Original Article

Production, Purification, and Characterization of Laccase Enzyme from Local Isolate Bacillus Cereus TY10 Isolated from Soil Samples

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Abstract

The local isolate TY10 was derived from a soil sample that was polluted with oil in a previous investigation. The study focused on evaluating the isolate's capacity to manufacture the laccase enzyme. The *Bacillus cereus* isolate was identified based on the findings of morphological and biochemical testing, as well as the VITEK-2 tests. The synthesis of laccase by the *Bacillus cereus* TY10 isolate was assessed using qualitative and quantitative methods, with guaiacol serving as a substrate indicator. The qualitative investigation revealed that the local isolate TY10 exhibited a strong capacity for laccase enzyme production in the solid medium. Simultaneously, it was shown that the indigenous strain *Bacillus cereus* TY10 exhibited an enzyme activity of 0.98 units/ml when the broth of the indigenous strain was utilised in quantitative measurements for enzyme synthesis. The impact of different durations of incubation, pH values, and the current research focused on examining temperatures and their impact on the ability of a local entity *Bacillus cereus* TY10 Extract laccase enzyme by isolation. The study discovered that laccase production reached its peak of 1.61 Unit/ml occurred after 48 hours of incubation at 35°C, using a growth medium with a pH of 7. The crude laccase enzyme underwent a purification procedure consisting of three sequential steps: Ammonium sulphate precipitation and two gel filtration steps using Sephadex G-25 and G-100. This process resulted in a 6.52 times purification of the laccase enzyme, with a recovery rate of 90.65% and a specific activity of 39.98 unit/mg protein. All purification steps contributed to this outcome.

Keywords: Bacillus Cereus, Laccase Enzyme, Guaiacol, Optimization, Purification.

Introduction

Enzymes are widely employed in business strategies as a substitute for chemical catalysts because of their high efficiency and environmentally friendly nature. Out of all the enzymes, the laccase enzyme stands out for its exceptional versatility and numerous possible applications. Traditionally, it has been noted to occur in fungus. However, recent research has revealed its presence in bacterial isolates, particularly those that belong to the *Bacillus* genus.[1,2] These bacterial isolates possess multiple characteristics that render them suitable for producing laccase enzymes. Therefore, these isolates of *Bacillus* spp. Have become prominent manufacturers for biotechnology applications.[3] Bacterial laccase is a multifunctional enzyme with numerous applications. Multiple recent research have demonstrated that bacterial species classified under the genus *Bacillus* may be extracted from the majority of soil samples analysed and gathered from various sites.[4,5] Smith and his team, together with other researchers, launched a study to acquire novel bacterial isolates from the genus Bacillus that have the ability to produce the enzyme laccase in significant amounts. This study demonstrated that *Bacillus* isolates possess significant enzymatic production capabilities and play a crucial role in the respective soil ecosystems they inhabit.[6,7] Another study conducted in 2020 by Kumar and Parshad confirmed that

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many species belonging to the genus *Bacillus* possess the ability to produce laccase enzymes, as they were isolated from different types of soil.

Additionally, it was shown that these isolates exhibit the capacity to generate various forms of laccase enzymes, making them significant in numerous biological applications (13). The production of the laccase enzyme by bacterial isolates is influenced by many growth conditions, including carbon and nitrogen supplies, as indicated by numerous studies pH, temperature, and aeration, each of these factors plays a significant role in the production of bacterial isolates by influencing their ability to enhance gene expression and produce the enzyme. Consequently, recent research has concentrated on improving the productivity of the laccase enzyme from bacterial isolates. This has been achieved by enhancing the optimal conditions for the fermentation process or employing genetic engineering techniques to enhance production efficiency. It is therefore essential to comprehend the regulatory mechanisms that impact laccase production in bacterial isolates of the genus *Bacillus* in order to develop efficient biological methods for scaling up laccase enzyme production. In 2018, Patel and Patel did a study to identify the most effective *Bacillus* isolates that produce laccase enzymes of superior quality. They then proceeded to analyse these enzymes in terms of their substrate specificity, stability, and possible uses.

Extensive study has been carried out to examine the methods and factors that contribute to the heightened activity of laccase enzymes produced by Bacillus isolates. These studies have mostly focused on investigating the biochemical, kinetic, and structural aspects of the enzyme in order to enhance its effectiveness. Additionally, these investigations involve the determination of the substrate specificity of enzymes, as well as their optimal pH and temperature conditions.[8]

Materials and Methods

TY10 Isolation and Identification

A prior study discovered that the local bacterial isolate TY10 was obtained while investigating the capacity of certain bacterial isolates to degrade old motor oil. This particular isolate demonstrated a notable proficiency in decomposing used motor oils, including its capacity to generate laccase enzymes.[9] The local TY10 isolates were cultured using nutritional agar medium to examine their colonies and various parameters, including gramme staining, endospore staining, and biochemical testing such as catalase, oxidase, and other assays.[10,11] The diagnosis of the TY10 isolate was confirmed using the VITEK 2 system. A sterile column was utilised to transfer a sufficient number of pure isolate colonies into a glass tube 3.0 ml of sterile sodium chloride solution (0.45-0.50%) with a temperature pH 4.5-7.0.

The Ability of TY10 to Produce Laccase Enzyme

1- Qualitative Test for Laccase Enzyme

The isolate TY10 was evaluated using the clear zone method, in which a loopful of the solid culture was collected and streaked over the surface of LB-agar that containing 0.1% of a A particular substrate known as guaiacol was used, and thereafter, all plates were placed in an incubator between 24 to 48 hours at 37 °C. If the TY10 The colonies exhibited a distinct brown colour zone, indicating that the isolate possesses a high proficiency in producing the laccase enzyme.[12]

2- Quantitative Test for Laccase Enzyme

0.1 ml of fresh culture from the TY10 isolate was added to a conical flask containing 100 ml of LB broth, pH 7. The flasks were placed in a shaker incubator and incubated for 24 hours at 37 ºC with a shaking speed of 150 rpm. After incubation, the culture was centrifuged at 6000 rpm for 20 minutes. The pellets were discarded, and the remaining supernatant was collected and tested for laccase activity.[12] The ability of local isolate TY10 for extracellular laccase production was evaluated according to the laccase activity using a quantitative method, where the guaiacol was used as substrate. Therefore, when the enzyme laccase oxidizes the guaiacol in the reaction medium, this leads to the production of a reddish-brown color that can be measured at 450 nm. To perform this assay, 2 mM guaiacol in 10 mM sodium acetate buffer (pH 5.0) as a substrate was used. A reaction mixture was prepared to contain 1 ml of guaiacol, 3 ml of acetate buffer (pH 5.0), and 1 ml of crude laccase enzyme (supernatant), which was then incubated at 30 C for 15 min. The absorbance was read at 450 nm. In the blank test, the enzyme amount was replaced with distilled water. The formula described by Desai[13] was used to calculate the enzyme activity (U/ml) of the laccase enzyme by the following equation:[13]

Enzyme activity (U/ml) = (A x V) / (t x e x v)

Where:

1. A represents the absorbance at 450 nm.
2. V represents the volume of the reaction mixture in milliliters.
3. t represents the incubation time in minutes.
4. e represents the extinction coefficient, which is 12,100 m-1 cm-1.
5. v represents the volume of enzyme in milliliters.

Optimum Conditions for Laccase Production by TY10

To optimize the cultural conditions for enzyme production, some experiments were conducted to determine the ideal parameters that would result in the highest laccase production. Each parameter was examined separately to determine its effect and then fixed the optimum. The experiments focused on studying the effects of various incubation periods (24, 48, 72, and 96 hrs,), temperature values (25, 30, 35, 40, and 45 °C), and pH levels (4, 5, 6, 7, and 8) that adjusted using either HCl or NaOH. After each experiment, the crude laccase (supernatant) was obtained from the culture flasks of the TY10 isolate, and used to assay the laccase activity according to the procedure described previously.[13,14]

Protein Estimation

The Lowery method was employed to determine the protein concentration in every fraction throughout the

purification process. The measurement of absorbance was performed at a wavelength of 600 nm. The values of protein concentration were determined by utilising the standard curve of bovine serum albumin.[15]

Laccase Enzyme Purification

Three steps were used to purify the crude laccase enzyme from TY10 isolate. The inoculated LB-broth was centrifuged at 10,500×g under 4 °C for 10 min, and the supernatant that represents the crude laccase was precipitated using different saturation percentages (60%, 70%, and 80%) of solid ammonium-sulfate. After each precipitation process, the fractions were collected separately and then 10 ml of 0.1 M potassium phosphate buffer (pH 6) was used to re-dissolve the precipitate and then dialysis. The laccase activity and protein concentration in the dialysate parts were determined.

For the purification of laccase at 4°C, two steps were taken using gel filtration columns of Sephadex G-25 and Sephadex G-100 columns. The first glass column size 35 x 1.5 cm was packed with Sephadex G-25 prepared with 0.1 M potassium phosphate buffer of pH 6. Where the partially purified laccase from the first step was added to the top surface of the gel column and eluted with the same buffer at a flow rate of 30 mL/h. To analyze laccase activity and protein content, fractions of 3.0 ml from each were collected after elution.

Following the purification step by Sephadex G-25, the most active fraction was used in the second step with a Sephadex G-100 column (35 x 1.5 cm). The column was washed with 0.1 M potassium phosphate buffer (pH 6) at a flow rate of 15 mL/h. A 3.0 ml fractions were collected and analyzed for laccase activity and protein content, as in the previous step with Sephadex G-25.[16-18]

Results and Discussion

The TY10 isolate was obtained from a soil sample that was contaminated with oil. It was identified as *Bacillus cereus* after conducting cultural, microscopic, and biochemical examinations. Additionally, the VITEK 2 system's results confirmed that the TY10 isolate belongs to *Bacillus cereus.* A wide range of gram-positive and gram-negative isolates that produce laccase enzymes were obtained from soil samples that were polluted with organic contaminants. Various species, including bacteria, fungus, and cyanobacteria, have the ability to synthesise laccase enzymes utilising different types of substrates.[19,20] Bacterial laccases exhibit superior thermal and pH stability compared to fungal and plant laccases, rendering them more efficient.[21] Furthermore, they exhibit a wide spectrum of substrate specificity and are economically efficient.[22]

Laccase is a significant enzyme in practical applications due to its ability to facilitate a diverse array of enzymatic reactions involving both natural and synthesised organic substrates.[23] Hence, it is beneficial in the process of breaking down numerous polymers, including intricate aromatic compounds. In recent decades, researchers have directed their attention towards these enzymes because of their capacity to oxidise both phenolic and nonphenolic chemicals, as well as complex environmental pollutants.[24]

Ability to Produce Laccase

The isolate Bacillus cereus TY10 was confirmed to produce the enzyme laccase through qualitative and quantitative tests. Results of the qualitative test by using the solid LB medium supplemented with guaiacol show the isolate TY10 gave a clear brown color surrounding the bacterial colonies after only 24 hours of incubation at 37°C. Also, the results of the quantitative test on the isolate TY10 confirmed that it has a high potential to produce the laccase enzyme in liquid LB medium after 24 h. of incubation at 37°C, as the produced enzyme gave an enzymatic activity of 0.98 units/ml.

A variety of qualitative and quantitative methodologies were employed to examine the capacity of microbial isolates to generate the enzyme laccase. The goal was to identify the most effective isolate for manufacturing, based on its ability to catalyse the oxidation of various substrates, including guaiacol, syringaldazine, ABTS, and others.[5,25]

Optimal Conditions for Laccase Production

To improve the productivity of the laccase enzyme from isolate *Bacillus cereus* TY10, the effect of incubation time, temperature values, and pH level of the growth medium on the amount of enzyme produced and thus on the enzyme activity was studied. The results in Figure (1) indicate that the isolate TY10 showed the ability to produce the laccase enzyme on all days of the experiments, with different enzymatic activity. In contrast, the second day of experiments gave the highest enzyme production with an enzyme activity of 1.23 units/ml.

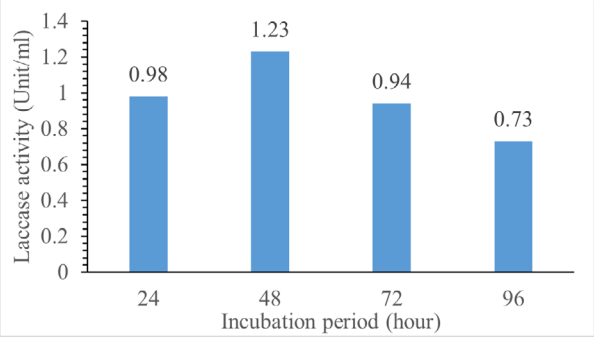

Figure 1: Effect of Different Incubation Periods On Laccase Production from *Bacillus Cereus* TY10 Inoculated in the Liquid LB Medium with pH 7 and Incubated at 37 C˚.

Figure 2 shows the results of the effect of temperature on the production of the laccase enzyme, where it is noted that the isolate TY10 showed the highest ability to produce the laccase enzyme when incubated at a temperature of 30 and 35°C, but the best production of enzyme was obtained at 35°C, with an enzyme activity of 1.61 Unit/ml, while it was noted that temperatures of 25 and 45°C are not suitable for laccase activity due to low enzyme productivity.

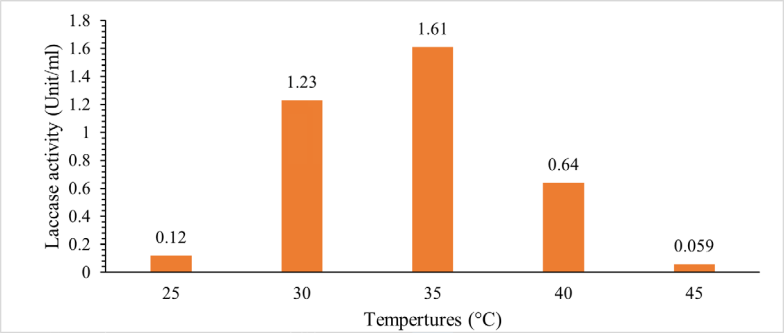

Figure 2: Effect of Different Temperature Values On Laccase Production from *Bacillus Cereus* TY10 Inoculated in the Liquid LB Medium with pH 7 and Incubated for 2 Hours.

The results of the effect of pH values on the production of the laccase enzyme by TY10 isolate, shown in Figure (3), also indicated that the best productivity appeared in the growth medium with a pH of 7, with an enzymatic activity of 1.61 units/ml. It is also noted from Figure (3) that the enzyme was produced well in a wide range of pH values ranging from 6-8.

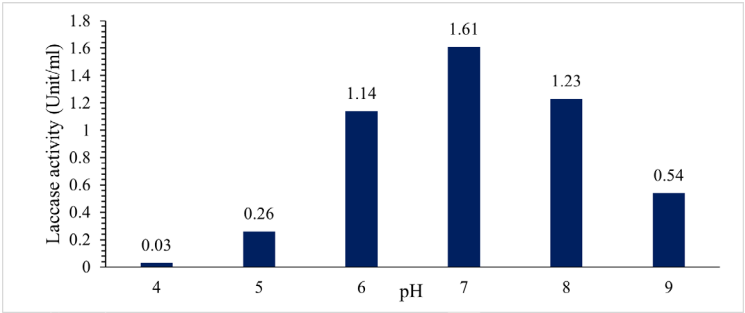

Figure 3: Effect of Different pH Values On Laccase Production from *Bacillus Cereus* TY10 Inoculated in the Liquid LB Medium and Incubated for 2 Hours at 35 C˚.

Many factors can affect laccase production by microorganisms such as duration of incubation, temperature, pH, type of substrate, and others because laccase enzyme is considered a secondary metabolism product and their production is influenced by these factors and in addition to some factors inducers as the genes encoding laccase.[26] Lorenzo and colleagues also indicated that the amount of laccase enzyme produced in the growth medium is affected by some cultural factors such as the components of the medium, time, pH, temperature, and type of carbon and nitrogen source.[27]

Several studies indicate that *Bacillus* species are widely distributed in nature due to their ability to survive in harsh environments in addition to their ability to consume many environmental pollutants as a carbon source.[28] Several studies on improving the production of the laccase enzyme by bacterial isolates belonging to the genus *Bacillus* showed an increase in productivity when using optimal culture conditions, and that the best productivity of the laccase enzyme was obtained after 2-5 days of incubation at a temperature of 30-35°C in a growth medium with a pH of 7-8 only according to the type and nature of Bacillus isolates.[16,29-31]

Laccase Enzyme Purification

The method of precipitation with ammonium sulfate was used at saturation rates of 60, 70, and 80% for the partial purification step of the laccase enzyme. The results appear in Table (1) that precipitation with 70% gave the highest specific activity of the enzyme, reaching 9.65 unit/mg protein, with a recovery rate of 74.37% with fold purification reaching 1.57 times. Hence, this fraction of the enzyme was dialysis and chosen to perform the remaining purification steps.

Table 1: Effect of the Different Ammonium Sulfate Saturations On Partially Purifying the Laccase Enzyme Produced from *Bacillus Cereus* TY10.

Fraction	Total Protein (mg)	Enzyme Activity (U/ml)	Total Activity (Unit)	Specific Activity (U/mg protein)	Recovery (%)	Purification (fold)
Crude extract	5.25	1.61	32.2	6.13	100	1
Precipitation with 60% ammonium sulphate	2.16	2.76	13.8	6.38	46.29	1.041
Precipitation with 70% ammonium sulphate	2.48	4.79	23.95	9.65	74.37	1.57
Precipitation with 80% ammonium sulphate	3.82	4.99	24.95	6.53	77.48	1.06

The partially purified laccase from 70% ammonium sulfate precipitation was loaded on the Sephadex G-25 column and then eluted as the 3 ml fractions for each. The results, as shown in Figure (4), indicated that fractions between 19-24 have good enzymatic activity, therefore these six fractions containing the most laccase activity were combined and then this collected solution appeared a specific activity of 27.7 U/mg protein with 110.12 % recovery and 4.52-fold purification as shown in Table (2).

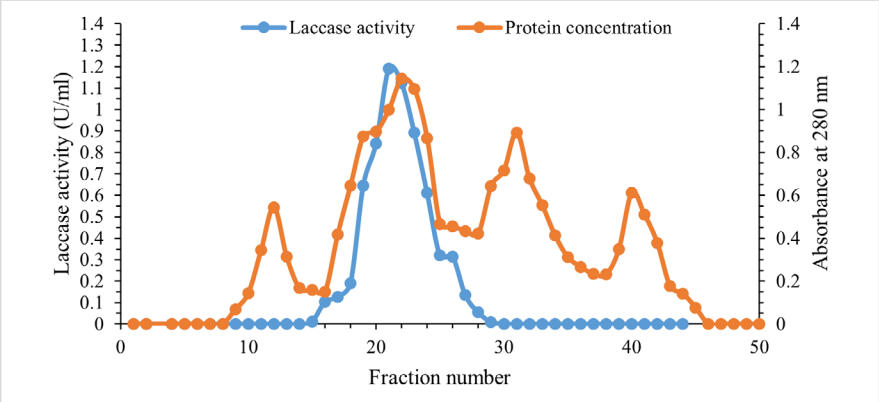

Figure 4: Elution Step of Laccase Enzyme Produced by *Bacillus Cereus* TY10 Through the Sephadex G-25 Column with Size 35 x 1.5 Cm After Precipitated by 70% Ammonium Sulfate.

After that, the eluted fractions that had the best laccase activity from the Sephadex G-25 column were used in the second step of purification by re-loading them in the Sephadex G-100 column. Figure (5) displayed one peak for both the laccase activity and protein concentration after eluting the combined fractions from the Sephadex G-100 column, and the presence of one peak for the laccase enzyme indicates the purity of the laccase enzyme studied. Also, Figure (5) shows that fractions 21-27 gave the best enzymatic activity, as it was found that the specific activity was calculated as 39.98 U/mg protein with 90.65% recovery and 6.52-fold purification as shown in Table (2).

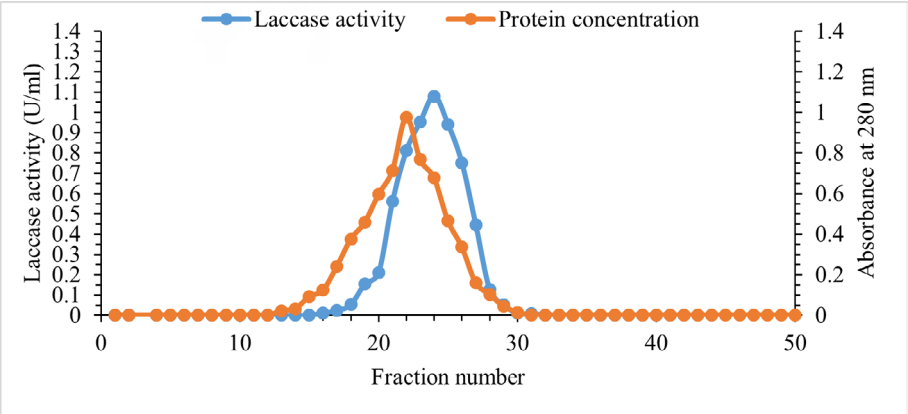

Figure 5: Elution Step of Laccase Enzyme Produced by *Bacillus Cereus* TY10 Through the Sephadex G-100 Column with Size 35 x 1.5 Cm After the Second Purification Step by the Sephadex G-25 Column.

Table 2: The Summary of Purification Steps for the Laccase Enzyme Produced by the Local Isolate *Bacillus Cereus* TY10.

Fraction	Total Protein (mg)	Enzyme Activity (U/ml)	Total Activity (U)	Specific Activity (U/mg protein)	Recovery (%)	Purification (fold)
Crude extract	5.25	1.61	32.2	6.13	100	1
Precipitation with 70% ammonium sulphate	2.48	4.79	23.95	9.65	74.37	1.57
Sephadex G-25	1.28	1.97	35.46	27.7	110.12	4.52
Sephadex G-100	0.73	1.39	29.19	39.98	90.65	6.52

Many techniques have been used to purify bacterial laccase enzymes such as ammonium sulfate precipitation, gel filtration, ion exchanges, ultrafiltration, and others. [32,33] Where precipitation of enzymes is achieved by

agents such as organic solvents, pH values adjustment, and some salts, like ammonium sulfate, which lowers the solubility of the enzymes and leads to precipitate them. Also, high polymers are used to separate many enzymes according to their charges or molecular weight.[34] Ammonium sulfate and organic solvents such as ethanol and acetone are mostly used to precipitate the enzymes. Sondh in 2020 used various acetone concentrations between 50-80% to precipitate the laccase enzyme and the maximum laccase activity was obtained at 65%.[35] Other researchers used various ethanol concentrations between 70-95% to precipitate the laccase enzyme and the maximum laccase activity was obtained at 80%.[36] The chromatography method by ion exchange and gel filtration was used to purify the laccase enzymes also, where DEAE-cellulose was widely employed in the purification of laccases, while other researchers purified the laccase using CM-Cellulose, Sepharose, and DEAE-Sepharose CL-6B.[37] On the other hand, all types of Sephadex like Sephadex G-25, Sephadex G-75, SephadexG-100, and SephadexG-200 are widely used in gel filtration method to purify the laccase enzymes.[38]

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