcribed spacer (ITS) region of the ribosomal DNA. Amplification of the target region was carried out using the fungal specific primers ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATGC). Amplified products were analyzed by electrophoresis on 1% agarose gel, then purified using PCR purification kit (Sigma-Aldrich, India) and sequenced. Sequencing was carried out in an Applied Biosystems (ABI) 3730 DNA Stretch Sequencer, with the XL Upgrade and the ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit. Sequence obtained was checked for PCR and sequencing errors and deposited in the GenBank under accession number KY235268 – KY235281. The sequence for each isolate was queried against NCBI-GenBank using BLASTn program for identification of its closest fungal taxa.

Culture conditions

Fungal isolate F-38, which showed maximum activity in plate assay was chosen for further experiments. They were grown in BK broth (20 ml in 100 ml Erlenmeyer flasks) for 7 days, homogenized mechanically in sterile sea water using sterile glass beads and the resulting suspension was used at 10% concentration (v/v) for inoculating 50 ml of BK broth in 250 ml Erlenmeyer flasks. Different concentrations of Poly-R were added to the BK broth for decolorization studies. The flasks were incubated under static conditions at room temperature in

triplicates for 15 days. At the end of the experimental period, the mycelia was harvested on pre-weighed filter paper (Whatman No. 1) and washed with distilled water. The dry biomass was determined by drying these filter papers in a freeze-dryer till the weight was stabilized. Filtrates were centrifuged at 10,000 rpm for 12 min to remove debris and suspended material, the recovered supernatant was used for quantifying enzyme activity and decolorization rate at the end of the experimental set-up.

Estimation of Poly-R decolorization

Poly-R was added separately to attain a final concentration of 40 mg L⁻¹, 80 mg L⁻¹ and 120 mg L⁻¹ to 3 day old cultures grown in BK broth and was considered to be the 0 day. Decolorization of Poly-R was monitored every third day till 15 days by determining the ratio of absorbance at 513 nm versus 362 nm. The final concentration of the dye in the medium on day 0 was considered to be 100%. The extent of decolorization was calculated from the mean values of triplicate readings¹⁰.

Approximately 5 g of the finely chopped corncobs moistened with 5 ml of BK broth was sterilized in 250 ml Erlenmeyer flasks. 50 ml of half-strength BK broth or sterile distilled water containing 40 mg L⁻¹ of Poly-R was added to three days old fungal culture grown on corncobs. The enzyme activity and decolorization rate was determined periodically as mentioned earlier till 15 days.

Quantitative estimation of the enzyme activity

Laccase activity was assayed in the culture supernatant by measuring oxidation of 1mM of 2,2'-azinobis-(3-ethylbenzothiazoline) (ABTS)

substrate buffered with 0.2 M Glycine-hydrochloride buffer, pH 3 at 405 nm. Laccase units (U) were calculated as micromoles of the substrate transformed per minute per liter of culture supernatant and expressed as UL⁻¹. Manganese-dependent peroxidase (MnP) assay was determined by measuring the rate of oxidation of veratryl alcohol to veratraldehyde in the presence of Mn. Units were calculated as micromole of the substrate transformed per minute per litre of culture supernatant expressed as UL⁻¹. Lignin peroxidase (LiP) activity was determined by the oxidation of veratryl alcohol to veratraldehyde¹⁰.

Results

Isolation and screening of fungi for lignin-degrading enzymes

Forty-six marine-derived fungi with representatives from mycelia forms and yeasts were isolated from coral reef regions. Fungal isolates were grouped into 15 distinct genera based on the BLASTn analysis of their internal transcribed spacer region of the rDNA (ITS rDNA) gene sequence. Maximum representation was by *Aspergillus* sp. (30%) followed by *Engyodontium* sp. (23%), *Acremonium* sp. (9%) and *Cladosporium* sp. (7%) (Table 1). Fungal cultures isolated from the sediment and water column accounted for 39 and 36% respectively. The relative abundance of fungal cultures was restricted to 11 and 14 % from coral mucus and sea grass samples.

Six mycelial fungi were selected based on their growth characteristics and screened for their efficiency to produce lignolytic enzymes by plate assay method. Of all the cultures screened F-9 showed week cellulolytic activity but prominent

Table 1-List of isolates and their closest identified taxa based on the ITS rDNA BLASTn analysis and its relative abundance

Isolate No.	Name	Closest identified taxa				Total (%)			
		Accession number	Identity (%)	Relative abundance in substrates (%)					
				Mucus	sediment	water	sea grass		
F-1	<i>Aspergillus</i> sp. [€]			-	20	7	2	30	
F-6	<i>Acremonium</i> sp.	JX262800.1	94	-	5	2	2	9	
F-7	<i>Camarosporium</i> sp.	JQ388925.1	93	-	-	2	-	2	
F-9	<i>Humicola</i> sp.	HQ608016.1	88	2	-	-	-	2	
F-11	<i>Cladosporium</i> sp.	AB572900.1	85	-	5	2	-	7	
F-13	<i>Rhodospiridium</i> sp.	JN383910.1	85	2	-	-	-	2	
F-15	<i>Nectria</i> sp.	JX868649.1	98	-	-	5	-	5	
F-17	<i>Engyodontium</i> sp.	KC311469.1	81	5	5	12	2	23	
F-18	<i>Mycosphaerella</i> sp.	JQ732916.1	99	2	-	-	-	2	
F-23	<i>Gymnoascus</i> sp.	AJ315836.1	99	-	-	-	2	2	
F-25	<i>Gliomastix</i> sp.	AB540561.1	99	-	-	-	2	2	
F-28	<i>Trichoderma</i> sp.	JN704354.1	99	-	-	-	2	2	
F-33	<i>Lepidosphaeria</i> sp.	GQ203760.1	88	-	-	5	-	5	
F-38	<i>Pseudolagarobasidium</i> sp.	JQ731604.1	99	-	2	2	-	5	
F-46	<i>Venturiaceae</i> sp.	HQ634611.1	91	-	2	-	-	2	
Total (%)					11	39	36	14	100

[€]Morphological identification

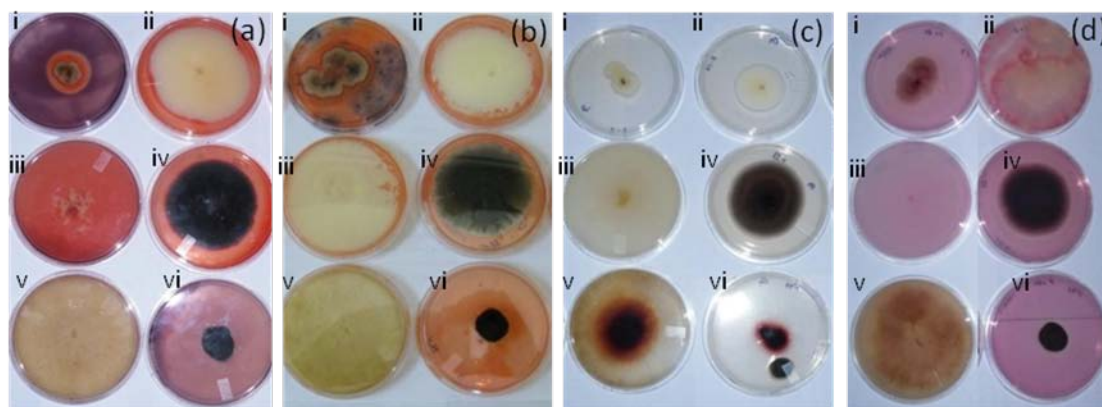


Fig.1—Plate assay for lignin-degrading enzymes using model compounds carboxy methyl cellulose (a), birch wood xylan (b), guaiacol (c) and Poly-R (d) for the isolates F-9 (i), F-17 (ii), F-23 (iii), F-33 (iv), F-38 (v) and F-46 (vi).

clearance zones were evident in F-17 and F-38 plates (Fig. 1a). Xylonolytic activity could be observed in four cultures (F-9, F-17, F-23 and F-38) and the maximum activity was by F-38 (Fig. 1b). Browning of media due to oxidation of guaiacol could be clearly seen in F-38 plates and F-46 showed some weak activity (Fig. 1c). Prominent decolorization activity was observed only in isolate F-38 from day 5 onwards and by Day-15 the plate was almost completely decolorized (Fig. 1d). Fungal isolate F-38, a basidiomycete showed activity on all the four plates used for qualitative analysis showing that it synthesized all the major lignin degrading enzymes such as LiP, MnP and Laccase. Such a potential fungal isolate which can produce a wide range of enzymes and exhibits synergistic effect has a huge potential for bioremediation of industrial effluents. Hence, the isolate F-38 was selected for further studies to quantify their enzyme activity and decolorization potential.

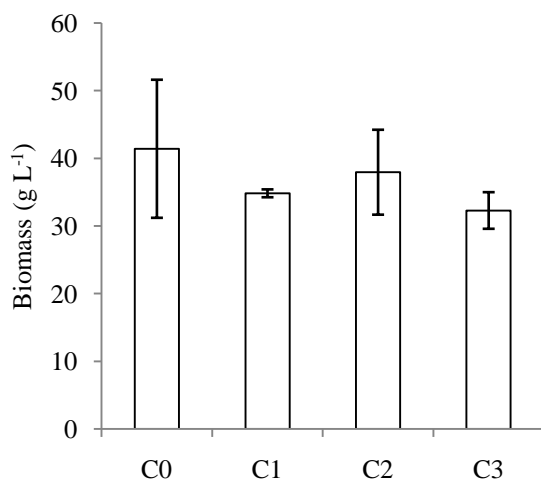


Fig. 2—Growth of F-38 as dry weight (g L⁻¹) in BK (C0), BK with 40 (C1), 80 (C2) and 120 (C3) mg L⁻¹ Poly-R.

Growth and decolorization of Poly-R dye in liquid media

Poly-R is an anthraquinone-based polymeric dye and structurally resembles certain polycyclic aromatic compounds that are substrates of ligninolytic peroxidases. It has been used widely as an effective screening method for determining the ability of the cultures to degrade coloured compounds. The growth of F-38 in BK and in the broth with Poly-R supplement was estimated as dry weight (g L⁻¹). The biomass values showed that there was about 25 % decrease in biomass when the culture was grown with Poly-R (Fig.2). F-38, was able to decolorize Poly-R at three different concentrations; 40 mg L⁻¹ (C1), 80 mg L⁻¹ (C2) and 120 mg L⁻¹ (C3) upto 82%, 35% and 30% respectively. The culture could grow on corncobs moistened with BK broth and their decolorization efficiency increased up to approximately 90% (Fig.3). The results obtained shows that the F-38 could decolorize Poly-R (40 mg L⁻¹) dissolved in half-strength BK broth (C4) or sterile distilled water (C5) by utilizing the nutrients from the corncobs.

Enzyme activity

Dye decolorization process involves the activity of extracellular enzymes such as LiP, MnP and Laccase activity. These were estimated in the crude culture filtrate of F-38 isolate grown in BK broth with and without Poly-R 478 supplementation. F-38 produced only a small amount (0.17) of laccase when grown on plain BK broth. With Poly-R addition, the enzyme activity increased to 1.77, 1.65 and 1.54 UL⁻¹ (Table 2). MnP and LiP activity in BK broth was 78.84 and 6.45 UL⁻¹ respectively; however there was a reduction in activity with Poly-R addition (Table 2). In this study we also attempted to study the efficiency of the isolate F-38 to use the

Table 2—Extra cellular enzyme activity of isolate F-38 in the crude culture filtrate

Growth condition	Enzyme activity (UL ⁻¹) (standard deviation values)		
	Laccase	MnP	LiP
BK broth	0.17 (0.10)	78.84 (19.98)	6.45 (2.60)
BK broth + Poly-R (40 mg L ⁻¹)	1.77 (0.85)	40.00 (8.81)	3.22 (1.48)
BK broth + Poly-R (80 mg L ⁻¹)	1.65 (0.31)	15.38 (1.38)	1.29 (0.96)
BK broth + Poly-R (120 mg L ⁻¹)	1.54 (0.43)	3.07 (1.86)	0.86 (1.37)
Corncob + BK broth ^ε + Poly-R (40 mg L ⁻¹)	281 (48.4)	17.82 (11.01)	3.02 (2.28)
Corncob + DW + Poly-R (40 mg L ⁻¹) ^ε half strength	988.04 (256.24)	17.09 (10.12)	4.36 (11.28)

agricultural waste corncobs as a substrate and synthesize enzymes. Corncobs, rich in cellulose served as a nutrient source for the culture and also acted as an inducer (Table 2). The nutrient source was gradually reduced by flooding the flask containing F-38 grown on corncobs with half-strength BK broth supplemented with Poly-R (C4). The laccase activity increased to 281 UL⁻¹ and MnP and LiP activity recorded was 17.82 and 3.02 UL⁻¹ respectively. The external nutrient source was further reduced and the culture was exposed to Poly-R dissolved in sterile distilled water (C5). Laccase activity increased greatly to 988 UL⁻¹ and moderate MnP (10.12 UL⁻¹) and LiP (4.32 UL⁻¹) production was observed. This was accompanied by decolorization activity which was equivalent to the activity when F-38 was grown in a BK broth supplemented with the same concentration of Poly-R (Fig. 3).

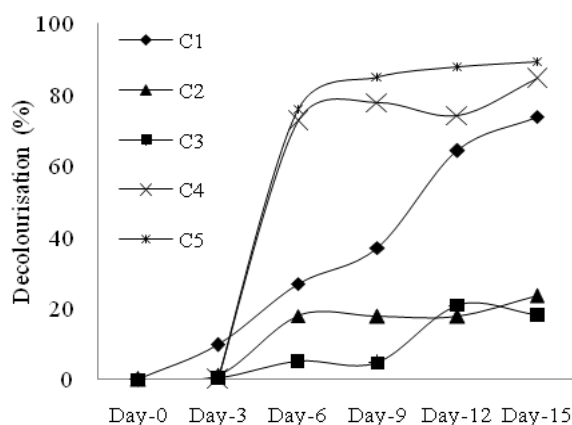


Fig. 3—Decolorization of Poly-R by F-38 grown in BK supplemented with 40 (C1), 80 (C2), 120 (C3) mg L⁻¹ poly-R, on corncobs flooded with half strength BK broth supplemented with 40 mg L⁻¹ poly-R (C4), on corncobs flooded with water containing poly-R 40 mg L⁻¹ (C5), mean values (n=3).

Discussion

Association of fungi with the corals and its skeleton has been largely considered to be pathogenic in nature^{12,13}. However, the symbiotic relationship of fungi in the coral reef ecosystem and their role in the nutrient cycling is becoming

evident with recent developments and application of molecular tools for the studies^{14,15}. Many novel and diverse fungal cultures with very high biotechnological potential have been isolated from the coral reef ecosystem and associated macroorganisms. Elucidation of fungal diversity using molecular tools from these regions has shown that they are metabolically active and phylogenetically diverse¹⁶. The fungal cultures from this study shows a high frequency of terrestrial cultures and included many infectious and pathogenic forms. Their distribution was higher in sediment, water samples followed by sea grass samples and the least in coral mucus (Table 1). The cultures isolated from the coral mucus included the *Humicola* sp., *Rhodospiridium* sp. and *Mycosphaerella* sp., which were mostly non-pathogenic, saprophytes and *Engyodontium* sp. was the only pathogenic form. The ecological role of the pathogenic or parasitic forms in the coral reef system was not determined. The reduced number of these infectious isolates in the coral mucus shows that they have a defense mechanism and can choose their microbial associates. Fungi are opportunistic pathogens and these species may have existed as saprophytes thriving on the organic matter present in the reef system. Marine-derived fungi from the coral reef regions are known as an important source for a number of extracellular enzymes and secondary metabolites which has a wide range of biotechnological applications.

In our study, we screened six fungal isolates which included F9 and F17, isolated from the coral mucus and F23 from the sea grass. F33, F46 isolated from water and sediment respectively and F-38 which was retrieved from both sediment and water samples were screened for their lignolytic activity by plate assay method. F-38 showed maximum activity on all the four media plates, showing the white-rot fungi had a greater potential than other isolates in their production of lignolytic enzymes. This isolate had maximum similarity with basidiomycete taxa *Pseudolagarobasidium acaciicola*, which was first described from South Africa as a plant

pathogen. *P. acaciicola* is also widely used as a mycoherbicide, to control the invasion of *Acacia* species in South Africa¹⁷. However, this basidiomycete fungus has not been isolated from any of the marine ecosystems and has not been so far tested for its lignolytic properties or for its bioremediation potential.

Isolate, F-38 grew in BK broth prepared in sea water and synthesized lignolytic enzymes Laccase at very low levels (0.17 UL^{-1}) and produced good concentration of MnP (78.84 UL^{-1}) and LiP (6.45 UL^{-1}) which has a greater potential for bioremediation application. The isolate was able to decolorize Poly-R (40 mg L^{-1}) up to 80% of and the enzyme activity of Laccase, MnP and LiP was 1.77, 40 and 3.22 UL^{-1} respectively. The culture was also able to grow on corncobs flooded with half-strength BK and decolorize the same concentration of Poly-R (40 mg L^{-1}) up to 84% (Fig.3). Isolate is also capable of growing on corncobs as the sole nutrient source and decolorize the same concentration of Poly-R supplemented by dissolving it in distilled water (C5). In this growth condition the culture showed a thousand fold increase in laccase activity, a marginal increase in MnP activity, 50% LiP activity and decolorization of Poly-R upto 89% (Fig.3). This clearly shows that the isolate can be utilized for bioremediation of coloured effluents.

Conclusion

Remediation of the textile effluents using fungi and their enzymes has been a popular method. The molecular mechanism involved in the breakdown of lignin by lignolytic enzymes of fungi is also greatly understood⁴. However, these enzymes which have wide biotechnological applications are still not commercially available and utilised. The basidiomycete fungus identified from this study with its ability to grow on agricultural waste and decolorize textile dyes proves to be a candidate species. Such potential isolates can be effectively optimized for industrial applications.

Acknowledgment

The first author thanks the Director, CSIR-National Institute of Oceanography, for providing all the required facilities to carry out the dissertation work during his post graduate program. This is NIO's contribution number 5960.

References

- 1 Francisco J R-D & Martinez AT, Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this, *Microb Biotechnol*, 2 (2009) 164-177.
- 2 Arora D S & Sharma R K, Ligninolytic fungal laccases and their biotechnological applications, *Appl Biochem Biotechnol*, 160 (2010) 1760-1788.
- 3 Harms H, Schlosser D & Lukas Y W, Untapped potential: exploiting fungi in bioremediation of hazardous chemicals, *Nat Rev Microbiol*, 9 (2011) 177-192.
- 4 Vilaseca M, Gutie M C, Grimau VL, Mesas M L & Crespi M, Biological treatment of a textile effluent after electrochemical oxidation of reactive dyes, *Water Environ Res*, 82 (2010) 176-181.
- 5 Khanam R & Gyana Prasuna R, Strain improvement of white rot fungi *Pycnoporus cinnabarinus* with the influence of physical and chemical mutagens for enhancing Laccases production, *J Sci Ind Res*, 73 (2014) 331-337.
- 6 D'Souza-Ticlo D, Verma A K, Mathew M & Raghukumar C, Effect of nutrient nitrogen on laccase production, its isozyme pattern and effluent decolorization by the fungus NIOCC No. 2a, isolated from mangrove wood. *Indian J Mar Sci*, 35 (2006) 364-372.
- 7 Hossain S M & Anantharaman N, Effect of wheat straw powder on enhancement of lignolytic enzyme activity using *Phanerochate chrysosporium*, *Indian J Biotechnol*, 7 (2008) 502-507.
- 8 Brijwani K, Oberoi H S & Vadlani V P, Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran, *Process Biochem*, 45 (2010) 120-128.
- 9 Bills G F & Polishook J D, Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica, *Mycologia*, 86 (1994) 187-198.
- 10 D'Souza-Ticlo D, Tiwari R, Sah AK & Raghukumar C, Enhanced production of laccase by a marine fungus during treatment of colored effluents and synthetic dyes, *Enzyme Microb Technol*, 38 (2006) 504-511.
- 11 Teather R M & Wood P J, Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen, *Appl Environ Microbiol*, 43 (1982) 777-780.
- 12 Yarden O, Ainsworth T D, Roff G, Leggat W, Fine M & Hoegh-Guldberg O, Increased Prevalence of Ubiquitous Ascomycetes in an Acropoid Coral (*Acropora formosa*) Exhibiting Symptoms of Brown Band Syndrome and Skeletal Eroding Band Disease, *Appl Environ Microbiol*, 73 (2007) 2755-2757.
- 13 Vega Thurber R, Willner-Hall D, Rodriguez-Mueller B, Desnues C, Edwards RA, *et al*, Metagenomic analysis of stressed coral holobionts, *Environ Microbiol*, 8 (2009) 2148-2163.
- 14 Wegley L, Edwards R, Rodriguez-Brito B, Liu H & Rohwer F, Metagenomic analysis of the microbial community associated with the coral *Porites astreoides*, *Environ Microbiol*, 9 (2007) 2707-2719.
- 15 Raghukumar C & Ravindran J, Fungi and their role in corals and coral reef ecosystems, in: *Biology of Marine Fungi*, edited by C. Raghukumar, (Springer, Berlin) 2012, pp. 89-113.

- 16 Amend A S, Barshis J D & Oliver T A, Coral-associated marine fungi form novel lineages and heterogeneous assemblages, *ISME J*, 6 (2012) 1291–1301.
- 17 Impson F A C, Kleinjan C A, Hoffmann J H, Post J A & Wood A R, Biological Control of Australian *Acacia* Species and *Paraserianthes lophantha* (Willd.) Nielsen (Mimosaceae) in South Africa, *Afr Entomol*, 19 (2011) 186-207.