

Original Research Article

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Identifying the Strains having Ability to Produce Ligninolytic Enzyme Laccase from the Plethora of Actinomycetes Isolated from Soil and Mangrove Samples

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ABSTRACT

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To identify laccase producing actinomycete strains a different approach was used in this study. Actinomycete strains were first grown in the presence of an inducer to better the enzyme induction. Later these strains were screened for the production of laccase enzyme which is very scarce in this group of bacteria. 62 actinomycete strains were isolated from the soil and mangrove samples collected from various locales. Aromatic inducers like guaiacol, 2,5 xylidine and tween 80 at the concentrations of 1mM were incorporated in the liquid medium. Also a lignin rich natural inducer pearl millet (bajra) powder at the concentration of 8% was incorporated in the liquid medium. The induction process with the variety of inducers was carried out for all the isolates for 2 weeks. After induction, enzyme activity was detected qualitatively by incorporating guaiacol in the medium. One isolate showed a reddish brown halo around the colony after 72 hours of incubation at 37⁰C indicating laccase positive activity. Laccase activity of this isolate was further confirmed by decolorization of two dyes RBBR and congo red.

Introduction

Actinomycetes are organisms having morphological characteristics similar to fungi. But they are prokaryotic in nature and taxonomically categorized as filamentous bacteria. On culture media they grow slowly giving rise to colonies with powdery consistency sticking firmly to the agar surface.

Actinomycetes are abundant in soil giving a count upto 10⁸ cells per gram of soil. They are aerobic, heterotrophic and mostly

mesophilic with some of the strains showing thermophilic growth (Hildén *et al.*, 2009). Actinomycetes play a very significant role in the degradation of lignocellulosic material (Claus 2003). They have attracted the attention owing to their ability to degrade lignin from lignocellulosic biomass. Lignin, a component of the lignocellulosic material, which is the most 'difficult to degrade', is degraded by three types of enzymes namely lignin peroxidase, Mn peroxidase and laccase (Claus 2004). Laccases are of importance as they can degrade polyphenol,

the principal recalcitrant component of lignin (Niladevi *et al.*, 2008). Laccase (benzenediol: oxygen oxidoreductases, E.C 1.10.3.2) is a multi-copper enzyme belonging to the group of blue oxidases. Laccase is described as oxidoreductase in Enzyme Commission (EC), which oxidizes diphenol and allied substances and use molecular oxygen as an electron acceptor (Tatiana *et al.*, 2010).

Actinomycetes as a Source of Laccase

Until recently laccases were found only in eukaryotes, e.g. fungi, plants, insects. Now it is evident that multi-copper oxidase proteins do exist in actinomycetes indicating laccase enzyme activity (Alexandre *et al.*, 2000).

Laccase is an inducible enzyme and shows enhanced expression if grown in the presence of inducers (Gnanamani *et al.*, 2006). List of inducers for laccase production includes variety of sources, rich in lignin &/or polyphenols. Lignin rich natural sources include wheat bran, flax seeds, bajra (pearl millet), barley and fruits such as kiwi, apple, black grapes etc. Some of the aromatic inducers are guaiacol, xylidene, tween 80 etc (Sampoorna *et al.*, 2010).

This study has used the fact that laccase is an inducible enzyme and the experiment was designed in such a way that it has allowed the growth of actinomycete strains in the presence of inducers, thus helping in the induction and detection of enzyme laccase (Kiiskinen *et al.*, 2004).

Materials and Methods

Sample Collection and Isolation of Actinomycetes

38 samples were collected from different geographical locations, to name a few, Kerala coffee plantation, Bandipur reserve, Dudhsagar forest, compost, vermi-compost,

Aarey river sediments, Borivali national park mangrove areas in Versova (Mumbai), Airoli (New Mumbai) and Goa (Rajkumar *et al.*, 2012). Samples were collected in zip lock polythene bags using alcohol sterilized spoons/scalpels. Samples once collected were kept in the refrigerator and processed within 24-48 hours. All the samples were air dried for 24 hours before processing. Air-dried samples were homogenized using mortar and pestle and sieved through 2mm sieve. Ringer's solution (g/L- NaCl 8.5, KCl 0.2, CaCl₂ 0.2, NaHCO₃ 0.01, Distilled water 1000ml) was used for sample dilution. Sterile 50% sea water was used as a diluent for samples collected from mangrove regions. Ten fold serial dilutions were carried out upto 10⁻⁷. Actinomycete Isolation Agar at pH 8 (Hi-media) was used for the isolation of actinomycetes from the samples. Antibiotics Cycloheximide – 50ug/ml and Streptomycin 100ug/ml were added to the medium. Plates were incubated at 37^oC and checked for growth. Incubation period varied from 1-3 weeks. Actinomycete colonies were identified by their morphological characteristics and microscopy.

Design of the Basal Medium

50ml of basal medium was taken in each flask.

Different inducers were incorporated in the medium keeping the composition of the basal medium constant (Susana *et al.*, 2002).

Composition of the medium(g/L)- (NH₄)₂SO₄ 2.6, K₂HPO₄ 1, KH₂PO₄ 0.5, MgSO₄ 0.2, yeast extract 1, Maltose 20mM, Asparagine 2. Asparagine is added as the source of organic nitrogen. High nitrogen concentration has found to improve the laccase production.

Sterile trace solution 0.1% v/v was added to the basal medium after autoclaving.

Composition of the trace solution (g/L)-
CuSO₄.7H₂O-0.025, CaCl₂.2H₂O-1.5,
ZnSO₄.7H₂O-0.3, FeSO₄.7H₂O-0.25,
MnCl₂.5H₂O-0.2, COCl₂.6H₂O-0.05,
MgSO₄.7H₂O-2.5. Trace solution was
sterilized separately by autoclaving.

Inducers used:

- (1) 2,5 xylidene dissolved in 95% ethanol, filter sterilized. Added in the autoclaved medium to get the final concentration 1mM.
- (2) Tween 80 dissolved in 95% ethanol, filter sterilized. Added in the autoclaved medium to get the final concentration 1mM.
- (3) Guaiacol dissolved in 95% ethanol, added to the medium to get the concentration of 1mM before autoclaving.
- (4) Bajra (Pearl millet) powder (8 gm%) added to the medium before autoclaving.

For mangrove isolates above media were prepared in 50% sea water (Kizhakkedathu *et al.*, 2005).

Inoculation and incubation of the Culture for Inducing the Enzyme Production

For all the actinomycete strains inoculation in 50ml of broth with inducer was done by picking up the actinomycete colony from the agar surface with the help of sterile toothpick.

Incubation was done at 37⁰C under static condition for 2 weeks.

Plate Assay Using Guaiacol

Medium used for the qualitative laccase plate assay had the basal agar with trace solution and 0.01% guaiacol added to it.

Confirmation of Laccase Activity by RBBR Decolorization and Congo Red Decolorization

RBBR decolorization: Basal medium with filter sterilized RBBR was used to get the final concentration of 100ppm. Sterile RBBR was added to the medium after autoclaving. Culture was spot inoculated on the agar surface and incubated at 37⁰C for 7 days (Palmieri *et al.*, 2005).

Congo red decolorization was carried out in nutrient broth. 100ppm concentration of congo red was added in nutrient broth before autoclaving. Culture was inoculated by picking up a colony with a sterile toothpick and incubated at 37⁰C for 3 days (Pointing *et al.*, 2000).

Results and Discussion

Isolates were numbered as A-1 through 62. Out of 62 isolates screened using 4 inducers, only one isolate A-25 indicated laccase activity. Positive laccase activity was indicated by the reddish brown halo formed around the colony when guaiacol was incorporated in the medium.

Positive laccase activity by A-25 could be detected for 3 inducers - guaiacol, Tween 80 and 2,5 xylidene. Activity could be seen on the medium containing guaiacol after 72 hours of incubation on all the 3 guaiacol plates from the 3 respective inducers. (Vaidyanathan *et al.*, 2011).

Laccase production was further confirmed by the use of RBBR dye (Kátia *et al.*, 2005). Positive result was shown by the RBBR decolorization indicated by clear zone around the colony after 7 days of incubation at 37⁰C (Margot *et al.*, 2013).

Table.1 Congo Red Decolorization was Detected Using UV Spectrophotometer (Shimadzu).
Medium used – Nutrient broth (NB)

Azo dye	Absorption maxima	Concentration of the dye used	Un-inoculated control (NB+Dye)	Test (after 72 hours)
			Absorbance at 480nm	
Congo red	480nm	100ppm	2.33	0.93
Isolate gave 60% Congo red decolourization				

Fig.1 Laccase Positive Activity on Agar with Guaiacol



Fig.2 Laccase Positive Activity in the Broth with Guaiacol



Fig.3 RBBR Positive Activity by A-25

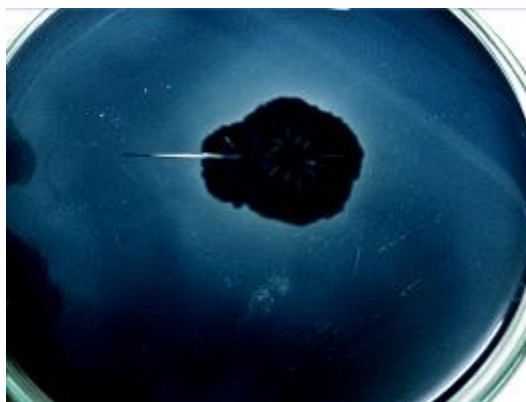
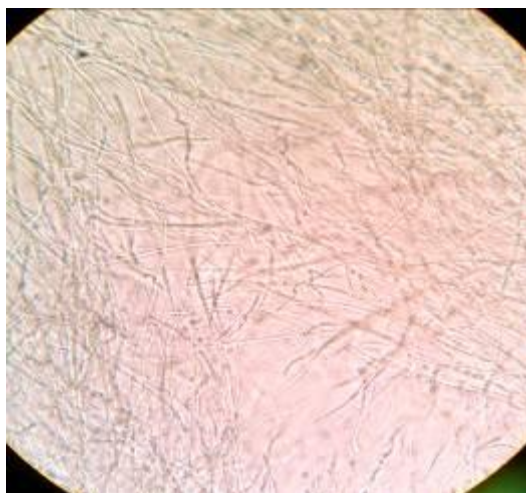


Fig.4 Morphology of the Laccase Positive Isolate A 25 Observed Under Phase Contrast Microscope



Laccases from actinomycetes are scarcely found in nature. Literature has indicated the wide spread distribution of laccase genes in prokaryotes but expression of these genes needs an inducer molecule. Many studies have been carried out making use of inducer molecules for improving the fungal as well as bacterial laccase production during submerged fermentation.

In this study one naturally available inducer and three aromatic inducers were used prior to screening to induce the enzyme production thus making its detection possible. Starting with 62 isolates and each being exposed to 4 inducers, one isolate

could be obtained as a laccase producer. This indicated rare occurrence of laccase expression by actinomycetes among prokaryotes.

Currently, less than 10 enzymes from actinobacteria have been properly characterized, all belonging to genus *Streptomyces*, but it is noteworthy that all of them have exhibited industrially important properties. Furthermore, studies with enzymes from this phylum revealed a novel molecular structure of laccases, providing the basis for a distinct family, the two-domain laccases. The commercial potential of actinomycete laccases emphasize the

need for more studies involving the isolation of this bacterial group from lignin-rich environmental samples, detection of their laccase activity and thereafter, characterization of the proteins and related genes.

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