Production of laccase by the phytopathogenic fungus *Rhizoctonia solani*

By

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Declaration

I hereby declare that this thesis is my own account of my research. The content of this research has not previously been submitted for a degree at any tertiary educational institution.

Place:

Date: ***Pranjal Bora***

Dedication

I dedicate this thesis to my dearest parents and my wife for going through so much hardships while I was undertaking this work. Without your help and support I could not have achieved this.

ABBREVIATIONS

A_{280}	Absorbance at 280 nm
Å	Angstrom unit (10^{-1} nm).
ABTS	2',2'-Azino-Bis (3-Ethylbenzthiazoline-6-sulphonic acid) Diammonium salt.
AG	Anastomosis group
bp	Base pair
BSA	Bovine serum albumin
ºC	Degree centigrade
CDOX	Czapek Dox growth medium
cm	Centimetre
cm^2	Square centimetre
CMC	Carboxymethyl cellulose
Co	Consensus
DE 52	Diethyl fifty two (anion exhchange chromatography medium)
DNS	Dinitrosalicyclic acid
dNTP	Deoxynucleotide triphosphate
Fig.	Figure
HV	Highly virulent
IPTG I	isopropyl-β-D-thiogalactoside
L	Litre
LB	Luria-Bertani medium
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agarose
PGA	Polygalacturonic acid
RAPD	Randomly amplified polymorphic DNA
SDS	Sodium dodecyl sulphate
Tween-20	Polyoxyethylenesorbitan monolaurate

ABSTRACT

This thesis is an investigation into the production of laccases by the phytopathogenic soil fungus *Rhizoctonia solani*. The fungus causes maceration of plant tissue by the production of a variety of plant cell wall degrading enzymes. Whilst most attention has focused on the role of pectinases in maceration, the laccases which degrade lignin are likely to be important in this process.

The production of laccase by the AG-11 isolate VR20 in V8 medium reached a maximum after 6 days incubation. Laccase activity was unaffected by variation in temperatures over the range 4-15°C, but as the temperature increased the activity increased to a maximum at 25°C. This high level activity was maintained as the temperature was increased to 37°C. The effects of pH on laccase activity was also determined. Activity was stable over the pH range 4.5-6. Outside this range the activity decreased significantly.

The composition of the growth medium also had a significant effect on laccase production. Similar levels of activity were observed during growth in V8, apple pectin media, or in media containing ground up lupin hypocotyls as a carbon source. However, approx 20 fold higher levels were obtained after growth in Czapek-Dox medium. Different laccase activity band patterns were obtained by zymogram analysis of culture supernatants. The production of the other cell wall degrading enzymes pectinase,

xylanase and cellulase in these media was assessed for comparison. Whilst all three were produced in V8 and apple pectin media, cellulase was not produced in lupin medium, and none of these were produced in Czapek-Dox medium.

Attempts to increase laccase production by the addition of the reported laccase inducers $CuSO_4.5H_2O$, *p*-anisidine, ethanol, $MnSO_4.7H_2O$, resveratrol, and tannic acid to the growth medium showed mixed results. The only case where enhancement of synthesis was observed was with the addition of $MnSO_4$ to Czapek-Dox medium. This compound did not enhance production in the other media tested. With the exception of *p*-anisidine, the other inducers had minimal effect in V8, apple pectin or lupin media. Para anisidine completely inhibited production in lupin medium. With the exception of $MnSO_4$, all inducers inhibited laccase production in Czapek-Dox medium, with *p*-anisidine causing complete inhibition.

The production of xylanase and cellulase was also inhibited by these inducers but in a growth medium dependent manner. Cellulase production in V8 medium was inhibited by ethanol, $MnSO_4$, resveratrol, and tannic acid whilst only the latter two inhibited xylanase production and none of these inhibited pectinase production. In contrast, *p*-anisidine had a greater inhibitory effect on pectinase and xylanase production in V8 medium than on cellulase production. Para-anisidine also inhibited xylanase (and laccase) but not pectinase production in lupin medium but not in apple pectin medium. Resveratrol and tannic acid also inhibited xylanase production in lupin medium.

The effects of the inhibitory compounds EDTA and SDS on laccase activity was determined. With SDS the % inhibition increased as the concentration of inhibitor decreased from 5% to 0.5%. With EDTA the opposite trend was observed. The effects of arginine on laccase activity was also tested. At concentrations of 0.5 to 5% arginine, laccase activity was completely inhibited.

Laccase activity was purified from the culture supernatant by anion exchange chromatography, and by electroelution from a native-PAGE gel. The degree of purification by each method was greater than 50 fold. Electrophoresis of the purified protein on an SDS-PAGE gel followed by staining with Coomassie blue showed two protein bands of 66 and 38 KDa. Measurement of the absorption spectrum of the purified protein showed two absorbance maxima, at 240 and 340nm.

Laccases produced by isolates from different anastomosis groups were analysed by staining gels for laccase activity. Variations in the band pattern were observed both between and within anastomosis groups. Analysis of single spore isolates from AG-8 and AG-11 showed segregation of band patterns. However the sample sizes were too small to make conclusions about the numbers of laccase enzymes produced by or the number of genes in the parent field isolates.

The role of laccases in maceration of lupin radicle tissue was investigated. Microscopic staining showed the presence of lignin in radicle tissue, and when incubated in the fungal enzymes the tissue lost integrity, characteristic symptoms of maceration. Maceration was

readily observed as discolouring when the radicle was incubated in a solution of fungal enzyme. The degree of maceration was quantified by measuring the length of the discoloured region. Enzymes from all of the isolates tested caused maceration of lupin radicle. The degree of maceration ranged from 80-100%. No maceration was observed when agrinine was included in the reaction mixture. Arginine does not inhibit the activity of pectinases, xylanases, or cellulases. In maceration assays with potato tuber tissue which does not contain lignin, the addition of arginine to the reaction did not inhibit maceration. The results show that laccases are required for maceration of lignified tissue, but not for non-lignified tissue.

Laccase gene sequences were cloned from three isolates, SCR122 (AG-6), 11034 (AG-8), and VR20 (AG-11) using degenerate primers to conserved sequences to amplify the gene sequences. The amplicons were cloned and sequenced. A BLAST search of the NCBI database with the derived amino acid sequences confirmed that the sequences were from laccase genes.

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