Materials and Methods

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MATERIALS AND METHODS

Laccases are widely distributed in nature and have been described in fungi plants, insects, and, more recently, in bacteria (Guijarro *et al.*, 2009).

The successful use of laccases in bioremediation processes is based both on obtaining organisms that produce enzymes with the best catalytic properties and on establishment of the conditions for development of strains that produce high levels of these enzymes. Concomitantly, over expression of laccase in suitable host organisms would provide means to achieve high titers. Use of inducers could also enhance production capabilities. To make laccases available for industrial applications, methods to reduce costs include fermentation media optimization, novel fermentation methods, and genetic modification for large scale production via eukaryotic recombinant strains. Much research has been done to identify effective methods for mass production of laccase (Diaz *et al.*, 2011).

The present study was focused on to determine the media components critical to laccase production and optimize their concentration and cultivation conditions that support high laccase yield from the isolated bacterial strain. The critical components for laccase production were first identified by "one time one factor" method. The concentrations of these components were further optimized using the statistical method namely Plackett -Burman Design. The methodology adopted to present study is discussed under the following headings.

3.1 CULTURE MAINTENANCE

3.2 OPTIMIZATION OF CULTURE CONDITIONS FOR LACCASE PRODUCTION

3.2.1 One Time One Factor approach

3.2.2 Screening of variables by Plackett-Burman Design.

3.3 STATISTICAL SOFTWARE

3.1 CULTURE MAINTENANCE

The bacterial strain was isolated from textile industry effluent. The isolated bacterial species were identified as Bacillus sp. based on biochemical tests. The species were selected for studying the laccase production and its optimization using statistical design of experiments. The stock culture was maintained on nutrient agar slants at 4°C with periodic transfer. For production of inoculum, the culture from the slant was transferred to Luria-Bertani (LB) Broth (Appendix-1) and incubated at 37°C. 5 ml of overnight grown culture was added to 25 ml of LB broth incubated at 37^0C for 12- 16 hrs. From this 1 ml was inoculated into Minimal Salt Medium. The Composition of Minimal Salt Medium is given in Appendix - II.

3.2 OPTIMIZATION OF CULTURE CONDITIONS FOR LACCASE PRODUCTION

3.2.1 One Time One Factor approach

The optimization of physicochemical conditions was carried out by varying the levels of one independent variable by fixing other variables at a certain constant level. The selected variables were inoculum age, inoculum size, carbon sources, nitrogen sources, metal ions, inducers, surfactants and temperature. The bacterial growth in terms of biomass production

(Appendix-III) and laccase activity (Appendix-IV) were determined under each set of experimental conditions.

Effect of inoculum age

The effect of age of inoculum on laccase production by the strain was studied using inocula aged 6, 12, 24 and 48 hrs. These differently aged cultures were inoculated into 100 ml of Minimal Salt Medium. The flasks were incubated at 37^0C for 24 hrs. The treatments were carried out in duplicates.

Effect of inoculum size

Ten different inocula size of 0.1 to 1 ml of bacterial culture were inoculated into each of 100 ml of Minimal Salt Medium. The flasks were incubated at 37^0C for 24 hrs. The treatments were carried out in duplicates

Effect of carbon sources on bacterial growth and enzyme production

The isolated bacterial sp. were grown in 250 ml Erlenmeyer conical flasks each containing 100 ml of Minimal Salt Medium with different carbon sources viz., glucose, starch, sucrose, maltose, lactose, sodium acetate and sodium citrate at 1 per cent level. The flasks were incubated at 37^0C for 24 hrs. The treatments were carried out in duplicates.

Effect of nitrogen sources on bacterial growth and enzyme production

The bacterial isolates were grown in 250 ml Erlenmeyer conical flasks each containing 100 ml of Minimal Salt Medium with 1% glucose and different nitrogen sources namely yeast extract, peptone, ammonium sulphate, potassium nitrate, urea and casein at 0.5 percent level. The flasks were incubated at 37^0 C for 24 hrs. The experiments were carried out in duplicates.

Effect of various metal ions on bacterial growth and laccase production

The isolated bacterial strain was grown in 250 ml Erlenmeyer conical flasks each containing 100 ml of Minimal Salt Medium with 1% glucose and 0.5% yeast extract and supplemented with 10 mM concentration of various metal ions namely $BaCl_2$, $CaCl_2$, $MgCl_2$, $FeCl_2$ $LiCl_2$ and KCl and incubated at 37^0C for 24 hrs.

Effect of various inducers on bacterial growth and laccase production

Each 100 ml of Minimal Salt Medium containing 1%glucose and 0.5% of yeast extract were inoculated with bacterial isolate and supplemented with different inducers viz., ABTS, phenol, phenol red, m-cresol, quercetin, remmazol brilliant blue-R, ferulic acid, 2, 5-Xylidine, galic acid, guaiacol at a concentration of 1 mM. The inducers were filter sterilized in 0.45 μm filter membrane. These were incubated at 37^0C for 24 hrs.

Effect of various surfactants on bacterial growth and laccase production

The effect of surfactants on growth and laccase production was tested by inoculating the bacterial culture in the minimal medium with Tween 80, SDS and triton-X 100 at a concentration of 1% and incubated for 24 hrs at 37^0C.

Effect of Temperature on bacterial growth and laccase production

The isolated bacterial sp. were grown in 250 ml Erlenmeyer conical flasks each containing 100 ml of Minimal Salt Medium and incubated at various temperatures namely 27^0C, 37^0C and 47^0C for 24 hrs.

3.2.2 Screening of variables by Plackett-Burman Design.

The classical method of medium optimization involves changing one variable at a time, keeping the others at fixed levels. Being single

dimensional, this laborious and time consuming method often does not guarantee determination of optimal conditions. On the other hand carrying out experiments with every possible factorial combination of the test variables is impractical because of the large number of experiments required In the first screening, it is recommended to evaluate the result and estimate the main effects according to a linear model. After this evaluation, the variables that have the largest influence on the result are selected for new studies. Thus, a large number of experimental variables can be investigated without having to increase the number of experiments to the extreme (Montgomery, 1997).

The Plackett-Burman design is a type of two-level screening design which allows for the study of 'n' number of factors with 'n+1' +'2' trials The Plackett-Burman design is orthogonal in nature, thereby the effect of each component worked out are pure in nature and are not cofounded with interaction among components for further optimization.

In the Plackett-Burman design to make the factors computationally equivalent, low and high factor settings corresponding to the low and high concentration of a particular medium component are coded as -1 and +1, respectively. Each factor is tested equal number of times at its low and high settings. Because of this equal allocation within each factor, balance exists between each and every pair of factors throughout the design.

The eleven components used to develop the statistical design were glucose, pH, incubation period, inoculums size, Magnesium sulphate, trace element solution, phenol, agitation, yeast extract, sodium acetate and ammonium sulphate. Low and high settings of eachof these components

Table 1

Plackett - Burman Design for the standardization of variables

Designation	Std Order	Run Order	Pttype	Blocks	Inoculum size	pH	$MgSO_4$	Incubation period	Glucose	Yeast extract	Ammonium sulphate	Sodium acetate	Phenol	Agitation	Trace element solutions
A	4	1	1	1	1	-1	1	1	-1	1	-1	-1	-1	1	1
B	3	2	1	1	-1	1	1	-1	1	-1	-1	-1	1	1	1
C	11	3	1	1	-1	1	-1	-1	-1	1	1	1	-1	1	1
D	9	4	1	1	-1	-1	-1	1	1	1	-1	1	1	-1	1
E	1	5	1	1	1	-1	1	-1	-1	-1	1	1	1	-1	1
F	12	6	1	1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
G	6	7	1	1	1	1	1	-1	1	1	-1	1	-1	-1	-1
H	2	8	1	1	1	1	-1	1	-1	-1	-1	1	1	1	-1
I	10	9	1	1	1	-1	-1	-1	1	1	1	-1	1	1	-1
J	7	10	1	1	-1	1	1	1	-1	1	1	-1	1	-1	-1
K	8	11	1	1	-1	-1	1	1	1	-1	1	1	-1	1	-1
L	5	12	1	1	1	1	-1	1	1	-1	1	-1	-1	-1	1

All the variables were studied at two levels (Low and high levels). The experiments were performed in Duplicates and the variables are Inoculum size (1ml and 5ml), pH(4 and 8), $MgSO_4$(1mM and 2mM), Incubation period(24 and 72 hours),Glucose(0.5% and 1.5%),Yeast extract(0.5% and 1.5%),Ammonium Sulphate(0.4mM and 1.2mM), Sodium acetate(2omM and 4omM),Phenol(With out and 1mM),agitation(100 and 180rpm), (Trace element solutions(0.5 and 1.5ml)(Appendix-VI)).

were used to prepare a combination of twelve trials of production medium, whose compositions are specified in Table 1. Laccase activity (Appendix - IV) and intracellular protein (Appendix-V) were determined for each trial.

3.3 STATISTICAL SOFTWARE

The statistical analysis for one time one factor approach was carried out using Sigma Stat 3.1. The design of Plackett – Burman method was carried out using Minitab 15.