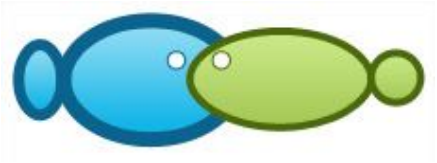

Decolorization of synthetic dyes by ligninolytic *Lysinibacillus sphaericus* JD1103 isolated from Thai wetland ecosystems

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Abstract. Ligninolytic bacterium JD1103 was isolated from soil samples collected from wetland ecosystems in Rayong Province, Thailand and identified as *Lysinibacillus sphaericus* JD1103 based on 16S rRNA sequence analysis. Its ligninolytic performance for crude laccase and lignin peroxidase was determined and showed enzymatic activity by 1.13±0.06 U mL^{-1} and 0.90±0.01 U mL^{-1}, respectively. Its ability to decolorize various synthetic dyes under aerobic conditions was assayed and showed decolorization of Congo Red and Remazol Brilliant Blue R by 84.38±0.50% and 50.00±0.10% within 72 h, respectively. This result indicates the effectiveness of *L. sphaericus* JD1103 for treatment of industrial effluent containing synthetic dyes.

Key Words: Congo Red, laccase, lignin peroxidase, Remazol Brilliant Blue R, wastewater treatment.

Introduction. Textile industries have been increasing proportionally with world population growth; about 5-10% of synthetic dyes from textile production are released as industrial effluent into aquatic ecosystems (Ciullini et al 2008), presenting environment problems due to their cytotoxicity and genotoxicity (Sandhya et al 2008). Treatment of wastewater containing synthetic dyes has conventionally been carried out by effective classical methods (physical or chemical), but may generate significant amounts of chemical sludge which increases processing costs (Rajeswari et al 2014). Therefore, novel and more eco-friendly methods are preferred for wastewater treatment. There are several studies describing decolorization of industrial effluents by ligninolytic microorganisms containing oxidative enzymes such as laccases and lignin peroxidases (Babu et al 2013; Lončar et al 2014; Mathews et al 2016). For this reason, the isolation and characterization of novel or effective bacteria from various environments are important challenges. Wetland ecosystems offer a promising habitat for the isolation of ligninolytic bacteria. Wetlands are defined as complex ecosystems with characteristic interactions between terrestrial and marine systems, providing many ecological services (Chantarasiri et al 2017). Microbial communities in wetland ecosystems have played vital ecological roles in biotransformation and biodegradation of various compounds (Truu et al 2009).

The aim of this study was to isolate ligninolytic bacteria from wetland ecosystems and characterize their ligninolytic activity. Efficient ligninolytic bacteria were identified and applied for the decolorization of various synthetic dyes.

Material and Method

Isolation, screening and identification of bacteria. Twenty-one soil samples were randomly collected from wetland ecosystems (Bueng Samnak Yai Wetland, Prasae River Estuary and Rayong River Estuary), located in Rayong Province, Thailand between July 2014 and December 2015. One gram of each collected sample was suspended in 10 mL of BSGYP medium (pH 6.0), conforming to previously described methods (Chang et al

2014). All cultures were incubated at 28°C, the average temperature of sampling sites, for 72 h on a shaking incubator at 120 rpm. The culture media were serially diluted and spread plated on Tryptone Soya Agar (TSA) medium (HiMedia, India). Morphologically dissimilar colonies were picked and streak plated on TSA medium to obtain pure bacterial isolates.

Bacteria containing laccase (EC 1.10.3.2) and lignin peroxidase (EC 1.11.1.14) were determined for ligninolytic performance as previously described by Chantarasiri et al (2017). Bacterial isolates were grown in BSGYP medium (pH 6.0) supplemented with 0.005% (w/v) of lignin powder (Sigma-Aldrich, USA) at 28°C for 72 h on a shaking incubator. BSGYP medium was centrifuged at 12,000×g for 10 min at 4°C and the supernatant was collected as crude enzyme solution. Enzymatic activity in 1 mL of reaction mixture containing substrate, crude enzyme solution and 50 mM sodium phosphate buffer (pH 8.0) was assayed. Laccase reaction was monitored at 440 nm using 75 mM 1,2-dihydroxybenzene (Sigma-Aldrich, USA) as a substrate. Lignin peroxidase was monitored at 310 nm using 2.5 mM 3,4-dimethoxybenzyl alcohol (Sigma-Aldrich, USA) as a substrate. Reaction mixture was incubated at 28°C for 10 min and assayed in triplicate. One unit (U) of ligninolytic activity was defined as the quantity of enzyme which required the release of 1 µmol of substrate per minute of reaction (Yang et al 2011).

The bacterial 16S rRNA gene was amplified for species identification using universal primers (Sigma-Aldrich, USA) including 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) primers. Genomic DNA was extracted using ZR Bacterial DNA MiniPrep (Zymo Research, USA). Polymerase chain reaction (PCR) was performed in a Mastercycler® Nexus (Eppendorf, Germany) and cycling conditions were set up as previously described (Ferbiyanto et al 2015). The PCR product was sequenced by MU-OU:CRC, Faculty of Science, Mahidol University, Thailand. The resulting 16S rDNA sequence was compared against sequences in the NCBI database using the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was constructed with the MEGA6 program (Tamura et al 2013) by using the Neighbor-Joining algorithm.

Decolorization assay. The dye decolorization study was performed with five synthetic dyes (Sigma-Aldrich, USA) as follows: Brilliant Blue R (BB), Bromocresol Green (BG), Congo Red (CR), Reactive Blue (RB) and Remazol Brilliant Blue R (RBBR). Fifty microliters of bacterial suspension were inoculated in 5 mL of BSGYP medium (pH 6.0), supplemented with 0.005% (w/v) of synthetic dye and incubated at 28°C, with an agitation rate of 120 rpm, in dark conditions for 72 h. Bacterial cells were discarded from the medium by centrifugation at 12,000×g for 10 min. Decolorization of dye was observed in the cell-free supernatant using a GENESYS™ 10S UV-Vis spectrophotometer (Thermo Scientific, USA) at λmax of the synthetic dyes as follows: BB (595 nm), BG (615 nm), CR (470), RB (595 nm) and RBBR (592 nm). All experiments were assayed in triplicate and the decolorization percentage was calculated using the following formula:

Decolorization (%) = (Initial absorbance-Final absorbance) / Initial absorbance] × 100

Results and Discussion. Ninety-eight bacterial isolates with distinct colony morphology were isolated. Only one effective isolate, bacterium JD1103, contained ligninolytic activity effectively with a laccase activity of crude enzyme by 1.13±0.06 U mL^{-1} and lignin peroxidase activity by 0.90±0.01 U mL^{-1}. It could be concluded that this bacterium plays an important function influencing lignin and phenolic degradations in the wetland ecosystem by using its laccase and lignin peroxidase. Morphological examination showed that bacterium JD1103 had a cream-color, was circular, was entire and formed a raised colony (Figure 1). Genotypic characterization of 16S rRNA gene provided evidence that the bacterium JD1103 is closely related to *Lysinibacillus sphaericus* BR2308 (GenBank Accession number KX447404) at 99% identity and the phylogenetic relationship indicated its taxonomic position, as shown in Figure 2. The nucleotide sequence of 16S rRNA gene (1,444 bp) in this study was deposited in the GenBank database under the GenBank

Accession number KY234386. Based on such data, this ligninolytic bacterium was designated as *Lysinibacillus sphaericus* JD1103.

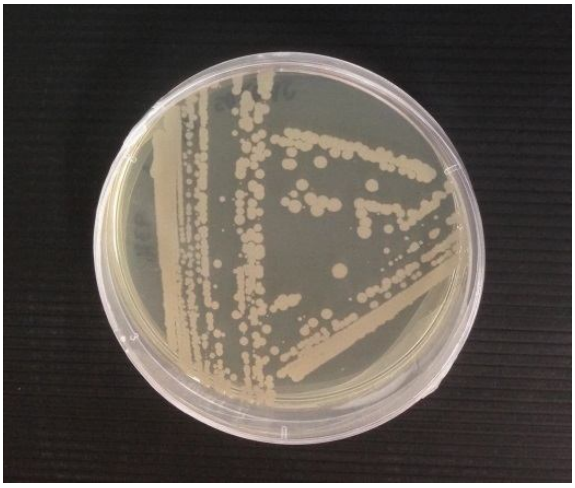

Figure 1. Colony morphology for ligninolytic bacterium JD1103 (original).

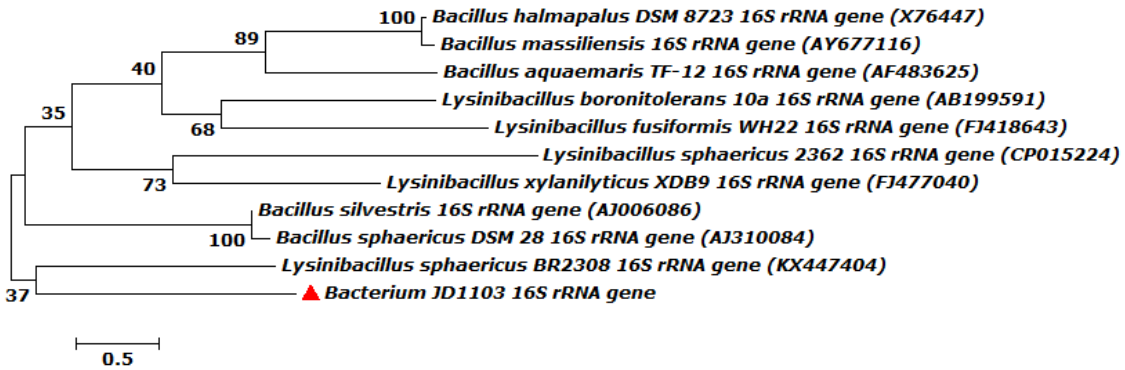

Figure 2. Phylogenetic tree of ligninolytic bacterium JD1103 and related taxa based on 16S rRNA gene sequences.

Decolorization assays were performed to quantify synthetic dye degradation by *L. sphaericus* JD1103 at 72 h of incubation time. BB, BG and RB dyes showed little or no decolorization, whereas CR and RBBR dyes were decolorized by 84.38±0.50% and 50.00±0.10%, respectively (Table 1). The results of CR and RBBR decolorization by *L. sphaericus* JD1103 are shown in Figure 3 and 4. Decolorization performance indicated that *L. sphaericus* JD1103 can utilize CR and RBBR as the sole carbon source, and some dyes (BB, BG and RB) are toxic to *L. sphaericus* JD1103 under aerobic condition.

Table 1

Decolorization of synthetic dyes by *Lysinibacillus sphaericus* JD1103 at 72 h of incubation time

Synthetic dyes	*λmax*	*Decolorization (%)*
Brilliant Blue R (BB)	595	1.65±0.43
Bromocresol Green (BG)	615	No decolorization
Congo Red (CR)	470	84.38±0.50
Reactive Blue (RB)	595	No decolorization
Remazol Brilliant Blue R (RBBR)	592	50.00±0.10

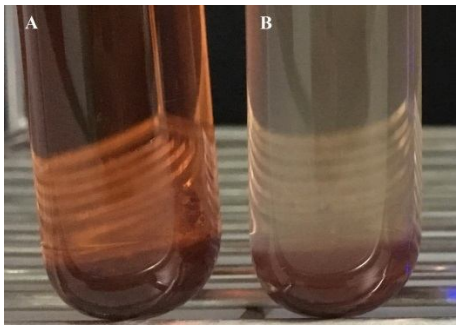

Figure 3. CR decolorization by *Lysinibacillus sphaericus* JD1103 cultured in BSGYP medium: (A) Uninoculated, (B) Inoculated with *L. sphaericus* JD1103 (original).

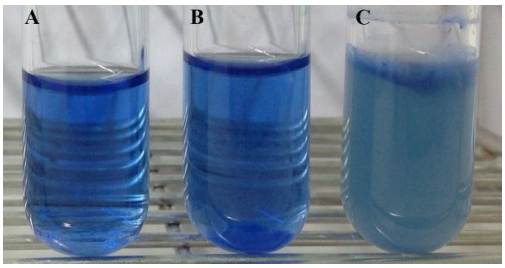

Figure 4. RBBR decolorization by *Lysinibacillus sphaericus* JD1103 cultured in BSGYP medium: (A) Uninoculated, (B) Inoculated with *Escherichia coli* (negative control), (C) Inoculated with *L. sphaericus* JD1103 (original).

In recent years, aerobic and anaerobic bacteria containing ligninolytic enzymes have been reported and developed for dye degradation and wastewater reutilization (Elisangela et al 2009). Most ligninolytic enzymes such as laccases and peroxidases are produced from bacteria belonging to the genera *Bacillus*, *Kerstersia* and *Xenphylus* (Elisangela et al 2009; Lončar et al 2014). In this study, *L. sphaericus* JD1103 was shown to be a novel and effective bacterium for degrading CR and RBBR dyes under mild conditions (pH 6.0 and 28°C) within 72 h. CR dye is a kind of azo dye regularly used in the cellulose and textile industries. It is a potential carcinogen and highly recalcitrant (Wang et al 2017). RBBR dye is one of the most important anthraquinone-based dyes used in the textile industry and is also toxic and recalcitrant (Sumandono et al 2015). The decolorization of various synthetic dyes by *Lysinibacillus* sp. is shown in Table 2.

Table 2

Decolorization performance of *Lysinibacillus sphaericus* JD1103 and related species in the *Lysinibacillus* genus

Bacteria	*Synthetic dyes*	*Decolorization (%)*	*Decolorization conditions*	*References*
Lysinibacillus sp. AK2	Metanil Yellow	100	pH 7.2 and 40°C	Anjaneya et al 2011
L. fusiformis RGS	Mixture of dyes	87	pH 6.6 and 30°C	Saratale et al 2013
L. fusiformis	CR	77	pH 6.0 and 30°C	Mehta et al 2015
L. sphaericus BR2308	RBBR	58	pH 6.0 and 30°C	Chantarasiri et al 2017
L. xylanilyticus	CR	70-76	pH 6.0 and 35°C	Mehta et al 2015
L. sphaericus JD1103	CR	84	pH 6.0 and 28°C	Present study
L. sphaericus JD1103	RBBR	50	pH 6.0 and 28°C	Present study

Degradation performance of *Lysinibacillus* sp. was reported earlier with the ability to utilize a wide range of chemical compounds, pesticides, heavy metals and sulfonated azo dyes (Saratale et al 2013). It indicates the applicability of *L. sphaericus* JD1103 for the treatment of industrial wastewater containing synthetic dyes under aerobic and mild conditions. However, the application of *L. sphaericus* JD1103 for treatment of real textile effluents remains to be demonstrated. To our knowledge, the decolorization of CR and RBBR dyes by *L. sphaericus* isolated from wetland ecosystems in Thailand has not been reported on previously.

Conclusions. This study has described the decolorization ability of ligninolytic *L. sphaericus* JD1103 isolated from wetland ecosystems on various synthetic dyes. The results demonstrate that *L. sphaericus* JD1103 could decolorize highly recalcitrant and toxic dyes including Congo Red and Remazol Brilliant Blue R by its ligninolytic enzymes. Therefore, *L. sphaericus* JD1103 shows promise as a potential microbe for the application in the treatment and bioremediation of dye-containing wastewater effluent from the textile industry.

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