

Isolation, screening and induction of mutation in strain for extra cellular lignin peroxidase producing bacteria from soil and its partial purification

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ABSTRACT:

The Lignin peroxidase enzyme production was carried out through bacteria isolated from sample soil collected around decayed wood at Sulur, Coimbatore. In this report, a mutant of *Bacillus subtilis* LPTK was obtained after chemical mutagenesis with EMS and screened for better Lignin peroxidase production. In the production optimization studies, the bacterial strain needs temperature around 37°C, pH.6, lactose as a carbon source, peptone as nitrogen source and incubation time for 36 hours for its higher enzyme productivity. The partial purification was also done.

Keywords:

Lignin, LP, *Bacillus subtilis* and enzyme activity.

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Introduction

Lignin is the most abundant renewable carbon source on earth (Bo Zhang *et al.*, 2008). When plants die, it will rot and degrade in soil. Researches are widely done on fungi which degrade lignin. While bacteria grow and multiply faster, it is anticipated to be better in production of lignin degrading enzymes. There are many types of lignin degrading enzymes such as lignin peroxidase, manganese peroxidase, laccase and glyoxal oxidase are produced by fungi as well as bacteria such as lignin peroxidase by the bacteria *Streptomyces viridosporus* (Ramachandra *et al.*, 1987).

Lignin peroxidase is an enzyme that is used to degrade lignin. It was first discovered in 1983. Lignin peroxidases are produced by many wood degrading fungi as a family of isoenzymes (Kirk and Farrell, 1987). Recent researches also showed that it can be produced from bacteria such as *Streptomyces viridosporus*. These hemoproteins are similar to the more familiar plant peroxidases in structure and mechanism, and utilize hydrogen peroxide and organic peroxides to oxidize a variety of substrates (Tien *et al.*, 1985, 1986). Some of the most important features distinguishing these enzymes from other oxidoreductases (such as horseradish peroxidase), for example, are their very low pH optima and much higher redox potentials.

The substrates of lignin peroxidase include both phenolic and non-phenolic aromatic compounds; the phenolic substrates are oxidized to yield products similar to those produced by classical peroxidases, while the oxidation of the non-phenolic methoxybenzenes are unique to the lignin peroxidases (Korsten L and Cook N, 1996), the oxidation of these substrates yield aryl cation radicals. Lignin peroxidase can catalyze the oxidation of substrates with a reduction potential greater than 1.3 volts. The enzyme is capable to oxidizing lignin monomers, dimers and trimers as well as polycyclic aromatic compounds such as benzopyrene (Haemmerli *et al.*, 1996). The powerful and relatively non-specific nature of this enzyme has lead to the investigation of its potential use in diverse fields of biodegradation of toxic chemicals, pulp, paper processing, and in the textile industry.

METHODS AND MATERIALS

Sample Collection and Isolation of Bacteria

The sample soil was collected around decayed wood at Sular, Coimbatore in a sterile container. The collected sample was serially diluted

up to 10^7 dilutions using sterile saline as a blank and the diluted samples were plated into the sterile nutrient agar using spread plate method. The nutrient agar plates were incubated at 37°C for 24 hours. The isolated colonies were further purified by streak plate method using sterile media plates. The pure cultures were inoculated into sterile nutrient agar slants and nutrient broth for further use.

Screening for the Enzyme Production

The isolated pure strains were screened for the production of extra cellular lignin peroxidase using screening medium which contains lignin as a substrate (Yeoh Teik Loon *et al.*, 2006). The pure cultures were streaked at the lignin agar plates and the plates were incubated at 37°C for 24 hours. The observation was made to see the substrate utilized zone around the colony. Only the strain showing positive and better zone was taken for further study.

Strain Improvement by Mutation

The chemical mutagenesis was carried out according to Kotchoni and D. Bartels (2003). Ethyl methanesulphonate (EMS) was used as the mutagenic agent. The better zone formed strain was grown to mid logarithmic phase of growth in LB broth and from that cells were centrifuged at room temperature. The pellet was re-suspended in normal saline and a suspension of (v/v) of EMS was made. Appropriate dilutions were made and incubated overnight at room temperature to allow mutational segregation. The culture was then spread on agar media and incubated overnight at 37°C. Single mutant clones were further grown in new plates till the third generation. The third generations (M3) of the mutants were replica-plated in nutrient agar plates. The wild and mutant strain was screened again using lignin screening medium. The selection of mutants was based on the diameter of the clear zone surrounding the colonies.

Enzyme Production

Preparation of Inoculum

The inoculum for further production of enzyme and other studies was prepared using Luria broth (Zolta'n Pra'Gai, 2002). The pure culture was inoculated into sterile inoculum broth and was incubated at 37°C in a rotary shaker for overnight. The fresh over night culture was used as inoculum for the production of enzyme.

Production

The enzyme production was carried out by shake flask fermentation using production medium with pH 8. The inoculated medium was incubated at 37°C for 48 hours. The medium was agitated at



200 rpm for better aeration and growth of the organism.

Lignin peroxidase Assay

Bacterial crude culture preparation

Aliquots of 10ml of the culture suspension were centrifuged at 5000 rpm for 15 minutes and cell free extract was subjected to enzyme assay. The extract was stored at 4°C for further analysis.

Plate Assay

The plate assay was performed using agar plates amended with Lignin. The agar plates were prepared by mixing of 1% Lignin with 1.7% agar. After solidification of agar, around 10 mm diameters of wells were cut out aseptically with the help of cork-borer. The well was filled with the culture filtrate and incubated at 37°C for overnight. The observation was made to see the hydrolytic zone around the wells. Two wells were filled with distilled water and production medium without culture acts as a control (Yeoh Teik Loon *et al.*, 2006)

Chemical Assay

Lignin peroxidase activity was determined by estimating the reducing sugar produced during enzymatic reaction by dinitro salicylic acid method. Assay for lignin peroxidase were performed using substrate composed of alkaline lignin dissolved in 0.1M phosphate buffer with pH of 7.0. After incubation at 33°C for 24 hours, the broth is transferred for centrifugation. Centrifugation was done for 5 minutes. The supernatant obtained was used as a crude enzyme. The crude enzyme was incubated with substrate at 33°C. The reaction mixtures were tested for its simple sugar using DNS method.

Estimation of Total Protein

The chemical assay for the total protein content from the sample was determined using Bradford method (Bradford, 1976). To the culture filtrate Bradford reagent was added. The tube was gently tilted once for mixing and the absorbency was taken at 595nm in UV- VIS spectrophotometer. The blank was prepared by mixing distilled water reagent. The protein concentration was determined by comparing the value with standard graph prepared using Bovine serum albumin.

PARAMETER OPTIMISATION STUDIES

Effect of incubation time on lignin peroxidase production

Around 500ml of sterile production medium for bacterial were prepared and 5% bacterial inoculum was added aseptically. The inoculated

medium was incubated at 37°C temperature with shaking around 150 rpm. After incubation, around 20 ml of culture was aseptically withdrawn periodically at 6 hours intervals up to 48 hours. The culture filtrate was tested for the total protein content and lignin peroxidase activity (Tuncer *et al.*, 2004).

Temperature

Hundred ml of sterile production medium for bacteria was prepared in different conical flask at pH 8.0 and inoculated with 5% inoculum. Each flask was incubated at different temperatures such as 28°C, 32°C, 37°C, 42°C, 47°C, and 52°C for 36 hours. The protein estimation and enzyme activity were estimated followed by Tuncer *et al.* (2004).

pH

Hundred ml of sterile production medium was prepared for bacterial strain in different conical flasks and each flask was adjusted to different pH such as 4, 5, 6, 7, 8, 9 using 0.1N NaOH and 0.1N HCl. After sterilization, flasks were inoculated with 5% inoculum. The flasks were incubated at 37°C for 36 hours. The protein estimation and enzyme activity were estimated (Tuncer *et al.*, 2004).

Carbon Sources

Hundred ml of sterile production medium (pH.6) for bacteria was prepared in different conical flasks. Each flask was amended with different carbon sources such as Glucose, Fructose, Maltose, Lactose, Sucrose and Mannitol. The flasks were inoculated with 5% inoculum and incubated at 37°C for 36 hours. The culture filtrate was collected for protein estimation and enzyme activity (Tuncer *et al.*, 2004).

Nitrogen source

Hundred ml of sterile production media for bacteria (pH 6) was prepared in different conical flasks. Each flask was amended with different nitrogen sources such as Beef extract, Casein, Peptone, Gelatin, Ammonium chloride, Ammonium sulphate and Potassium nitrate. The flasks containing bacterial medium were inoculated with 5% inoculum and incubated at 37°C for 36 hours. The culture filtrate was collected for Protein estimation and Enzyme activity (Tuncer *et al.*, 2004).

Partial Purification

Ammonium Sulphate Fractionation of Proteins

The enzyme separation from the exhausted medium was done by 70% Ammonium sulphate saturation (Elba *et al.*, 1999). The mixture was then

stored in a cold room for 24 hours to precipitate all the proteins and the precipitation was separated by centrifugation for 10 minutes. The supernatant was discarded and the remaining precipitate was dissolved with 5 ml of 1M-citrate phosphate buffer (pH.8). Then the mixture was subjected to dialysis.

Dialysis

The purpose of dialysis is to remove undesired small molecular weight molecules from a mixture in which the desired species of molecules are too large to travel across the membrane. Ordinarily this process is utilized during protein purification in which salting out procedure has been employed as the initial step with ammonium sulphate. After the protein is precipitated from the initial source, it is re-dissolved in buffer and then poured into a dialysis bag.

RESULTS

Naturally occurring microorganisms are having the ability to produce various enzymes. Now a day's most of the enzymes are important for human welfare and industry. In this study, the bacterial strains were isolated from the sample soil at Sulur area, Coimbatore because most of the natural wastes are degraded by the native microbes that are growing over that waste. In such a way, it is fact that the microbes which are isolated from decayed wood soil may have ability to produce lignin peroxidase. From the samples, around 15 bacterial strains were isolated and screened; it was found that four bacterial strains showed positive results on lignin peroxidase production. The better zone formed bacterial strain was considered for further strain improvement study.

Strain improvement was done by Kotchoni and Shonukan (2002) method. In this experiment the better positive strain for lignin peroxidase was treated with Ethyl methanesulphonate and the M3 generation mutant strain produced much better yellow zone then compared to wild strain. This strain was used for further studies.

In plate assay the lignin peroxidase activity was identified by a clear zone when compared with the control and the chemical assay determined by DNSA (3, 5- dinitro salicylic acid) method using starch as a substrate.

Effect of Time

The growth study of the organism is essential for the production of enzyme because most of the extra cellular enzymes are produced during log phase of the organisms. Generally during growth, the bio mass of the cells was estimated. The

culture was withdrawn for enzyme activity. The bacterial cultures were withdrawn once in every six hours. The results revealed that there is gradual increasing of production has occurred from 18th hour to 36th hours and higher production has occurred at 36th hours (**Table.1**). This shows that bacterial isolate should have maintained its log phase from around 18th hour to 36th hour. Besides, it is believed that the higher production of lignin peroxidase has occurred in extreme log phase because even though the log phase was maintained around 18th to 36 hours, the followed drop of production has indicated that the organism should have entered into stationary phase of growth (**Fig 1**).

Effect of Temperature

The environmental parameters are showing great influence in the growth of the organisms and the production of enzymes. The optimum temperature for the better production was made in various temperatures. It was found that like other mesophilic organisms, the higher lignin peroxidase activity was found (41U/ml) at 37°C from bacteria

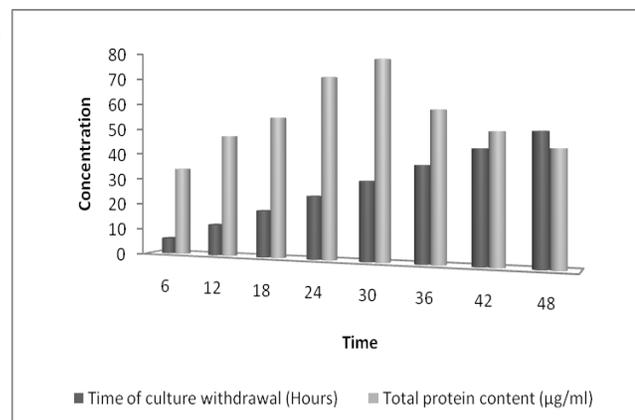


Figure 1. Enzyme production and total protein content of bacterial isolate at different incubation time

(**Table.2**). These indicate that the optimum temperatures for better production of bacterial isolates are 37°C (**Fig.2**). The temperature requirement of the organism is based on the nature of organisms. Many thermophilic bacteria like *Clostridium* sp. needs 55°C for better production of lignin peroxidase (Tuncer *et al*, 2004).

Effect of pH

The pH is the important parameter which determines the growth of the organism and lignin peroxidase production. The study results showed that the optimum pH around six is better for bacterial isolate (**Table. 3 & Fig.3**). Like



temperature, different organisms need different pH ranges for its lignin peroxidase production. The Thermophilic bacterium has produced high amount of lignin peroxidase between pH 4-6 (Tuncer *et al*, 2004).

Effect of Carbon source

Table.2 Effect of Temperature on total protein and enzyme production by bacterial isolate

| S. NO | Temperature (°C) | Total protein content (µg/ml) | Enzyme activity (U/ml) |
|-------|------------------|-------------------------------|------------------------|
| 1 | 28 | 49 | 15 |
| 2 | 32 | 50 | 20 |
| 3 | 37 | 52 | 41 |
| 4 | 42 | 40 | 26 |
| 5 | 47 | 31 | 12 |
| 6 | 52 | 28 | 4 |

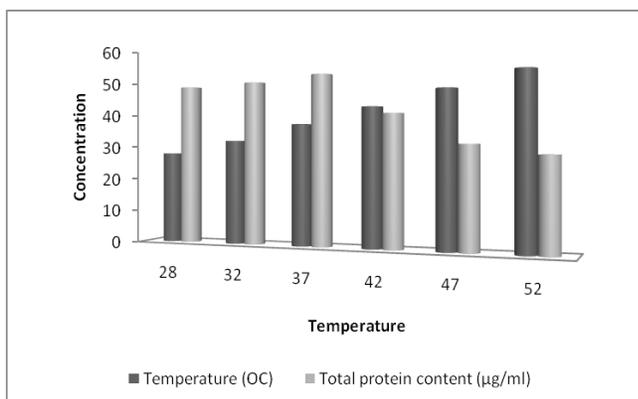


Figure 2. Effect of Temperature on total protein and enzyme production by bacterial isolate

The carbohydrates are soul energy source for most of the heterotrophic organisms. These shows great influence on the production of many enzymes. In this study, lactose was found to be a right carbon source for bacterial strain for higher production of lignin peroxidase (Table. 4 & Fig.4). Lignin peroxidase production will be low in the medium amended with glucose, fructose, maltose,sucrose and mannitol when compare to lactose (Tuncer *et al*, 2004).

Effect of Nitrogen source

The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the production. The nature of the compound and the concentration that we are using may stimulate or down modulate the production of enzymes. The

Table.3 Effect of pH on total protein and enzyme production of bacterial isolate

| S. NO | pH | Total protein content (µg/ ml) | Enzyme activity (U/ml) |
|-------|----|--------------------------------|------------------------|
| 1 | 4 | 9 | 4 |
| 2 | 5 | 31 | 21 |
| 3 | 6 | 73 | 41 |
| 4 | 7 | 92 | 33 |
| 5 | 8 | 70 | 15 |
| 6 | 9 | 62 | 12 |

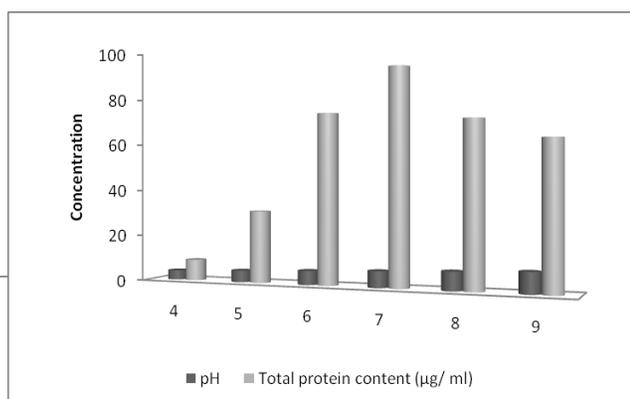


Figure.3 Effect of pH on total protein and enzyme production of bacterial isolate

nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the production (Table.5 & Fig.5).

DISCUSSION

The Lignin peroxidase enzyme production

Table. 4 Enzyme production and total protein production on different carbon sources

| S.No | Carbon sources | Bacteria | |
|------|----------------|--------------------------------|------------------------|
| | | Total protein content (µg/ ml) | Enzyme activity (U/ml) |
| 1 | Glucose | 48 | 6 |
| 2 | Fructose | 57 | 18 |
| 3 | Maltose | 38 | 9 |
| 4 | Lactose | 86 | 31 |
| 5 | Sucrose | 51 | 15 |
| 6 | Mannitol | 47 | 10 |

was carried out through bacteria isolated from sample soil collected around decayed wood at Sultur. *Trichoderma harzianum* isolates (D1/4) proved to be the highest producer for the two enzymes such as 160% and 186% of CMCase and β

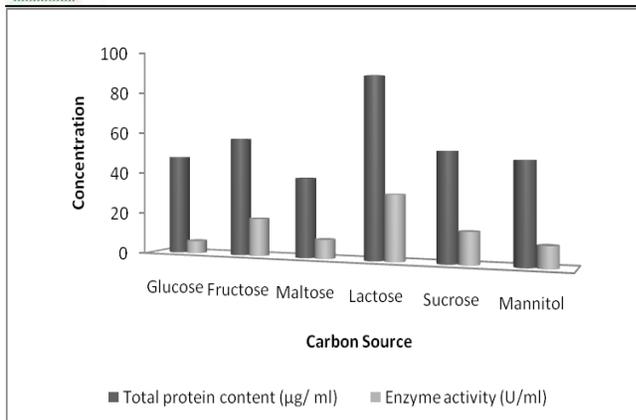


Figure.4 Enzyme production and total protein production on different carbon sources

-glucosidase respectively, more than the original strain (Ahmed M. El-Bondkly *et al.*, 2010). In this report, a mutant of *Bacillus subtilis* LPTK was obtained after chemical mutagenesis with EMS and screened for better Lignin peroxidase production. The effect of carbon and nitrogen sources on lignin peroxidase (LiP) activity of *Aspergillus sp.*, showed that glucose (1%) and ammonium sulphate were the best carbon and nitrogen sources (Ahammed S and Prema P,2002). In the production optimization studies, the bacterial strain needs temperature around 37°C, pH.6, lactose as a carbon source, peptone as nitrogen source and incubation time for 36 hours for its higher enzyme productivity.

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Table.5 Enzyme and total protein production on different nitrogen sources

| S.No | Nitrogen sources | Bacteria | |
|------|-------------------|-------------------------------|------------------------|
| | | Total protein content (µg/ml) | Enzyme activity (U/ml) |
| 1 | Beef extract | 46 | 24 |
| 2 | Casein | 98 | 32 |
| 3 | Peptone | 90 | 41 |
| 4. | Gelatin | 94 | 34 |
| 5 | Ammonium chloride | 38 | 24 |
| 6 | Ammonium sulphate | 36 | 22 |
| 7 | Potassium nitrate | 31 | 23 |

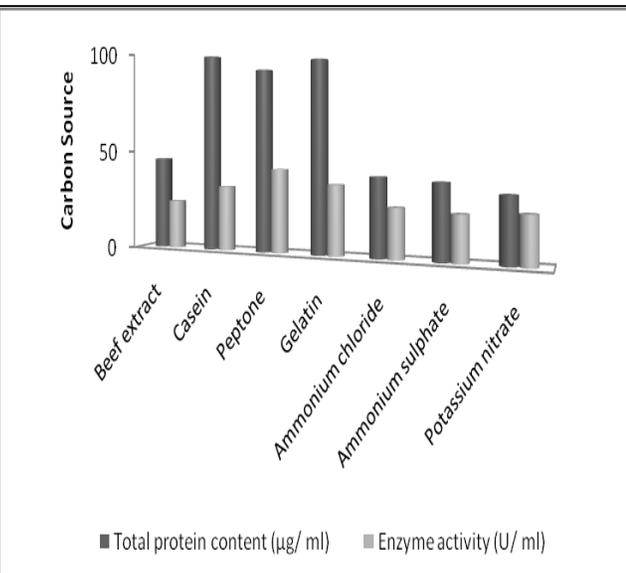


Figure. 5 Enzyme and total protein production on different nitrogen sources

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