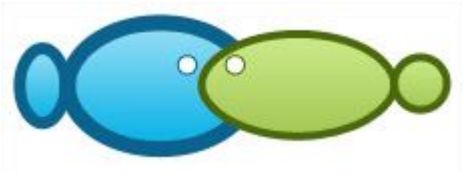

Isolation and characterization of *Lysinibacillus sphaericus* BR2308 from coastal wetland in Thailand for the biodegradation of lignin

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Abstract. One hundred and forty-five bacterial isolates were obtained from forty-two muddy sediment and soil samples of the Bueng Samnak Yai coastal wetland in Thailand. All isolated bacteria were screened for ligninolytic characteristics, with nine defined as ligninolytic bacteria based on the results of decolorization by Remazol Brilliant Blue R in BSGYP medium. The isolate BR2308 showed maximum degradation percentage of commercial lignin by 42.46±2.79 within 72 h. The optimum pH and temperature for commercial lignin degradation were determined to be 8.0 and 30°C, respectively. Its ligninolytic performance of crude enzyme for laccase, lignin peroxidase and manganese peroxidase was determined and showed productive laccase activity by 1.93±0.26 U mL^{-1}. The isolate BR2308 was identified as *Lysinibacillus sphaericus* based on its phenotypic and genotypic characteristics. For its application, it could be used in the biological pre-treatment of *Sesbania aculeata* for bioethanol production and promoted biological saccharification performance for bioethanol production by 66.67%.
Key Words: biological pre-treatment, decolorization, laccase, ligninolytic bacteria, saccharification.

Introduction. Lignocellulosic biomass or lignocellulose is a plentiful and renewable resource with tremendous value to humans as agricultural residues, municipal waste and pulp residues (Raddadi et al 2013). It is composed of complex carbohydrates and noncarbohydrate molecules including cellulose, hemicellulose and lignin. Biological conversion of lignocellulosic biomass into bioethanol has been garnering favorable interest and is still under development. Cellulases from cellulolytic microorganisms have become focal biocatalysts for the biological saccharification of cellulose to monomer sugars, with subsequent fermentation into ethanol (Chantarasiri 2015; Dyk & Pletschke 2012). This cellulase utilization requires a pre-treatment process to disrupt the lignin, lignin related compounds and the waxy materials that protect the cellulose within the plant biomass structure (Gunny et al 2015). However, the lignin polymer and related compounds are highly resistant to disruption (Bugg et al 2011). Lignin can be depolymerized by thermochemical methods. Many of these processes are inappropriate due to their harmful effects on the environment and excessive power requirements (Bandounas et al 2011). Therefore, friendlier methods using ligninolytic enzymes or lignin-degrading enzymes have been preferred for lignin degradation because they occur under mild conditions and have no environmental impact. Studies on ligninolytic enzymes from microorganisms have focused on fungal enzymes, but bacterial enzymes are not well understood (Bugg et al 2011; Chang et al 2014). Few soil bacteria and actinomycetes, including *Cellulomonas* sp., *Pseudomonas* sp., *Rhodococcus* sp. and *Streptomyces* sp., have been reported as lignin degrading microbes (Bugg et al 2011; Brown & Chang 2014; Woo et al 2014). For this reason, the isolation and screening of novel or effective bacteria from various environments are important and challenging.

One interesting environment for isolation of ligninolytic bacteria is the coastal wetland ecosystem. Coastal wetlands are defined as complex ecosystems with a

characteristic interaction between terrestrial and marine systems covering coastal lowlands, tidelands and shallow waters. Such areas provide many ecological services such as the storage of surface runoff, pollution trapping and remediation, nutrient cycling and habitats for wetland wildlife (Ryan & Ntiamoa-Baidu 2000; Yu et al 2012; Jiang et al 2015; Sun et al 2015). The microbial communities in coastal wetlands have played vital ecological roles in nitrogen removal, phosphorus transformation and organic matter degradation (Truu et al 2009).

The tropical legume *Sesbania aculeata* (Dhaincha) is an aquatic weed and a common noxious weed found widely in rice paddy fields and the lowlands of Thailand as well as several other Asian countries. *S. aculeata* is an inexpensive biomass and can be used as a potential material for bioethanol production because of its high cellulose contents. The chemical properties of dhaincha were 34.3-37.9% α-cellulose, 70.0-72.8% holocellulose, 21.9-23.0% lignin, 19.5-19.7% pentosan and 3.4-3.8% ash (Jahan et al 2009). For this study, ligninolytic bacteria were isolated and identified from Thai coastal wetland. The useful bacterial isolate was applied to the biological pre-treatment process of *S. aculeata* for bioethanol production.

The purpose of this study was to determine the beneficial ligninolytic bacteria from aquatic environment for possible use in pretreatment process of bioethanol production and related environmentally-friendly industries.

Material and Method

Sampling site. Muddy sediment and soil samples were collected from Bueng Samnak Yai coastal wetland (12° 39′ 39″ N, 101° 32′ 09″ E) in Rayong Province, situated on the east coast of Thailand (Figure 1). Bueng Samnak Yai coastal wetland is designated as a Wetland Reserve of Thailand, which is grown over by characteristic plants including *Melaleuca quinquenervia*, *Garcinia cowa*, *Cratoxylum formosum* and *Lepironia articulata* (Chantarasiri et al 2015). Muddy sediment and soil samples close to the roots of plants or beneath decayed plant residues were collected aseptically and kept in plastic bags. Samples were collected during the rainy season in Thailand, once in July 2014 and again in August 2014.

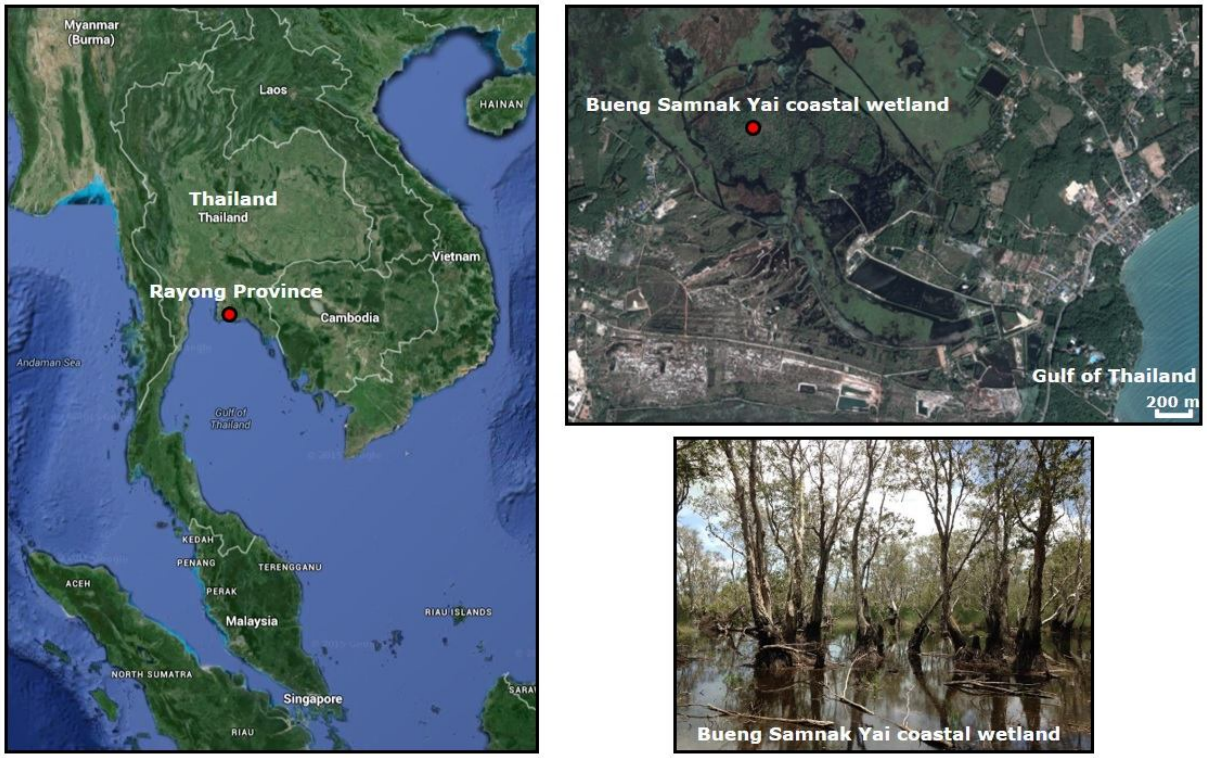

Figure 1. Map of Bueng Samnak Yai coastal wetland. The sampling site covered an area of 650,000 m^2. The figure was generated using the Google Maps service.

Isolation and purification of bacteria. One gram of each collected sample was enriched in 10 mL of Tryptone Soya Broth (HiMedia, India). All cultures were incubated at 30°C, the average temperature of sampling site, and agitated at 150 rpm for 72 h. The media were serially diluted with sterile deionized water to obtain 1:1,000 dilutions and spread plated on Tryptone Soya Agar (HiMedia, India) to isolate the bacterial colonies. The colonies were selected, investigated for morphology and streak plated on Tryptone Soya Agar to obtain pure bacterial isolates.

Screening of ligninolytic bacteria. The ligninolytic bacteria were determined by decolorization of Remazol Brilliant Blue R (Sigma-Aldrich, USA). The BSGYP medium supplemented with Remazol Brilliant Blue R conforming to previously described methods (Chang et al 2014). Bacterial isolates were inoculated in 10 mL of BSGYP medium and incubated at 30°C, with an agitation rate of 150 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 GenesysTM 10S UV-Vis spectrophotometer (Thermo Scientific, USA) at λmax of 592 nm. The decolorization percentage was calculated using the following formula:

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

The initial percentage was obtained from the absorbance of BSGYP medium supplemented with Remazol Brilliant Blue R measured before incubation. Positive control for screening was the moderately ligninolytic bacterium, *Bacillus methylotrophicus* RYC01101, isolated from Thai bovine feces (Chantarasiri 2014). All experiments were performed in triplicate. The effective isolates were studied further.

Biodegradation of commercial lignin. Biodegradation of commercial lignin by the effective bacterial isolates was modified from Chang et al (2014). Two milliliters of log-phased bacteria were cultured in 100 mL of BSGYP medium containing 0.005% (w/v) kraft lignin (Sigma-Aldrich, USA). All bacteria were cultured in bottom-baffled flasks (Duran, Germany) at 30°C, with an agitation rate of 150 rpm, in dark conditions. Bacterial suspensions were taken aseptically and centrifuged at 12,000×g for 10 min to discard any cells. Disappearance of kraft lignin in cell-free supernatants was determined by a GenesysTM 10S UV-Vis spectrophotometer at 280 nm (Yang et al 2011). The degradation percentage of kraft lignin was calculated using the following formula:

Degradation percentage of kraft lignin (%) =
[(Initial absorbance - Final absorbance) / Initial absorbance] × 100

Degradation percentage of kraft lignin was determined as a percentage of the obliterable kraft lignin after culturing of the ligninolytic bacteria. The initial percentage was obtained from the absorbance of kraft lignin in BSGYP medium before culturing. The degradation of kraft lignin was monitored for 96 h. All experiments were performed in triplicate with a positive control (inoculated with *B. methylotrophicus* RYC01101). The most effective isolates were studied further.

Effects of temperature and pH on commercial lignin biodegradation. The environmental parameters affecting commercial lignin degradation were determined by culturing the effective isolates in BSGYP medium at different pH values ranging from 5.0 to 10.0 and incubation at different temperatures ranging from 20°C to 45°C in bottom-baffled flasks. Log-phased bacterial culture of 2 mL was inoculated in 100 mL of BSGYP medium containing 0.005% (w/v) kraft lignin and agitated at 150 rpm in dark conditions for 72 h. Bacterial suspensions were taken aseptically for the disappearance of kraft lignin analyses using the method described above. All experiments were performed in triplicate.

Crude enzyme preparation. BSGYP medium containing 0.005% (w/v) kraft lignin (pH 8.0) was seeded with 2% (v/v) inoculum of the effective isolate in the log-phase. The bacterial cells were cultured in bottom-baffled flasks at 30°C with agitation of 150 rpm in dark conditions for 72 h. The bacterial culture was centrifuged at 15,000×g for 10 min at 4°C to obtain the cell-free supernate, which served as the crude enzyme solution.

Determination of crude ligninolytic enzyme activity. The ligninolytic activities of crude enzyme were assayed on the catalytic performance of three enzymes, including laccase, lignin peroxidase and manganese peroxidase. Laccase activity was determined as previously described by Chang et al (2014). The activity was measured in 1 mL of reaction mixture by incubating 0.2 mL of crude enzyme solution with 75 mM 1,2-dihydroxybenzene (Sigma-Aldrich, USA) in 50 mM Sørensen's phosphate buffer (pH 8.0). Laccase reaction was monitored at 440 nm for 10 min. The determination of lignin peroxidase and manganese peroxidase activity was modified from Yang et al (2011). Lignin peroxidase activity was measured by incubating 1.5 mL of crude enzyme solution with 0.5 mL of reaction mixture containing 2.5 mM 3,4-dimethoxybenzyl alcohol (Sigma-Aldrich, USA) and 0.5 mM H_2O_2 in 50 mM Sørensen's phosphate buffer (pH 8.0). Lignin peroxidase reaction was monitored at 310 nm for 5 min. Manganese peroxidase activity was measured by incubating 1.5 mL of crude enzyme solution with 0.5 mL of reaction mixture containing 0.1 mM $MnSO_4$, 0.1 mM H_2O_2 and 0.25 mM phenol red (LabChem, USA) in 50 mM Sørensen's phosphate buffer (pH 8.0). Manganese peroxidase reaction was monitored at 610 nm for 5 min. One unit (U) of ligninolytic enzyme activity was defined as the amount of enzyme catalyzing in 1 µmol of substrates oxidized per minute of reaction. All experiments were assayed in triplicate at 30°C.

Identification of ligninolytic bacteria. Phenotypic characterization of the most effective isolate was investigated using morphological examination and biochemical tests. The morphological examination was conducted from a formerly described method (Chantarasiri 2014) such as Gram staining, motility test, growth in anaerobic condition and growth in salinity condition. Biochemical tests were determined using KB013 HiBacillus Identification Kit (HiMedia, India) on twelve tests comprised of arginine test, catalase test, citrate test, malonate test, nitrate reduction test, ONPG test for β-galactosidase, Voges-Proskauer test and five separate carbohydrates (arabinose, glucose, mannitol, sucrose and trehalose) utilization tests.

Genotypic characterization of the isolate was analyzed by sequencing of 16S rRNA gene and phylogeny assay. The 16S rRNA gene was amplified by polymerase chain reaction using a set of primers as follows: 27F forward primer (5′-GAGTTTGATCATGGCTCAG-3′) and 1492R reverse primer (5′-CGGTTACCTTGTTACGACTT-3′). Nucleotide sequencing of the PCR products was carried out by the Mahidol University-Osaka University Collaborative Research Centre for Bioscience and Biotechnology, Thailand. The resulting 16S rDNA sequence was compared against the sequences present in the National Center for Biotechnology Information (NCBI) database using the Standard Nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree was conducted with a MEGA6 program (Tamura et al 2013) using the Neighbor-Joining algorithm (Saitou & Nei 1987). The tree was estimated by the bootstrap analysis (Felsenstein 1985) with 1,000 replications.

Application of biological pre-treatment for biological saccharification in bioethanol production. The application of biological pre-treatment on *S. aculeata* for bioethanol production with the most effective isolate was investigated. The bacteria of 2% (v/v) in log-phase were inoculated in a basal medium (the medium per liter contained 0.5 g of KCl, 1.0 g of K_2HPO_4, 0.5 g of $MgSO_4$, 2.0 g of $NaNO_3$, 0.2 g of peptone and pH 8.0) supplemented with 1% (w/v) dried *S. aculeata* powder of 500 µm particle size, cultured in bottom-baffled flasks at 30°C with agitation of 150 rpm in dark conditions for 96 h. The biological saccharification of pre-treated *S. aculeata* was followed by inoculating 1% (v/v) of cellulolytic *Bacillus cereus* (Chantarasiri et al 2015) in a basal medium and incubated at 30°C with agitation of 150 rpm in dark conditions for 48 h. The

biological saccharification performance was analyzed by determination of glucose release from hydrolyzed *S. aculeata* using the 3,5-dinitrosalicylic acid method (Miller 1959). The resulting biological saccharification performance was compared with the culture of cellulolytic *B. cereus* without biological pre-treatment of *S. aculeata* under similar experimental conditions. All experiments were performed in triplicate.

Results and Discussion. One hundred and forty-five bacterial isolates with distinct colony morphology were isolated, purified and named from forty-two coastal wetland samples. The Remazol Brilliant Blue R decolorizing performance of each isolate was investigated. Only nine isolates showed decolorization activity and could preliminarily be defined as ligninolytic bacteria. The colony morphology and decolorization activity of ligninolytic isolates are shown in Table 1. Three effective isolates, including BR0702, BR2304 and BR2308, exhibited Remazol Brilliant Blue R decolorizing activity of more than 50%, greater than the positive control bacterium by a factor of between 4.38-4.61 (Figure 2).

Table 1

Colony morphology and decolorization of Remazol Brilliant Blue R in ligninolytic bacteria

Bacterial isolate	*Shape*	*Margin*	*Elevation*	*Pigmentation*	*Decolorization percentage (%)*
BR0702	Circular	Entire	Raised	Cream	56.50±2.54
BR2304	Circular	Entire	Raised	Cream	55.28±1.86
BR2308	Circular	Entire	Raised	Cream	58.13±1.41
BR2503	Circular	Entire	Convex	White	30.49±1.22
BR2804	Circular	Entire	Raised	White	9.35±1.86
BR3104	Circular	Entire	Convex	White	18.70±5.08
BR3202	Circular	Entire	Raised	White	14.63±2.11
BR3402	Circular	Entire	Convex	White	36.99±1.86
BR3403	Circular	Entire	Convex	White	34.15±2.11
Positive control	Circular	Entire	Convex	White	12.60±4.93

Positive control was *B. methylotrophicus* RYC01101.

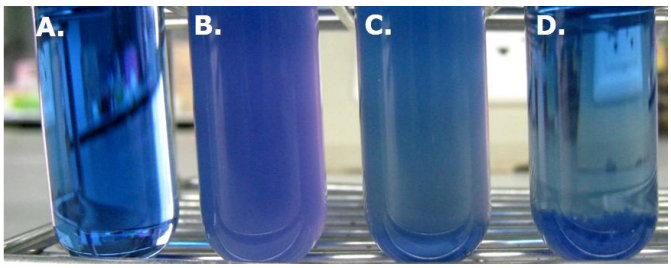

Figure 2. Remazol Brilliant Blue R decolorization by ligninolytic bacteria cultured in BSGYP medium. (A) Uninoculated. (B) Inoculated with positive control. (C) Inoculated with the isolate BR3402. (D) Inoculated with the isolate BR2308 (original).

The effective isolates were selected for determination of commercial lignin degrading performance. Commercial lignin (kraft lignin) degrading performance of the effective isolates is shown in Figure 3. Bacterial isolate BR2308 was considered the most effective isolate. It showed maximum degradation percentage of kraft lignin of 42.46±2.79 at 96 h of culture time. The degrading performance had increased slightly after 96 h of culture time. However, the degradation percentage did not reach 43.00 (data not shown). Until a few years ago, a number of reports have been elucidated about many bacteria isolated from environments that could breakdown lignin. In one interesting study, soil bacteria from several genera have been reported as ligninolytic bacteria by a degradation percentage of more than 43%, including *Arthrobacter* spp. (59% lignin degradation),

Bacillus sp. (95% lignin degradation), *Nocardia* spp. (49% lignin degradation) and *Sphingomonas* sp. (48% lignin degradation) (Chang et al 2014). There is also a study on ligninolytic bacteria isolated from rayon grade pulp industry that reported effective ligninolytic bacteria, including *B. subtilis* IITRC12 (36% lignin degradation) and *Klebsiella pneumoniae* IITRC13 (39% lignin degradation) (Yadav & Chandra 2015).

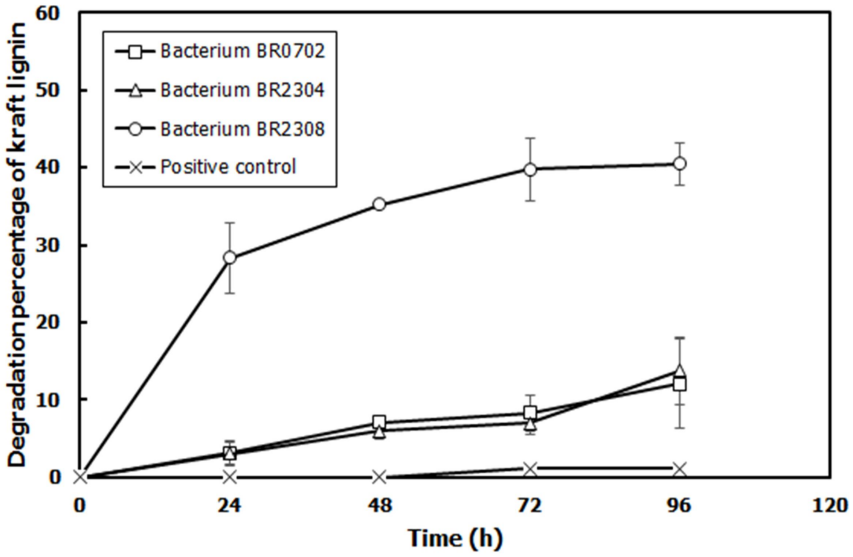

Figure 3. Biodegradation of commercial lignin (kraft lignin) by effective ligninolytic isolates with 0.005% (w/v) kraft lignin. Error bars represent the standard deviation of triplicate experiments.

The effect of environmental parameters on kraft lignin degrading performance of isolate BR2308 was observed. The results elucidated that temperature and pH conditions affect biodegrading performance considerably. A favorable temperature for kraft lignin degradation was determined to be between 30 and 35°C, with the optimum temperature found to be 30°C (Figure 3). Additionally, the results indicated that the pH for degradation of kraft lignin was alkali (pH range of 8.0-9.0) and the optimum pH for degradation was found to be 8.0 (Figure 4). These results correlated with a previous study that reported on the isolation of two ligninolytic *Bacillus* sp. from forest soil in Japan and their application in lignocellulosic biomass pre-treatment, in which a favored temperature of 37°C and alkali pH conditions (pH 8.0) to degrade alkali lignin were reported (Chang et al 2014).

Crude enzyme solution produced from isolate BR2308 was prepared and preliminarily characterized for catalytic performance of ligninolytic enzymes. The catalytic performance of three ligninolytic enzymes was examined, which showed that they could yield 0.67±0.08 U mL^{-1} of lignin peroxidase activity and 0.07±0.02 U mL^{-1} of manganese peroxidase activity. The bacterium produced effective performance for laccase activity of 1.93±0.26 U mL^{-1}. It could be concluded that this bacterium played an important function for lignin degradation in the coastal wetland ecosystem using its laccase and lignin peroxidase. For laccase, it is not a common enzyme from certain prokaryotic organisms (Shi et al 2015). The indication is that bacterial isolate BR2308 is attractive for laccase applications. In the last two decades, bacteria with effective catalytic performance of laccase could be applied biotechnologically in several fields such as the bioremediation, foodstuff industry, pulp industry and textile industry (Couto & Herrera 2006).

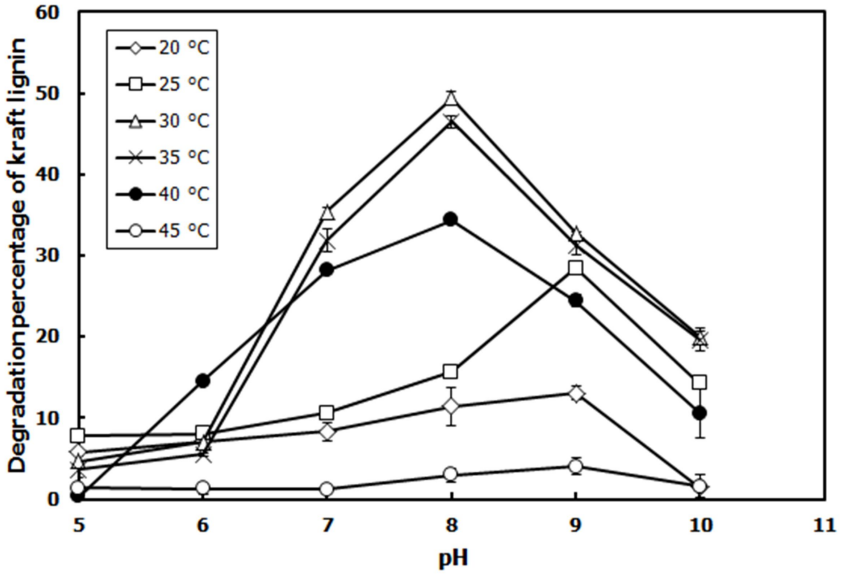

Figure 4. Effect of pH and temperature on biodegradation of commercial lignin (kraft lignin) by bacterial isolate BR2308. Error bars represent the standard deviation of triplicate experiments.

The isolate BR2308 was identified using phenotypic and genotypic characterizations. The phenotypic results of morphological examination showed that isolate BR2308 had a rod-shaped, Gram-positive and non-motile cell. It was an obligate aerobe bacterium growing at a pH of between 5.0 and 10.0 and a temperature ranging from 20 to 45°C under a NaCl concentration of 0-3% (w/v). Biochemical test results showed that the bacterium was negative for the citrate test, malonate test, nitrate reduction test, ONPG test and Voges-Proskauer test, but positive for the catalase test and weak positive for the arginine test. It could not ferment any carbohydrates including arabinose, glucose, mannitol, sucrose and trehalose. Genotypic characterization for 16S rDNA sequence analysis of 1,466 bp provided evidence that the bacterium was closely related to *Lysinibacillus sphaericus* strain 2362 (GenBank Accession number CP015224) at 99% identity and the phylogenetic relationship indicated its taxonomic position, as shown in Figure 5. The 16S rDNA sequence in this study was deposited in the GenBank database under the GenBank Accession number KX447404. According to a previous report, *Lysinibacillus* sp. is a group of bacteria with rod-shaped cells that are positive for a catalase test, but negative for ONPG and nitrate reduction tests (Ahmed et al 2007). Based on such data, this ligninolytic bacterium was designated as *L. sphaericus* BR2308. *L. sphaericus* have also been isolated from various environments and described as cellulolytic and xylanolytic bacteria (Ghio et al 2012; Gupta & Parkhey 2014; Jyotsna et al 2010). However, *L. sphaericus* BR2308 showed no correlation with cellulolytic and xylanolytic activities on lignocellulosic biomass degradation (data not shown). It is indicated that the type of microbial enzyme for organic matter degradation involving lignocellulosic biomass degradation might vary, despite the bacteria being of the same genus and species. To our knowledge, the ligninolytic bacterium isolated from the coastal wetland ecosystem in Thailand is reported for the first time.

The utilization of lignocellulosic biomass and agricultural waste for the production of reducing sugars and bioethanol by bioconversion technology is a worldwide concern nowadays (Chantarasiri 2015). In this study, dried *S. aculeata* powder was bioconverted to reducing sugars using the biological saccharification process of a cellulolytic *B. cereus*. After 48 h of incubation with cellulolytic *B. cereus*, 1% (w/v) of dried *S. aculeata* powder was bioconverted to reducing sugars of 0.27±0.00 mg mL^{-1}. To enhance the biological saccharification performance of cellulolytic *B. cereus* on dried *S. aculeata* powder, the biomass powder was pre-treated with *L. sphaericus* BR2308 before the saccharification process. At the end of incubation, 1% (w/v) of dried *S. aculeata* powder was converted to reducing sugar of 0.45±0.01 mg mL^{-1}, which was better than the saccharification without

pre-treatment process by 66.67%. In this study, *L. sphaericus* BR2308 was not a cellulolytic or xylanolytic bacterium. Therefore, the improved performance of cellulolytic *B. cereus* on saccharification mainly involved ligninolytic enzymes produced from *L. sphaericus* BR2308. This result suggested that ligninolytic *L. sphaericus* BR2308 could be applied in biological pre-treatment for degrading lignin in lignocellulosic biomass.

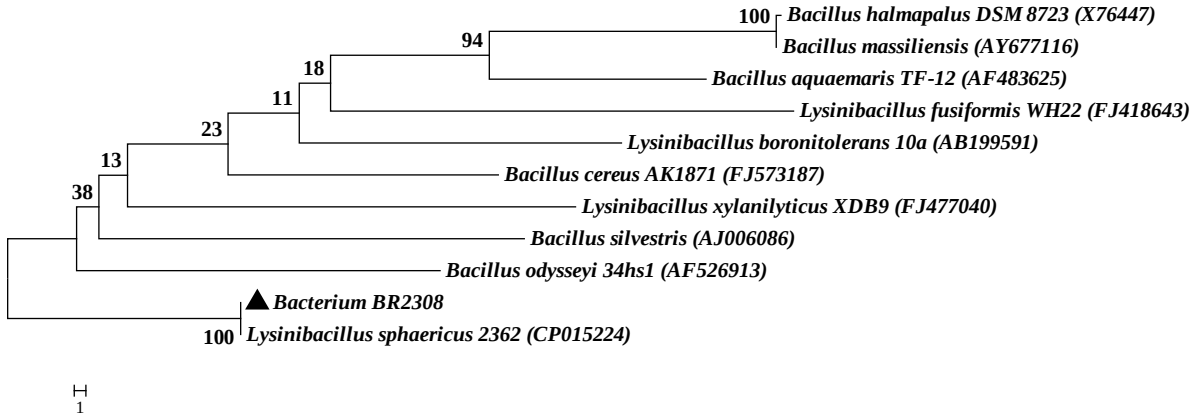

Figure 5. Phylogenetic tree of bacterial isolate BR2308 and related taxa based on 16S rDNA sequences.

Conclusions. Ligninolytic bacteria and lignin-degrading bacteria are not well understood. In this study, an effective ligninolytic bacterium strain BR2308 was isolated from a coastal wetland ecosystem, characterized and designated as *L. sphaericus* BR2308. The ligninolytic characteristics of *L. sphaericus* BR2308 have enhanced the cellulolytic performance of unrelated bacterium, *B. cereus*. The biological pre-treatment process of lignocellulosic biomass using *L. sphaericus* BR2308 will develop bioconversion technology for producing bioethanol. However, the purification procedures for ligninolytic enzymes and optimization of biological pretreatment conditions require further study.

Acknowledgements. This research was funded by King Mongkut's University of Technology North Bangkok. Contract No. KMUTNB-GEN-58-48.

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Received: 04 February 2017. Accepted: 10 March 2017. Published online: 15 March 2017.
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How to cite this article:
Chantarasiri A., Boontanom P., Nuiplot N., 2017 Isolation and characterization of *Lysinibacillus sphaericus* BR2308 from coastal wetland in Thailand for the biodegradation of lignin. AACL Bioflux 10(2):200-209.