KKU SCIENCE JOURNAL
Journal Home Page : https://ph01.tci-thaijo.org/index.php/KKUSciJ
Published by the Faculty of Science, Khon Kaen University, Thailand

การผลิตและคุณลักษณะของแลคเคสจาก *Streptomyces salinarius* และการตรึงเอนไซม์บนอนุภาคนาโนแม่เหล็ก

Production and Characterization of Laccase from *Streptomyces salinarius* and its Immobilization on Magnetite Nanoparticles

ปริญญา คำกอง[1] และ วิยะดา มงคลธนารักษ์[1*]

Parinya Khamkong[1] and Wiyada Mongkolthanaruk[1*]

[1]สาขาวิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น 40002

[1]Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand

บทคัดย่อ

แลคเคสเป็นเอนไซม์ที่มีคุณค่าในการย่อยสลายสารมลพิษทางชีวภาพในสิ่งแวดล้อม จึงศึกษาการผลิตแลคเคสจากเชื้อ *Streptomyces salinarius* ซึ่งถูกคัดแยกจากดินที่มีการปนเปื้อนสีย้อมมาก่อนหน้านี้ โดยศึกษาลักษณะทางสัณฐานวิทยาภายใต้กล้องจุลทรรศน์แบบใช้แสงและกล้องจุลทรรศน์อิเล็คตรอนแบบส่องกราดด้วยระบบสุญญากาศระดับสภาวะแวดล้อม พบลักษณะเส้นใยเป็น rectiflexibiles มีรูปร่างสปอร์แบบ doliform ขนาดเส้นผ่านศูนย์กลาง 2 ไมโครเมตร พื้นผิวของสปอร์แบบ parallel rugose และสปอร์เป็นแบบ polysporous ในการศึกษาการผลิตเอนไซม์แลคเคส มีการทดสอบชนิดและปริมาณของวัสดุเหลือใช้ทางการเกษตร เพื่อใช้เป็นสับเสตรตในอาหารเลี้ยงเชื้อ และศึกษาคุณสมบัติของเอนไซม์หยาบเพื่อดูความเสถียรและการเก็บรักษา พบว่ากิจกรรมของเอนไซม์แลคเคสสูงที่สุด (0.824 ยูนิตต่อมิลลิลิตร) ในอาหารเหลวที่เติมรำข้าวร้อยละ 1 เมื่อศึกษาการทำงานของเอนไซม์แลคเคส พบว่าแลคเคสจาก *S. salinarius* มีความคงตัวได้ดีในช่วง pH 4 - 8 โดยพบกิจกรรมคงเหลือร้อยละ 70 หลังจากการบ่ม และมีความคงตัวของเอนไซม์ที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง เมื่อทดสอบความคงทนต่อสารเคมี พบว่าสารเซทิล-ไตรเมทิลแอมโมเนียมโบรไมด์ (CATB) ช่วยเพิ่มการทำงานของเอนไซม์ ในขณะที่เฟอรัสคลอไรด์ ($FeCl_2$) ยับยั้งการทำงานของเอนไซม์ เมื่อศึกษาการตรึงเอนไซม์หยาบบนวัสดุพาหะที่เป็นวัสดุอนุภาคนาโนแม่เหล็ก (Fe_3O_4) เพื่อนำเอนไซม์กลับมาใช้ซ้ำได้ พบว่าเอนไซม์คงตัวอยู่ได้ โดยมีกิจกรรมของเอนไซม์ร้อยละ 70 และผลจากการตรึงได้โปรตีนที่ตรึงได้ร้อยละ 20 ซึ่งตรวจสอบการจับของโปรตีนด้วยเครื่อง Fourier-transform infrared spectroscopy (FTIR) จากผลการทดลองแสดงให้เห็นว่า *S. salinarius* เป็นแหล่งผลิตแลคเคสที่มีศักยภาพสามารถตรึงบนอนุภาคนาโนแม่เหล็กได้ เพื่อนำไปประยุกต์ใช้ในการบำบัดสารมลพิษ

ABSTRACT

Laccases are valuable enzymes for the biodegradation of environmental pollutants. This study investigates laccase production by *Streptomyces salinarius*, previously isolated from dye-contaminated soil. Morphological characterization using light and environmental scanning electron microscopy confirmed

*Corresponding Author, E-mail: wiymon@kku.ac.th

Received date: 18 December 2025 | Revised date: 16 April 2025 | Accepted date: 7 May 2025

doi: 10.14456/kkuscij.2025.12

typical *Streptomyces* features, including branched rectiflexibiles hyphae and rugose, doliform spores (2 µm) in polysporous chains. Various types of agricultural residues were evaluated at different concentrations to enhance laccase production. Furthermore, laccase properties related to stability and optimal storage were determined. Optimal laccase production (0.824 U/mL) was achieved in submerged fermentation using starch nitrate medium supplemented with 1% rice bran. The crude laccase exhibited stability between pH 4 and 8, retaining approximately 70% activity after storage, and was stable at 30°C for at least 24 hours. Activity was enhanced by Cetyltrimethylammonium bromide (CTAB) but strongly inhibited by ferrous chloride ($FeCl_2$). Immobilization onto magnetite nanoparticles (Fe_3O_4) is an approach to enable enzyme reuse; thus, this method was investigated using *S. salinarius* laccase. The results showed that the immobilized preparation retained 70% of its initial specific activity, although the overall protein recovery was 20%. Successful immobilization onto the nanoparticles was confirmed by Fourier-transform infrared spectroscopy (FTIR). These findings suggest that *S. salinarius* is a potential source of stable laccase suitable for immobilization on magnetic nanoparticles, offering promising source for bioremediation applications.

คำสำคัญ: แลคเคส การตรึงเอนไซม์ อนุภาคนาโนแม่เหล็ก สเตร็ปโตมัยซีท
Keywords: Laccase, Immobilization, Magnetic nanoparticle (MNPs), *Streptomyces*

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) oxidizes a broad range of substrates and is a versatile catalyst for the reduction of two oxygen molecules into water. Laccase has high redox potential and multicopper oxidoreductases on phenolic and non-phenolic aromatic compounds such as amino, methoxy monophenol, syringaldazine and 2,2'-azino-bis(3-ethylthiazoline-6-sulfonate) (ABTS). It is ubiquitous in nature, *e.g.* in fungi, insect, plant and bacteria (Coria-Oriundo *et al.*, 2021). Advantageously, laccase does not require cofactors, unlike another oxidoreductase of abundant copper protein family in microbial blue copper protein reported in a literature (Shraddha *et al*., 2011). Furthermore, bacteria have shown the potential to produce laccase at higher levels than plants, and bacterial laccase has demonstrated the ability to be thermostable and alkaline stable to a greater extent than fungal laccase, which is acid tolerant (Gogotya *et al.*, 2021; Liu *et al.*, 2020). These enzymes possess benefits in the paper and pulp, textiles, petrochemicals, pharmaceuticals, and food and beverage industries - including organic synthesis, biosensor, and in the detoxification, decolorization, bio-bleaching in industrial wastes (Ashraf *et al.*, 2020).

Azospirillum lipoferum is a laccase producing bacterium which was first detected in 1993, from rice rhizosphere. Recent research has expanded the bacterial laccase producers, identifying strains within genera such as *Bacillus*, *Marinomonas*, *Pseudomonas* and *Streptomyces* (Agarwal *et al.*, 2022). *Streptomyces* species, in particular, are recognized for their production of extracellular laccases and have been successfully employed in the degradation of micropollutants (Karuna and Poonam, 2020). The structural similarity of phenolic compounds and aromatic amines to lignin and its derivatives suggests their potential to activate secondary metabolic pathways, thereby stimulating the production of lignolytic enzymes.

Consequently, these compounds are frequently used as inducers to enhance laccase production. Furthermore, certain antibiotics have also demonstrated the ability to enhance laccase production without compromising enzyme activity (Debnath and Saha, 2020). *Streptomyces* laccases typically exhibit a small protein structure with two domains, forming a homotrimeric active form. This enzyme class is notable for its resilience to harsh conditions, including elevated temperatures and a broad pH range, characteristics that are highly desirable for biodegradation of phenolic pollutant. However, bacterial laccases are low expression levels for industrial applications. To address this limitation, various protein engineering strategies are being explored, including directed evolution, heterologous functional expression and immobilization.

Biomass residues obtaining cellulose, hemicellulose, and lignin can be depolymerized directly by laccase (Wang *et al.*, 2019) releasing carbon and nitrogen for biofuel technologies. In addition, agro-residues form beneficial substrates, promoting *Streptomyces* growth and laccase production. In a prior study (Mon *et al.,* 2022), bacterial strains were isolated from dye-contaminated soil in the Chonnabot District of Khon Kaen Province, Northeastern Thailand. *Streptomyces salinarius* was identified using 16S rRNA gene sequence (Sarnthima *et al.*, 2024) by whole genome sequencing which was submitted in NCBI database (accession number PRJNA1003678). This strain was capable of reducing silk dye pigmentation in wastewater and was subsequently shown to produce laccase (Sarnthima *et al.*, 2024). Consequently, this study investigated the optimization of laccase production in submerged culture, focusing on the identification of effective inducers derived from biomass residues. Furthermore, the biochemical properties of the laccase, including its stability across a range of pH levels, temperatures, and in the presence of organic solvents and metal ions, were characterized to assess its potential for the efficient biodegradation of phenolic pollutants. Finally, magnetite nanoparticles were explored as a matrix for the immobilization of crude laccase, with the goal of determining immobilization efficiency, protein recovery, and residual enzyme activity.

MATERIALS AND METHODS

Bacterial cultivation and morphological characterization

Streptomyces salinarius was studied for morphology on International Streptomyces Project (ISP) agar of ISP-2, ISP-3, ISP-4, ISP-5, ISP-6 and ISP-7 as described by Shirling and Gottlieb (1966) and incubated at 30°C. The morphology of *S. salinarius* was observed and determined under light microscope. Moreover, the cells were collected from ISP-2 broth by centrifugation, stored at -20°C overnight and then lyophilized at -80°C for 2 days (modified from Prakash and Nawani (2014)). The lyophilized cells were detected under environmental scanning electron microscope (E-SEM) (Quattro-S E-SEM, Thermo Fisher Scientific).

Optimization of substrates for laccase production

The bacterial inoculum (10% v/v) from 24 h culture of ISP-2 broth was transferred into starch nitrate solution (SNS) containing (g/L): soluble starch, 20; $NaNO_3$, 2; KCl, 0.5; K_2HPO_4, 1; $Mg_2SO_4.7H_2O$, 0.5; $CaCO_3$, 1; $FeSO_4.5H_2O$, 0.01 at pH 7.0 (Reda *et al.*, 2019). Different biomass residues (2%) were individually added into the medium, *e.g.* soybean meal, wood mill, rice husk, rice straw and rice bran. The media were incubated at 30°C with shaking of 150 rpm for 8 days. The supernatant was collected to measure laccase

activity. To test different substrate concentrations, biomass residue was added to the SNS medium at 0, 0.5, 1.0, 2.0, 3.0 and 4.0% (w/v). The bacterium was inoculated into the medium (10% v/v) and the cultures were incubated at 30°C with shaking at 150 rpm. The supernatant was collected daily for 8 days to measure laccase activity.

Laccase activity

Aliquots of crude enzyme (30 µL) were mixed with 100 µL ABTS solution (20 mM) in 870 µL sodium acetate buffer (0.1 M; pH 4.0). The reaction was held at 30°C for 10 min for measurement of the oxidation reaction at 420 nm ($\boldsymbol{\varepsilon}$ ABTS = 36000 $M^{-1}CM^{-1}$). One unit of enzyme was defined as the amount of enzyme oxidizing 1 µmol of ABTS min^{-1} (Jing, 2010). The protein content was determined by using Lowry method. The laccase activity was calculated using an equation.

$$Laccase\ activity = \frac{\Delta A_{420} \times test\ volume}{ABTS\ coefficient \times enzyme\ volume \times time}$$

Effects of pH and chemical compounds on stability of laccase

The crude enzyme was mixed with 0.1 M of glycine-HCl buffer (pH 3 and 6), sodium acetate buffer (pH 4) and glycine-NaOH buffer (pH 8) and then incubated at 4°C and 30°C; after which the enzyme was taken at various times for activity assay. Additionally, the crude enzyme was mixed with organic solvents (propanol and acetonitrile), chemicals (sodium citrate, tween-80, triton x-100, and CTAB) and ionic compounds (NaN_3, $NaNO_3$, $MgSO_4$, $FeSO_4$, $MnSO_4$, $FeCl_2$, $FeCl_3$, and Fe_3O_4) at 1 mM and 5 mM for 30 min at 30°C; the remaining activity was determined. Relative activity was expressed as a percentage of the maximum activity, which was set to 100%.

Immobilization process

The magnetic nanoparticle (MNP) was synthesized from iron precipitation of 0.6 M $FeCl_2$ and 0.72 M $FeCl_3$ mixing at the rate of 5.0 mL/min in 1.0 M NH_4OH (450 mL) in stirring condition at room temperature. The black iron precipitates were separated by magnet under magnetic field and washed with distilled water, followed by washing in phosphate buffered saline (PBS; 100 mM sodium phosphate, 150 mM NaCl, pH 7.4) 3 times, giving the product of MNPs contained with -OH group. Afterward, the amino group (-NH_2) was added into MNPs by incubation of 300 mg MNPs in 2% (v/v) 3-Aminopropyltriethoxysilane (APTS) of 30 ml at 70°C for 24 h. The MNP-NH_2 was washed 3 times with PBS and kept at 4°C until use (Taheran *et al.*, 2017).

The crude laccase was immobilized to MNP-NH_2 at a ratio of 0.2 mg protein/mg MNP-NH_2. The reaction was dissolved in 100 mM ammonium sulphate and 2.5 mM Triton X-100 with shaking at 30 rpm for 5 min. After that, a 2.5 M glutaraldehyde solution was added in the reaction at a final concentration of 200 mM and shaking continued for 5 h. Finally, the MNP-laccase particles were separated by a magnetic field and washed 3 times with PBS. The immobilized laccase in PBS was kept at 4°C until used. The concentration of ammonium sulphate (50 - 200 mM) and glutaraldehyde (50 - 300 mM) were investigated for optimal immobilization, including incubation time (1 - 24 h). Air-dry immobilized enzyme was analyzed the chemical group by FTIR (Fourier transform infrared spectroscopy, TENSOR27 Bruker) at the range of 500 - 4000 cm^{-1}.

Statistical analysis

All data are reported as mean ± standard deviation (SD). To compare means between samples for substrate optimization and chemical enzyme stability, a one-way ANOVA with a Least Significant Difference (LSD) post-hoc test was performed using Statistix 10.0 (Analytical Software).

RESULTS AND DISCUSSION

Morphological characterization

Streptomyces salinarius differentiated into substrate and aerial mycelium forming colony shape and color on different media (Figure 1). The observation of form, margin, elevation, and color on various visualizable culture plates were irregular, undulate, crateriform, and yellow on ISP-2; circle, umbonate, entire, yellow brownish and pink on ISP-3; irregular, undulate, filiform, light yellow and yellow brown on ISP-4; irregular, undulate, filiform, light yellow and yellow on ISP-5; irregular, undulate, crateriform, and whitish yellow on ISP-6; irregular, undulate, crateriform, and whitish cream on ISP-7.

Streptomyces is a member of actinomycetes as gram positive filamentous bacteria with a high guanine and cytosine (G + C) content. Morphologically, it has branches of mycelia like fungi, whereas chromosome, peptidoglycan and cell wall are similar to bacteria. Under E-SEM, the mycelium of *S. salinarius* was branched with rectiflexible spore chain; spore shape was doliform around 2 µm with polysporous in sporangium; the spore surface was parallel rugose (Figure 2). This strain showed some features similar to *S. salinarius* SS06011, another strain type of this species (Klanbut *et al.*, 2023).

The most basic characteristic for novel information on this taxon is morphological characterization such as mycelia, spore shape and chain, and pigment. Vegetative mycelium of *Streptomyces* was expanded from spore via tip growth at an early stage of the life cycle; later on, aerial mycelium developed into branches of hyphae in the air. The sporulation and spore maturation occurred as a response to nutrient sources. During vegetative (substrate) mycelium, *Streptomyces* were able to release secondary metabolites (bioactive compound) included antibiotics and enzymes until growth limitation; then the spore was produced. Thus, the investigation of laccase production should determine how and when nutrients play a beneficial role in the life cycle of this bacterium.

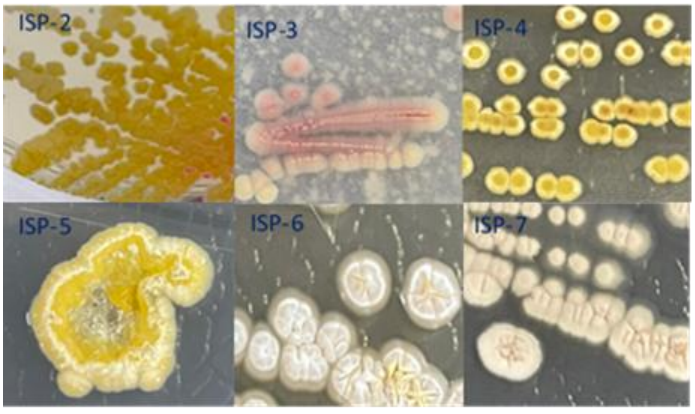

Figure 1 Morphology of *S. salinarius* on surface media at 30°C for 10 days.

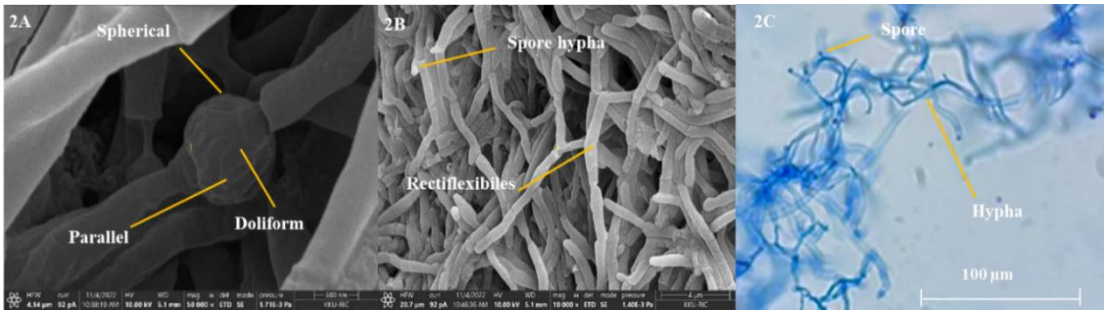

Figure 2 *S. salinarius* under microscopes; 2A spore feature under E-SEM showed spherical sporangium, doliform shape and parallel rugose surface; 2B mycelium and spore hypha in rectiflexibiles type under E-SEM; 2C spore and hypha under light microscope.

Optimal substrate for laccase production

Streptomyces salinarius was able to produce laccase enzyme; thus, the production medium with various substrates was determined for optimization of laccase production. Lignocellulose is a major agricultural waste providing complex nutrient sources, such as carbon and nitrogen, for laccase production. The optimum medium was rice bran supplemented in the starch nitrate solution, showing maximum enzyme activity at 0.262 U/ml. Other substrates, soybean meal, wood mill, rice husk, and rice straw were tested in the medium, resulting in 0.191, 0.087, 0.098 and 0.036 U/mL of laccase activity, respectively (Figure 3A). Rice bran contains neutral detergent fiber (NDF), hemicellulose, lignin, cellulose, acid detergent fiber (ADF), and ash (Muthukumarasamy *et al.*, 2015). Additionally, it includes proteins, amino acids, vitamins and minerals such as phosphorus and manganese (Araújo *et al.*, 2022), which have been found to be suitable for *S. salinarius* growth and laccase production.

Optimal conditions for laccase production were determined by varying the percentage of rice bran. Maximum activity (0.824 U/mL) was observed on day 7 in the medium containing 1% rice bran (Figure 3B). These results suggest that the bacterium utilized carbon sources from the rice bran which gave different sugars, such as xylose, arabinose for growth. Additionally, lignin and phenolic compounds present in the rice bran served as inducers for laccase production. Subsequently, the activity decreased, possibly due to nutrient limitation. Carbon sources, particularly phenolic compounds commonly found in agricultural residues, are known to be key substrates or inducers for laccase production. Different *Streptomyces* species have been reported to utilize various substrates for laccase production. For example, *S. cinnamomensis* achieved optimal production (0.0175 U/mL) in a medium containing 1.24% yeast extract, 2.3% coffee pulp, 1.46% wheat bran, 1.78% sawdust and 3.22% urea (Jing and Wang, 2012). Laccase production by *S. psammoticus* MTCC7334 was optimized, yielding 17.3 U/g in solid-state fermentation using rice straw (Niladevi *et al.*, 2007) and 15.2 U/mL in submerged fermentation using coffee pulp (Niladevi *et al.*, 2009). However, the culture of *S. salinarius* should be studied further for production with a combination of inducers.

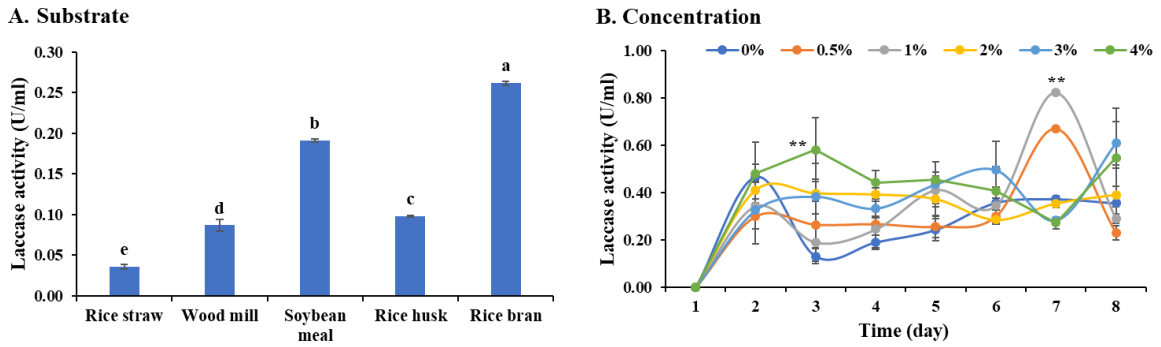

Figure 3 Effect of (A) substrate type and (B) concentration on laccase activity of *S. salinarius* at 30°C in starch nitrate medium; Laccase activity over time with various substrates (2% w/v). Letters denote significant differences between substrates (LSD, $p < 0.01$). Laccase activity over time with different rice bran concentrations (0 - 4% w/v). Asterisks (**) indicate statistically significant differences between concentrations within a specific day ($p < 0.01$).

Laccase stability

The stability of the laccase produced by *S. salinarius* was evaluated under various conditions, including temperature, pH and exposure to chemicals and organic solvents. Previous work established that this laccase exhibits significant thermal tolerance, retaining over 50% of its activity at 80°C. Its optimal pH for ABTS lies within the acidic range of pH 3 - 4, and it shows stability at pH 4 (Sarnthima *et al.*, 2024). Acidic conditions might affect protein structure in the long term; thus, pH stability was further investigated over 24 hours at two temperatures (4°C and 30°C) for storage conditions. At 4°C (Figure 4A), the enzyme activity decreased within the first 3 hours at acidic pH, retaining approximately 50% activity at pH 3 and 70% at pH 4, after which it remained relatively stable for up to 24 hours. At pH 6, the enzyme was stable initially but activity decreased to about 50% by 24 hours. Notably, at pH 8, the enzyme maintained nearly 100% activity throughout the 24-hour period.

A similar pattern was observed at 30°C (Figure 4B), although the activity loss was more pronounced under acidic conditions, particularly at pH 3 where activity dropped to only 20%. At pH 6 and 30°C, the enzyme activity fluctuated showing an increase after 3 hours. This may be related to conformational changes or the complex nature of laccase structures, which often contain multiple copper centers (T1/T2/T3) with potentially different pH profiles (Scheiblbrandner *et al.*, 2017). These results indicate that pH 8 at 4°C provides the highest stability for storage. While the enzyme functions optimally at acidic pH, storage at pH 4 (especially at 4°C) is feasible but may result in approximately 30% activity loss over 24 hours. The broad pH and temperature tolerance observed is consistent with laccases from other Streptomyces species, such as the heat-tolerant (90°C) alkaline laccase from Streptomyces sp. LAO (Gogotya *et al.*, 2021) and the enzyme from *S. sviceus* with a wide optimal pH range (pH 5 - 11) depending on the substrate (Gunne and Urlacher, 2012).

Streptomyces salinarius laccase demonstrated good tolerance to various chemicals and organic solvents tested at 10 mM concentration, generally retaining over 65% of its initial activity (Table 1). As metalloenzymes involved in redox reactions, laccases can be influenced by ionic compounds. Consistent with previous findings showing induction by $CuSO_4$, $ZnSO_4$ (Sarnthima *et al.*, 2024); this study found good activity retention in the presence of $MgSO_4$ and $MnSO_4$. Furthermore, cationic surfactant CTAB appeared to induce laccase activity similar to that of laccase from *Bacillus tequilensis* SN4 (Sondhi *et al.*, 2014). The interaction between this surfactant and the enzyme may alter its enzymatic and physical characteristics. Strong inhibition was observed with $FeSO_4$ and $FeCl_2$, indicating that iron (II) ions (Fe^{2+}) significantly reduce enzyme activity. Laccases, being multicopper oxidases, are susceptible to inhibition by agents that interfere with their copper cofactors. Fungal laccase of *Trametes polyzona* WRF03 was inhibited by Fe^{2+}, sodium azide and sodium cyanide (Ezike *et al.*, 2020); likely due to *T. versicolor,* laccase demonstrated an iron inhibitor. The possible reason was from Fe^{2+} oxidized into Fe^{3+} and then Fe^{3+} complex bound with laccase T1-Cu site impeding the single-electron transfer system (Li *et al.*, 2022). Interestingly, the *S. salinarius* laccase retained approximately 70% activity in the presence of $FeCl_3$ and Fe_3O_4 (magnetite). This tolerance to magnetite is particularly noteworthy, suggesting potential compatibility with immobilization strategies using magnetic nanoparticles.

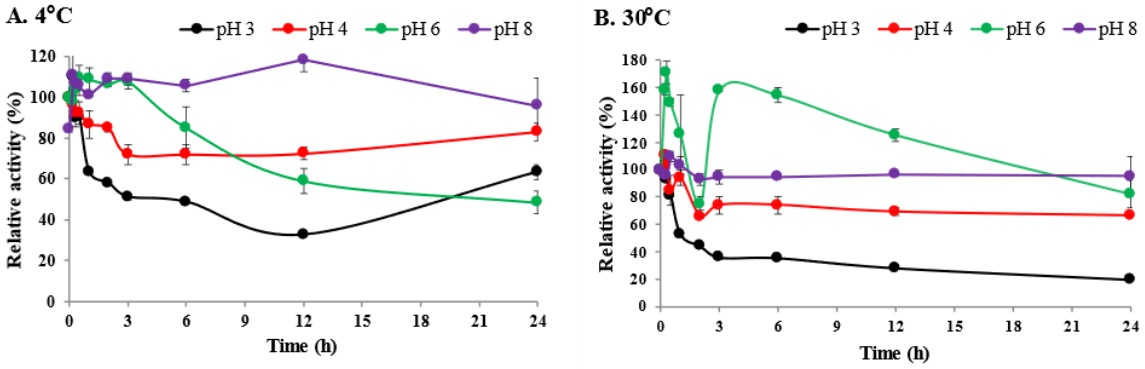

Figure 4 Stability of laccase in different pH at (A) 4°C and (B) 30°C for long storage.

Table 1 Effect of chemical compounds on laccase activity at 30°C for 30 min

Chemical compound		Relative activity (%)	
		1 mM	10 mM
Control		100 ± 0.014^{a}	100 ± 0.014^{b}
Organic solvent	Propanol	72 ± 0.012^{g}	65 ± 0.010^{f}
	Acetonitrile	72 ± 0.043^{g}	73 ± 0.005^{e}
Chemicals	Sodium citrate	91 ± 0.036^{bc}	91 ± 0.78^{cd}
	Tween-80	77 ± 0.022^{f}	87 ± 0.018^{d}
	Triton x-100	72 ± 0.012^{g}	71 ± 0.036^{e}
	CTAB	82 ± 0.006^{de}	127 ± 0.015^{a}

Table 1 Effect of chemical compounds on laccase activity at 30°C for 30 min (continued)

Chemical compound		Relative activity (%)	
		1 mM	10 mM
Ionic compounds	NaN_3	52 ± 0.028^{i}	15 ± 0.005^{g}
	$NaNO_3$	94 ± 0.009^{b}	87 ± 0.048^{d}
	$MgSO_4$	86 ± 0.022^{cd}	93 ± 0.005^{c}
	$FeSO_4$	70 ± 0.010^{g}	0 ± 0.000^{h}
	$MnSO_4$	78 ± 0.060^{ef}	91 ± 0.041^{cd}
	$FeCl_2$	61 ± 0.015^{h}	0 ± 0.000^{h}
	$FeCl_3$	96 ± 0.023^{ab}	68 ± 0.017^{ef}
	Fe_3O_4	81 ± 0.012^{ef}	73 ± 0.018^{e}

Different letters in each column indicate significant difference at $p < 0.05$ probability levels by least significant difference (LSD) test.

Laccase immobilization

The potential process for enzyme usage is immobilization enabling reuse of the enzyme. The properties of *S. salinarius* laccase suit immobilization for biodegradation, as the enzyme is stable across a wide range of environments, pH, temperatures and chemicals. The enzyme yield was low in fermentation; thus, it should be used efficiently. Magnetic nanoparticles (Fe_3O_4) were investigated for laccase immobilization. This approach offers several potential advantages, including facile recovery and reuse of the immobilized enzyme via magnetic separation, enhanced enzyme activity due to the increased surface area and inherent non-toxicity. Furthermore, the scalability of Fe_3O_4 nanoparticle synthesis makes this immobilization strategy attractive for large-scale wastewater treatment. In this study, ammonium sulfate was used to precipitate protein; the results showed 100 mM ammonium sulfate gave the highest protein recovery (Table 2). In the next step, glutaraldehyde, which designed the linkage of particles and proteins, showed optimal concentration at 200 mM, resulting in high activity and protein content. The time of reaction was determined and resulted at 10 hours of immobilization process with the highest activity and protein content.

Table 2 Immobilization conditions of laccase on Fe_3O_4

Ammonium Sulfate (mM)	Glutaraldehyde (mM)	Time (h)	Specific Activity (mU/mg)	Protein Recovery (%)
50	100	5	0.038 ± 0.008	14.47
100	100	5	0.028 ± 0.007	16.67
150	100	5	0.000	9.89
200	100	5	0.126 ± 0.006	7.36
100	50	5	0.000	0.00
100	100	5	0.073 ± 0.000	3.65
100	200	5	0.097 ± 0.000	4.87
100	300	5	0.000	0.00

Table 2 Immobilization conditions of laccase on Fe_3O_4 (continued)

Ammonium Sulfate (mM)	Glutaraldehyde (mM)	Time (h)	Specific Activity (mU/mg)	Protein Recovery (%)
100	200	1	0.072 ± 0.001	3.58
100	200	3	0.084 ± 0.001	4.22
100	200	5	0.350 ± 0.000	17.50
100	200	10	0.417 ± 0.000	20.83
100	200	24	0.258 ± 0.001	12.91

The surface functional group of laccase immobilized magnetite was determined by fourier transform infrared spectra (FTIR) shown in Figure 5. The results were interpreted according to related reports. The broad peaks at 3400 cm^{-1} and 3000 cm^{-1} were attributed to OH-stretching and NH-stretching, causing by the interaction of amino and hydroxyl groups. The peaks near 1633 cm^{-1} were related to OH-stretching vibration modes and asymmetric and symmetric stretching vibrations of the carbonyl group of organics (Liu *et al.*, 2016). The peak around 1300 cm^{-1} was the existence of amino groups (He *et al.*, 2018). The broad peaks in the range of 500 - 1000 cm^{-1} were attributed to the stretching vibration mode associated to the metal-oxygen (Fe-O bonds) contribution. This confirmed that the protein of crude laccase was immobilized to Fe_3O_4, presenting at 3000 cm^{-1} and 1000 - 1600 cm^{-1} with changing and bending peaks.

However, the protein recovery was insufficient, at lower than 50%. The process should be modifying the nanoparticle surface with functional groups to optimize enzyme binding and activity, such as adding other compounds, e.g. chelated Cu^{2+} of carbon shell, known Fe_3O_4@C-Cu^{2+} nanoparticles (Li *et al.*, 2020), chitosan (Zhang *et al.*, 2020). Moreover, other materials are of interest for laccase immobilization, for example; graphene, nanotube, nanofibers membrane (Taheran *et al.*, 2017; Xu *et al.*, 2014).

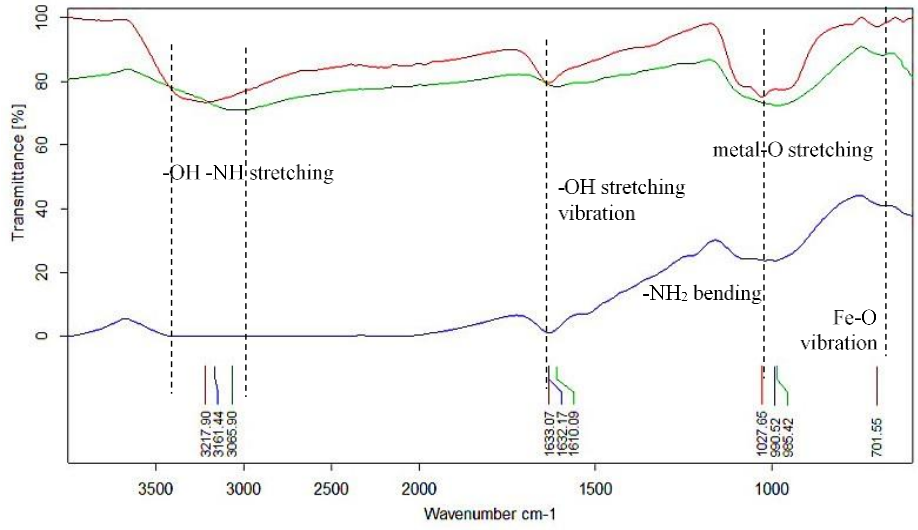

Figure 5 The spectra of Fe_3O_4 nanoparticles (red), Fe_3O_4-NH_2 (blue) and Fe_3O_4-NH_2-laccase (green) observed under FTIR.

CONCLUSIONS

Streptomyces salinarius represents a valuable new source for laccase production, offering a low molecular weight enzyme with practical advantages. The demonstrated ability to enhance production using inexpensive agricultural waste (rice bran) underscores its potential for sustainable enzyme sourcing. The resulting laccase displayed optimal storage condition at 4°C and 30°C and significant tolerance to magnetite nanoparticles as a key property for immobilization. Although immobilization was achieved, optimizing this process to improve protein recovery is necessary for developing reusable biocatalysts. Future research should focus on optimizing both laccase production and immobilization strategies to fully realize the biotechnological potential of this novel enzyme for applications such as bioremediation and biocatalysis.

ACKNOWLEDGMENT

This research was supported by the Fundamental Fund of Khon Kaen University from the National Science, Research and Innovation Fund (NSRF).

REFERENCES

Agarwal, N., Solanki, V.S., Gacem, A., Hasan, M.A., Pare, B., Srivastava, A., Singh, A., Yadav, V.K., Yadav, K.K., Lee, C., Chaiprapat, S. and Jeon, B.H. (2022). Bacterial laccases as biocatalysts for the remediation of environmental toxic pollutants: A green and eco-friendly approach-a review. Water 14: 4068.

Araújo, G.S., Ribeiro, G.O., Almeida de Souza, S.M., Paulo da Silva, G., Mafra de Carvalho, G.B., Bispo, J.A.C. and Martínez, E.A. (2022). Rice *(Oryza sativa)* Bran and Soybean *(Glycine max)* Meal: Unconventional Supplements in the Mead Production. Food Technology and Biotechnology 60(1): 89 - 98.

Ashraf, F., Irfan, M, Shakir, H.A., Ali, S. and Khan, M. (2020). An overview of production and industrial exploitation of bacterial laccases. Punjab University Journal of Zoology 35(1): 147 - 156.

Coria-Oriundo, L.L., Battaglini, F. and Wirth, S.A. (2021). Efficient decolorization of recalcitrant dyes at neutral/ alkaline pH by a new bacterial laccase-mediator system. Ecotoxicology and Environmental Safety 217: 112237.

Debnath, R. and Saha, T. (2020). An insight into the production strategies and applications of the ligninolytic enzyme laccase from bacteria and fungi. Biocatalysis and Agricultural Biotechnology 26: 101645.

Ezike, T.C., Ezugwu, A.L., Udeh, J.O., Eze, S.O. and Chilaka, F.C. (2020). Purification and characterisation of new laccase from *Trametes polyzona* WRF03. Biotechnology Reports 28: e00566.

Gogotya, A, Nnolim, N.E., Digban, T.O., Okoh, A.I. and Nwodo, U.U. (2021). Characterization of a thermostable and solvent-tolerant laccase produced by *Streptomyces* sp. LAO. Biotechnology Letters 43: 1429 - 1442.

Gunne, M. and Urlacher, V.B. (2012). Characterization of the Alkaline Laccase Ssl1 from *Streptomyces sviceus* with Unusual Properties Discovered by Genome Mining. PLoS One 7: e52360.

He, X., Yang, W., Li, S., Liu, Y., Hu, B., Wang, T. and Hou, X. (2018). An amino-functionalized magnetic framework composite of type Fe_3O_4-NH2@MIL-101(Cr) for extraction of pyrethroids coupled with GC-ECD. Microchimica Acta 185: 125.

Jing, D. (2010). Improving the simultaneous production of laccase and lignin peroxidase from Streptomyces lavendulae by medium optimization. Bioresource Technology 101(19): 7592 - 7597.

Jing, D. and Wang, J. (2012). Controlling the simultaneous production of laccase and lignin peroxidase from *Streptomyces cinnamomensis* by medium formulation. Biotechnology for Biofuels 5(15): 1 - 7.

Karuna, D. and Poonam, S. (2020). Production, partial purification and characterization of laccase from rhizospheric bacteria *Pseudomonas putida* strain LUA15.1. Research Journal of Biotechnology 15(2): 144 – 152.

Klanbut, K., Rattanakavil, T., Duangupama, T., Suriyachadkun, C., Herron, P.R. and Thawai, C. (2023). *Streptomyces salinarius* sp. nov., an actinomycete isolated from solar saltern soil. International Journal of Systematic and Evolutionary Microbiology 73(4): 005863.

Li, Z., Chen, Z., Zhu, Q., Song, J., Li, S. and Liu, X. (2020). Improved performance of immobilized laccase on Fe_3O_4@ C-Cu^{2+} nanoparticles and its application for biodegradation of dyes. Journal of Hazardous Materials 399: 123088.

Li, S., Liu, Q., Liu, J., Sun, K., Yang, W., Si, Y. and Gao, Y. (2022). Inhibition mechanisms of Fe^{2+}/Fe^{3+} and Mn^{2+} on fungal laccase-enabled bisphenol a polyreaction. Chemosphere 307: 135685.

Liu, Y., Luo, G., Ngo, H.H., Guo, W. and Zhang, S. (2020). Advances in thermostable laccase and its current application in lignin-first biorefinery: A review. Bioresource Technology 298: 122511.

Liu, Y., Yan, M., Geng, Y. and Huang, J. (2016). Laccase Immobilization on Poly(*p*-phenylenediamine)/Fe_3O_4 Nanocomposite for Reactive Blue 19 Dye Removal. Applied Sciences 6: 232.

Mon, W.P., Jantaharn, P., Boonlue, S., McCloskey, S., Kanokmedhakul, S. and Mongkolthanaruk, W. (2022). Enzymatic Degradation of Azo Bonds and Other Functional Groups on Commercial Silk Dyes by *Streptomyces coelicoflavus* CS-29. Environment and Natural Resources Journal 20(1): 19 - 28.

Muthukumarasamy, N.P., Jackson, B., Joseph, R.A. and Sevanan, M. (2015). Production of Extracellular Laccase from *Bacillus subtilis* MTCC 2414 Using Agroresidues as a Potential Substrate. Biochemistry Research International 2: 765190.

Niladevi, K.N., Sukumaran, R.K. and Prema, P. (2007). Utilization of rice straw for laccase production by Streptomyces psammoticus in solid-state fermentation. Journal of Industrial Microbiology and Biotechnology 34(10): 665 - 674.

Niladevi, K.N., Sukumaran, R.K., Nicemol, J., Anisha, G.S. and Prema, P. (2009). Optimization of laccase production from a novel strain-*Streptomyces psammoticus* using response surface methodology. Microbiological Research 164: 105 - 113.

Prakash, D. and Nawani, N.N. (2014). A rapid and improved technique for scanning electron microscopy of actinomycetes. Journal of Microbiological Methods 99: 54 - 57.

Reda, F.M., El-Mekkawy R.M. and Hassan, N.S. (2019). Detoxification and Bioremediation of Sulfa Drugs and Synthetic Dyes by *Streptomyces mutabilis* A17 Laccase Produced in Solid State Fermentation. Journal of Pure and Applied Microbiology 13(1): 85 - 97.

Sarnthima, R., Mongkolthanaruk, W., Sanachai, K. and Khammuang, S. (2024). Laccase from *Streptomyces* sp. CS29 and molecular insight of sulfamethoxazole degradation. Biologia 79: 311 - 320.

Scheiblbrandner, S., Breslmayr, E., Csarman, F., Paukner, R., Führer, J., Herzog, PL., Shleev, SV., Osipov, EM., Tikhonova, TV., Popov, VO., Haltrich, D., Ludwig, R. and Kittl, R. (2017). Evolving stability and pH-dependent activity of the high redox potential Botrytis aclada laccase for enzymatic fuel cells. Scientific reports 7: 13688.

Shirling, E.B. and Gottlieb, D. (1966). Methods for characterization of Streptomyces species[1]. International Journal of Systematic and Evolutionary Microbiology 16(3): 313 - 340.

Shraddha, SR., Sehgal, S., Kamthania, M. and Kumar, A. (2011). Laccase: microbial sources, production, purification, and potential biotechnological applications. Enzyme Research 2011: 217861.

Sondhi, S., Sharma, P., Saini, S., Puri, N. and Gupta, N. (2014) Purification and Characterization of an Extracellular, Thermo-Alkali-Stable, Metal Tolerant Laccase from *Bacillus tequilensis* SN4. PLoS ONE 9(5): e96951.

Taheran, M., Naghdi, M., Brar, S.K., Knystautas, E.J., Verma, M. and Surampalli, R.Y. (2017). Covalent Immobilization of Laccase onto Nanofibrous Membrane for Degradation of Pharmaceutical Residues in Water. ACS Sustainable Chemistry and Engineering 5(11): 10430 - 10438.

Wang, F., Xu, L., Zhao, L., Ding, Z., Ma, H. and Terry, N. (2019). Fungal Laccase Production from Lignocellulosic Agricultural Wastes by Solid-State Fermentation: A Review. Microorganisms 7(12): 665.

Xu, R., Bi, H., He, G., Zhu, J. and Chen, H. (2014). Synthesis of Cu-Fe_3O_4@graphene composite: A magnetically separable and efficient catalyst for the reduction of 4-nitrophenol. Materials Research Bulletin 57: 190 - 196.

Zhang, K., Yang, W., Liu, Y., Zhang, K., Chen, Y. and Yin, X. (2020). Laccase immobilized on chitosan-coated Fe_3O_4 nanoparticles as reusable biocatalyst for degradation of chlorophenol. Journal of Molecular Structure 1220: 128769.

❑❑❑❑❑