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Selection and characterization of ligninolytic *Bacillus albus* PA3/3 from fermented tea leaf and its use in industrial wastewater treatment

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Abstract

Lignin and lignin-related compounds are considered complex barrier substrates that are difficult for microorganisms to degrade. Therefore, the search for potential novel ligninolytic bacteria has become a focus in the field of biotechnology with the aim of making the process easy, effective, environmentally friendly and cost-effective. In this study, the ligninolytic *Bacillus albus* PA3/3 was isolated from lignin-containing fermented tea leaves, using guaiacol as a substrate, and identified based on 16S rRNA gene sequencing and phenotypic characterization. Bio-decolorization activities in synthetic dye and wastewater treatment were also determined. The results revealed that *B. albus* PA3/3 is a Gram-positive bacterium with 99.35 percent sequence identity to *B. albus*. The strain PA3/3 decolorized methylene blue and showed oxidative activity towards guaiacol, as a lignin-mimicking compound. *B. albus* strain PA3/3 produced laccase with peroxidase and extracellular xylanase, amylase, and protease. Moreover, strain PA3/3 showed dye-decolorization properties with a particular preference for the recalcitrant azo dye (Reactive Black 5, Reactive Green 19 and Reactive Red 120). These bacteria were used to treat textile and ink industry wastewater. The results demonstrate the potential of *B. albus* PA3/3 as a promising tool for decolorization and industrial wastewater bio-treatment.

Keywords: *Bacillus albus* PA3/3, Fermented tea leaf, Industrial wastewater treatment, Ligninolytic enzyme

1. Introduction

Lignin, the natural glue between plant cell wall components, is the second most abundant aromatic biopolymer on the planet [1]. Because it stiffens the plant stem and is difficult to degrade it is a problem in industrial pretreatment processes and wastewater management systems, and thus many chemical methods have been developed to degrade it [2]. However, the use of microorganisms would be a better way to degrade and valorize lignin because chemical and physical methods can be environmentally unsafe and can only be used under specific conditions [3].

Lignin degrading and valorization activities have been found in plants, fungi, and bacteria [4]. Bacteria are a robust bioresource for lignin degradation because of their high environmental and biochemical adaptability [5]. In a ligninolytic system, enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and laccase (Lac) can act together [6]. Promising bacterial lignin metabolizers include *Pseudomonas putida*, *Rhodococcus jostii*, *Rhodococcus opacus*, *Acinetobacter baylyi*, *Amycolatopsis* sp., *Spingomonas* sp. [5], *Bacillus* sp. A4 [7] and *Anoxibacillus ayderensis* SK3-4 [8].

Despite pretreatment of lignocellulosic biomass, oxidoreductive ligninolytic enzymes can degrade and transform partially degraded or oxidized polymeric substances that can be easily taken up by microbial cells where they are completely mineralized [9]. Industrial textile wastewater also contains a complex mixture of organochlorine-based pesticides and heavy metals associated with dyes and the dyeing process [10]. Therefore, finding novel ligninolytic bacteria is an important focus in the field of biotechnology and could lead to the cheap and effective treatment of industrial wastewater [11].

Along with the bacterial depolymerization of lignin and lignin-like substances that are less well studied, potential ligninolytic bacteria remain largely unexplored and novel ligninolytic enzymes could be discovered from these bacterial sources. They may show benefits regarding the depolymerization of modified lignin residues from the waste streams in different industries [12].

Camellia sinensis, used to make fermented tea, is normally fermented during manufacturing in Thailand, and microbial populations increase during this process. Among the microbial flora, *Bacillus* spp. play a role in the process through their polyphenol oxidase and peroxidase activities [13]. Therefore, fermented tea leaf can be considered as a reservoir for ligninolytic bacteria because most of the lignin is degraded naturally during the fermentation process.

Ligninolytic bacteria are a trending field in biotechnological studies due to their production of oxidoreductive ligninolytic enzymes, which can degrade lignocellulosic biomass and other lignin-like polymeric dyes found in industrial wastewater effluents. Moreover, bacteria can tolerate wide temperature, pH, and oxygen ranges and are easy to genetically manipulate. In this study, a potential ligninolytic *Bacillus albus* PA3 was isolated, identified, and characterized along with its lignin-degrading properties. Furthermore, its potential for use in the depolymerization of insoluble substrates in industrial wastewater was assessed.

2. Materials and methods

2.1 Materials

Salty fermented tea leaf (pH around 6.0) from Northern Thailand was used in this work to isolate potential ligninolytic bacteria. Luria Bertani (LB) medium (tryptone 10 g, yeast extract powder 5 g, NaCl 10 g, distilled water 1,000 mL) was prepared to isolate the bacteria. The minimal salt medium (MS) was prepared by mixing $NaNO_3$ 2 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, $MnSO_4.H_2O$ 0.02 g, $FeSO_4.7H_2O$ 0.02 g, $CaCl_2.2H_2O$ 0.02 g and distilled water 1000 mL to screen the isolates.

2.2 Isolation and screening of ligninolytic bacteria

Enriched medium was made by adding 0.5 g of tea leaves to 50 g of LB broth with 1 mM guaiacol and incubating at 200 rpm, 37 °C for 48 h. Five hundred microliters of each enriched sample were added to 5 mL broth for 48 h and subcultured three times. Serial dilution was done up to 10^{-10}. One hundred microliters of each dilution was spread on LB plates containing 0.025 mg/L methylene blue. Plates were incubated at 37 °C overnight and single colonies surrounded by a clear zone were restreaked several times to obtain purified colonies [14]. For further investigation, isolates were kept in 10% glycerol stock at -20 °C.

2.3 Selection of lignin-degrading bacteria

2.3.1 Alkali lignin degradation

For further screening, single isolates were inoculated in minimal salt (MS) broth containing 1% alkali lignin as the sole carbon source and incubated at 37 °C, 200 rpm for 7 days [14].

2.3.2 Guaiacol degradation

Single isolates were also inoculated in MS broth amended with 1 mM guaiacol. All cultures are incubated at 37 °C, 200 rpm for 7 days. Based on the intensity of color change, the best isolate was selected as a potential ligninolytic bacterium producing oxidoreductive enzymes [15].

2.4 Identification of bacterial strain by 16s rDNA sequencing

The complete genomic DNA of selected positive bacteria was extracted using the CTAB method. The partial 16S rDNA sequence was amplified via polymerase chain reaction (PCR) using Universal Primers 18F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3). The resulting sequences were analyzed and a phylogenetic tree was constructed using MEGA-X. Cultural characteristics and the morphology of the bacterial strain were identified by Gram staining methods under a light microscope.

2.5 Analysis of enzyme activity on plate assays

The plate assay method was used for the detection of extracellular cellulase, xylanase, amylase, and protease activities. The appearance of clear zones was noted for enzyme activities.

Extracellular cellulase and xylanase activity was tested on LB agar plates containing 1% CMC and 1% xylan from beech wood, respectively, and incubated at 37 °C. Congo red (0.1%) was used to stain the plates, and 1 M NaCl was used to wash the plates [16].

Amylase activity was tested on LB agar plates containing 1% soluble starch from rice as a substrate, incubated at 37 °C. Iodine solution was used to stain the plates and water was used to wash the plates [17].

Protease activity was tested on LB agar plates containing 2% skim milk as a substrate. The appearance of clear halo zones was detected after incubating the plates at 37 °C [18].

2.6 Crude enzyme production

The strain was cultured in LB broth and incubated at 37 °C, 200 rpm for 1, 3 and 5 days. Supernatants were collected by centrifugation at 8,000 rpm for 10 min and used to measure laccase activity.

2.7 Analysis of laccase enzyme activities

Laccase enzyme activity was measured spectrophotometrically at 465 nm with 2 mM guaiacol as a substrate in 50 mM phosphate buffer (pH 6.5) (e465 = 48,000 /M. cm). One unit was defined as the amount of enzyme that increased the absorbance by 0.001 units per minute at 55 °C. All assays were carried out in triplicate, and the results are shown as mean ± SD. The enzyme activity (mU/L) was calculated using the following equation [19]:

$$\text{Enzyme Activity (mU/L)} = \frac{\Delta A \times Vt \times 10^6}{\Delta t \times \varepsilon \times d \times Vs} \quad (1)$$

where,

ΔA = Final Absorbance – Initial Absorbance
Vt = Total Volume of the Reaction (mL)
Δt = Incubation Time
ε = Molar Extinction Coefficient (L/mol.cm)
d = Optical Path (1cm)
Vs = Sample Volume (mL)
10^6 = Correction Factor (μmol/mol)

2.8 Dye degradation studies

Three dye degradation studies were conducted using Reactive Black 5, Reactive Green 19 and Reactive Red 120 and 24-h culture broth of *B. albus* PA3/3. The dye was added to the broth at a constant concentration of 0.01% (w/v). Decolorization was done by three different methods as described below. The rate of decolorization in the broth was determined by measuring the absorbance of the culture supernatant at the respective maximum wavelengths for each dye (Reactive Black 5-596 nm, Reactive Green 19-630 nm, and Reactive Red 515 nm).

2.9 Industrial wastewater treatment

Two wastewater samples from the textile industry (pH 6.16) and ink industry (pH 2.22) were used to assess degradation activity. Both samples were diluted in a 1:2 ratio with distilled water and pH was adjusted to 7.0. After that, 10% (v/v) of bacterial starter was inoculated into the wastewater samples and incubated at 37 °C and 200 rpm. Reduction of color and precipitates was observed qualitatively 24 h after inoculation [20].

3. Results and discussion

3.1 Isolation of ligninolytic bacteria

Thirty-six bacterial isolates from fermented tea leaf that could grow and show clear zones on LB agar in methylene blue dye-based plate screening were recorded in a preliminary screening. The three best isolates with strong activity on methylene blue were chosen for further screening with 1% alkali lignin or 1 mM guaiacol broth.

The three promising isolates shown in Figure 1 and Table 1 are Gram-positive bacteria with a milky opaque color. All show differences in shape, margin, and elevation.

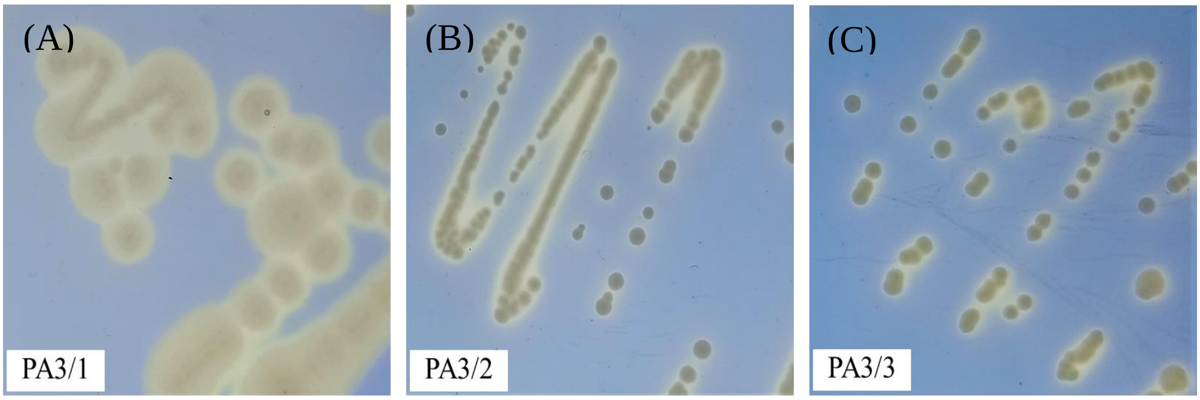

Figure 1 Three bacterial strains; isolated PA3/1 (A), isolated PA3/2 (B), isolated PA3/3 (C), showing clear zone on LB agar with methylene blue.

Table 1 Cultural characteristics and morphology of three promising isolates.

Isolates	Bacterial characteristics					Gram staining
	Shape	Margin	Opacity	Elevation	Color	
PA3/1	Irregular	Undulate	Opaque	Umbonate	Milky	Positive
PA3/2	Round	Entire	Opaque	Convex	Milky	Positive
PA3/3	Round	Entire	Opaque	Umbonate	Milky	Positive

3.2 Selection of lignin-degrading bacteria

There was no bacterial growth in MS broth containing 1% alkali lignin. However, all three bacterial isolates grew on MS broth with guaiacol and changed color to brown within 5 days due to guaiacol oxidation and the oxidoreductive activities of the ligninolytic enzymes. Isolate PA3/3 was selected for further study based on the intensity of the color change in MS broth with guaiacol (Figure 2).

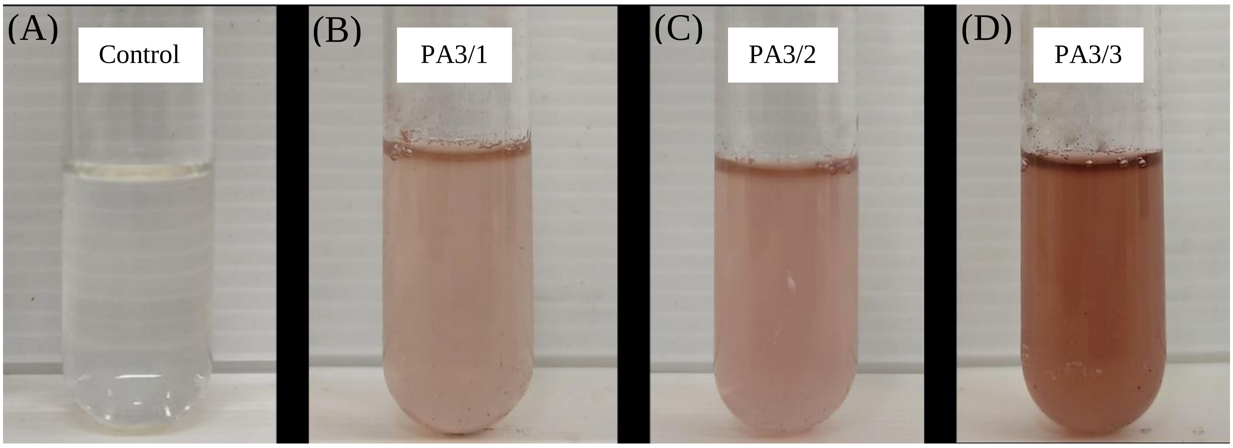

Figure 2 Bacterial culture in MS broth with guaiacol before inoculation (A) and after 5 days inoculation with isolated PA3/1 (B), isolated PA3/2 (C), isolated PA3/3 (D).

3.3 Identification of bacterial strain by 16s rDNA sequencing

Based on 16s rDNA sequencing, PA3/3 was similar to *B. albus* with 99.35 percent identity (Figure 3). *B. albus* is a novel species identified in 2017 and very few reports have been published about it [21]. It is a great source of potential novel ligninolytic enzymes. These novel enzymes are superior to their counterparts in their specificity, thermostability, and mediator dependency. They also show benefits regarding the depolymerization of modified lignin residues from industrial waste streams [12].

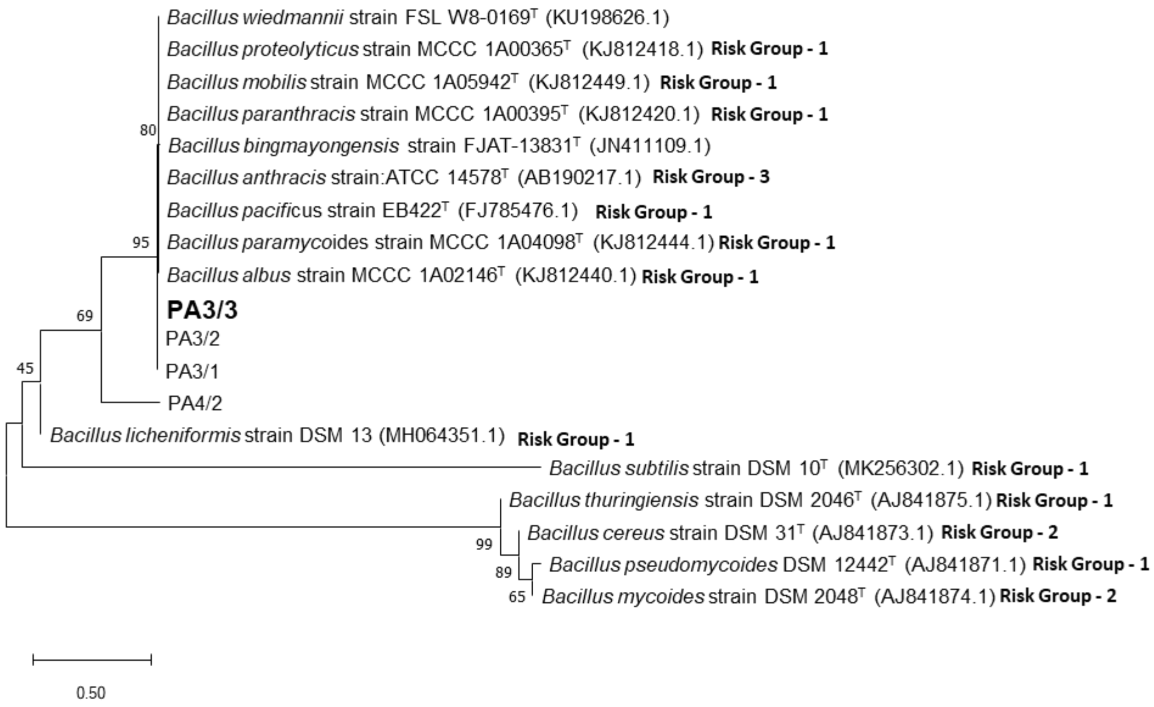

Figure 3 Phylogenetic tree of *B. albus* aligned with *Bacillus* type strains based on 16s rRNA gene sequences with neighbor-joining methods.

3.4 Analysis of enzyme activity on plate assays

After cultivation of strain PA3/3 on MS media supplemented with different carbon sources, no cellulase activity was detected 3 days after inoculation, while xylanase activity was detected along with the formation of a clear zone. Amylase and protease enzyme activity were observed after 48 h and 24 h, respectively (Figure 4). This positive extracellular hemicellulose, amylase, and protease activity means that the strain can promote the degradation of hemicellulose, starch and protein in wastewater effluents, making the strain attractive for bioremediation.

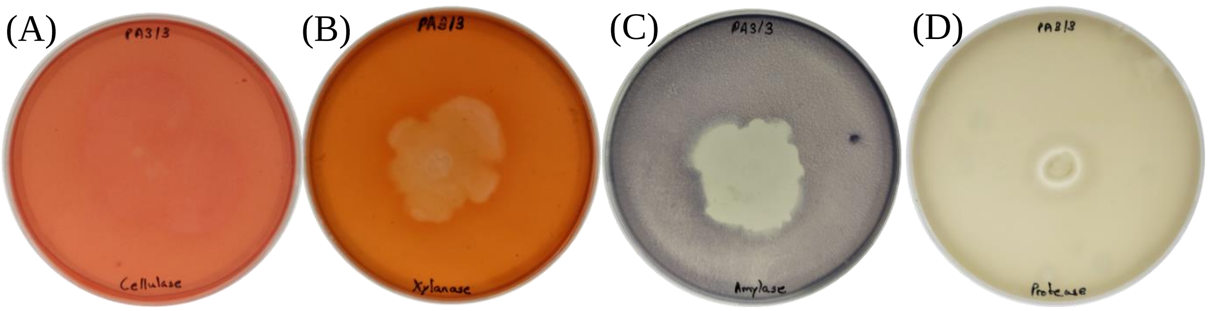

Figure 4 Plate assays for extracellular enzyme activity: for cellulase activity (A), xylanase activity (B), amylase activity (C) and protease activity (D) of *B. albus* PA3/3.

3.5 Laccase activity

Laccase activity was measured with guaiacol as a substrate and increased enzyme activity was found after 1 to 3 days of incubation. For *B. albus* PA3/3, the maximum laccase activity of 125 mU/L was reached 3 days after inoculation and this remained stable until 5 days after inoculation. Laccase is important for lignin degradation and decolorization (Figure 5). Moreover, extracellular laccase activity can be enhanced by the addition of growth factors for maximum enzyme production [22]. This is the first report of *B. albus* PA3/3 isolated from fermented tea leaf with laccase activity for the decolorization and degradation of wastewater precipitations. *B. albus* also showed other ligninolytic enzyme activity 3 days after inoculation, including manganese peroxidase (723 mU/L), lignin peroxidases (540 mU/L), and dye-decolorizing peroxidases (760 mU/L).

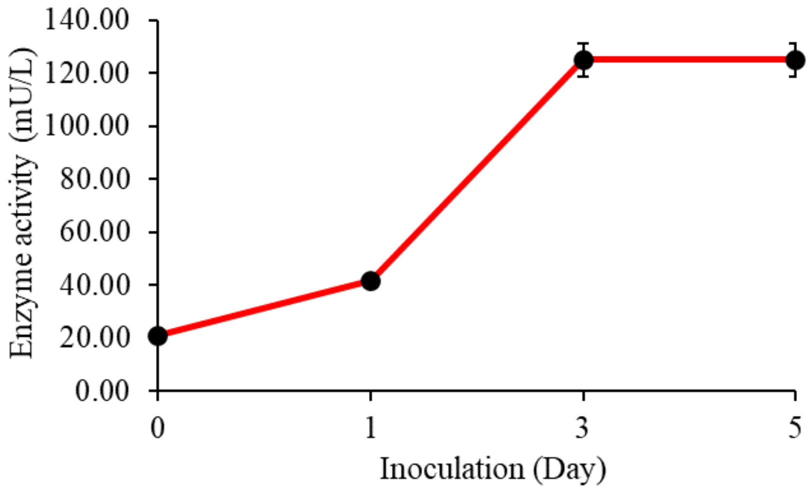

Figure 5 Laccase activity of bacterial isolates following inoculation.

Bacterial ligninolytic enzyme systems are increasing in importance due to their many remarkable features in comparison to fungal laccase from the industrial point of view, such as their ability to work in a wide temperature and pH range, their cost-effective production over short periods of time, their broad substrate specificity and their ease of cloning and expression in a host with suitable manipulation [23].

3.6 Dye-decolorization

The dye decolorization efficiency of *B. albus* PA 3/3 was assessed using three reactive azo dyes, namely Reactive Black 5, Reactive Red 120 and Reactive Green 19. A standard dye concentration of 0.1% (w/v) was used. The maximum degradation was 90% of Reactive Black 5 at 3 days followed by 60% of Reactive Red 120 and 35% of Reactive Green 19 over a similar period of time. The dye degradation percentage of Reactive Black 5 dye showed that it was more efficiently degraded by *B. albus* PA3/3 than the other dyes employed in the present study. Generally, the bacterial decolorize azo dyes and mineralize aromatic amines using peroxidase and that products could utilize as sole carbon source [24]. The bacterial decolorization and degradation of these dyes is of considerable interest since it can achieve a high degree of biodegradation and mineralization, is applicable to a wide variety of azo dyes, is inexpensive and environmentally friendly, and produces less sludge.

3.7 Wastewater treatment by B. albus PA3/3

Two wastewater samples from the textile and ink industries were investigated and their initial pH was 6.16 and 2.22, respectively. After adjustment to pH 7.0, *B. albus* PA3/3 (10% v/v) was applied as a starter culture. The bacteria utilized some sediment from the wastewater and produced oxidoreductive laccase enzymes and other extracellular hemicelluloses, amylases, and proteases to degrade organic matter.

Treatment of the wastewater from the textile industry with strain PA3/3 led to a decrease in color and precipitated particles after 24 h. The best improvement in water transparency was found in treated wastewater supplemented with glucose as a carbon source (Figure 6).

Treatment of the wastewater from the ink industry led to greater decolorization of precipitates (Figure 7). There was a significant reduction in precipitates with more than 50% dry weight loss in all treated water while some whitish precipitates were seen in treated wastewater supplemented with a carbon source. The mechanism and respective degraded products from wastewater biodegradation and the methodology needed to improve lignin degradation will be discussed in future work.

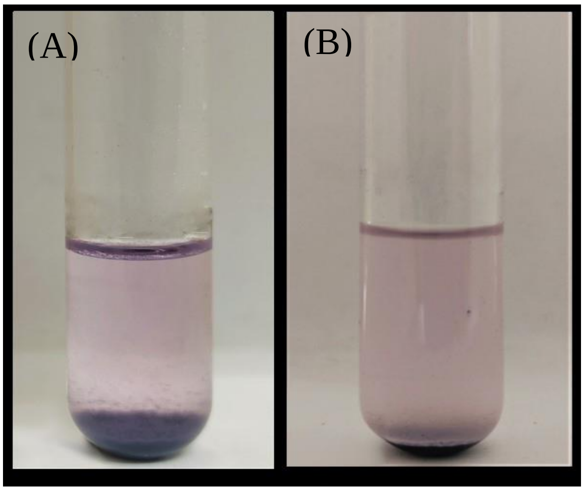

Figure 6 Untreated (A) and treated (B) wastewater samples from textile industry after incubating with *B. albus* PA3/3 at 37 °C, 200 rpm for 24 h.

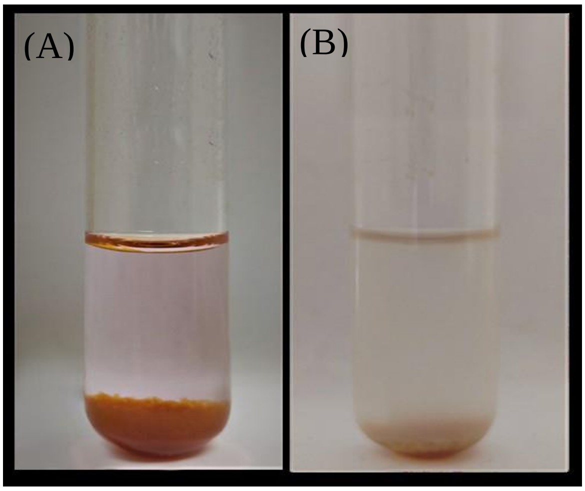

Figure 7 Untreated (A) and treated (B) wastewater samples from ink industry after incubating with *B. albus* PA3/3 at 37 °C, 200 rpm for 24 h.

4. Conclusion

In this work, a bacterial strain producing ligninolytic enzymes was isolated from fermented tea leaf and identified as *Bacillus albus* PA3/3 (Biosafety Level 1). The crude enzymes from the isolated strain included laccase, manganese peroxidase, lignin peroxidases, dye-decolorizing peroxidases, xylanase, amylase, and proteinase. The decolorization ability of PA3/3 confirmed its potential for use as an environmentally friendly treatment of industrial effluents.

5. Acknowledgements

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