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Biological Conversion of Agricultural Wastes into Indole-3-acetic Acid by Streptomyces lavenduligriseus BS50-1 Using a Response Surface Methodology (RSM)

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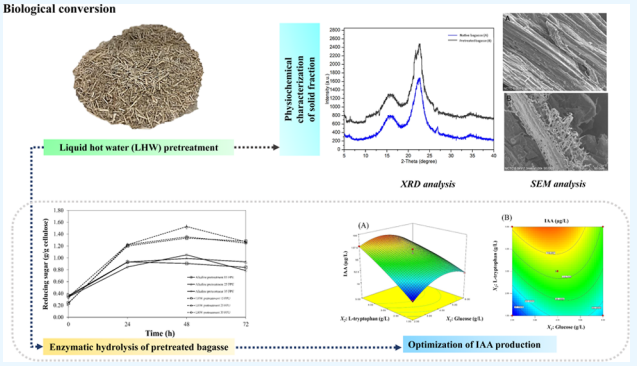
ABSTRACT: Agricultural waste is an alternative source for plant growth regulator biosynthesis by microorganisms. Actinobacteria are important soil microbes that significantly impact the soil as plant growth-promoting rhizobacteria and biofertilizers. This study focused on developing low-cost medium based on bagasse to improve indole-3-acetic acid (IAA) production by *Streptomyces lavenduligriseus* BS50-1 using a response surface methodology (RSM). Among 34 actinobacterial strains, *S. lavenduligriseus* BS50-1 produced the highest IAA level within the selected medium. An RSM based on a central composite design optimized the appropriate nutrients for IAA production. Thus, glucose hydrolysate and L-tryptophan at concentrations of 3.55 and 5.0 g/L, respectively, were the optimal factors that improved IAA production from 37.50 to 159.47 μg/mL within 168 h. This study reported a potential application of leftover bagasse as the raw material for cultivating actinobacteria, which efficiently produce IAA to promote plant growth.



1. INTRODUCTION

Currently, sugarcane is a major agricultural crop that is cultivated on a large scale in Thailand and other tropical countries. However, at the end of each cultivation, bagasse is openly burned, causing air pollution. In recent years, the replacement of chemical inputs with biotechnological products has attracted much attention in many research fields. This is a promising alternative to using chemicals that irreversibly deplete the environment and cause health concerns for both producers and consumers. Consequently, bagasse is used as a raw material to produce biochemicals such as bioethanol, polyhydroxyalkanoates (PHAs), biomethane, and xylitol.[1]

Implementing alternate strategies for soil biofertilization is urgently needed for sustainable agriculture with limited nutrient supplies. The rhizosphere is a rich source of microorganisms with various processes that promote plant growth.[2] Several microorganisms, including bacteria, fungi, and algae, produce plant growth regulators (such as auxin, gibberellin, and ethylene), siderophores, HCN, and antibiotics, which may considerably affect plant growth and development.[3−7] Actinobacteria are the most widely distributed microorganisms in nature. The genus *Streptomyces* is an important group of soil bacteria, 60% of which is a source of most bioactive compounds, including antimicrobial, antibiotic, and plant growth-promoting compounds.[8] *Streptomyces* enhance plant growth by producing phytohormones such as auxins or gibberellins.[9,10]

Indole-3-acetic acid (IAA) is the main member of the auxin family and is a common plant growth regulator (PGR) that can be synthesized by microorganisms. IAA is produced by several *Streptomyces* species (sp.), including *Streptomyces olivaceoviridis*, *Streptomyces rimosus*, and *Streptomyces viridis*.[11] IAA is the most effective metabolite for promoting plant development through various approaches. Additionally, it controls plant physiological processes, including photosynthesis, apical dominance maintenance, positive gravitropism (curvature of the roots toward gravity), pigment formation, and the biosynthesis of plant defense molecules such as phytoalexins, phenylpropanoids, and pathogenicity-related proteins.[12] A previous report described a

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secondary bacterial metabolite that regulates several biological processes, including cell division, growth, sprouting, gene regulation, root and shoot elongation, increasing plant weight, and the inhibition or promotion of flowering and fruiting.[13] IAA has a rising demand in the worldwide agricultural industry and could cost as much as US$ 150/kg due to obstacles in producing affordable PGRs. In the rhizosphere, bacterial populations form a great organic plant-specific microecosystem. Rhizospheric microbes develop complex connections with plants to increase their productivity by synthesizing various metabolites including phytohormones. Due to their significance in IAA generation and other aspects of soil fertilization, the use of phytohormone-producing microorganisms such as *Streptomyces* is being considered. *Streptomyces* sp. is used to evaluate plant growth promotion in chickpeas in vitro and in vivo. In the field, *Streptomyces* sp. result in increased nodule numbers, shoot weights, and yield by producing siderophores, cellulase, lipase, protease, chitinase, hydrocyanic acid, IAA, and β-1,3-glucanase.[13] Biotechnological IAA production is a clean technology that could replace chemical IAA synthesis. Chemical synthesis can be expensive and unstable and is performed under harsh conditions that generate highly toxic substances.[14] Although chemical and biotechnological IAA production strategies have been reported in the past decade, IAA synthesis from biomass pretreated with hydrothermal processes remains unexplored. Herein, we aimed to statistically optimize IAA production from pretreated bagasse by *S. lavenduligriseus* BS50-1. We believe that this study provides a reasonable approach for developing methods to synthesize bioproducts that would replace toxic substances in the agricultural sector.

2. RESULTS AND DISCUSSION

2.1. Composition of Bagasse. The native bagasse used in this study comprised 35.8 ± 0.51% cellulose, 24.2 ± 0.23% hemicellulose, 28.5 ± 0.41% Klason lignin, 5.3 ± 0.21% ash, and 6.24 ± 0.13% others on a dry basis. The carbohydrate content of native bagasse, which included cellulose and hemicellulose, was 60.0%. The other chemically bound components were water- and ethanol-soluble materials and proteins. The additional components of bagasse used in this experiment are displayed in Table 1. The physiochemical composition of bagasse, including other extractives, was determined through elemental analysis. Bagasse primarily included the following elements: total carbon (35.91%), total nitrogen (0.62%), calcium (2.6010 ± 0.021 g/kg), total Kjeldahl nitrogen (1.6180 ± 0.013 g/kg), potassium (0.9212 ± 0.018 g/kg), iron (0.5430 ± 0.001 g/kg), phosphorus (0.2594 ± 0.020 g/kg), aluminum (0.2060 ± 0.001 g/kg), and magnesium (0.1031 ± 0.002 g/kg). The total C:N ratio of the bagasse was 60%, as determined by elemental analysis. The moisture content of the bagasse was 50.23%, which was determined based on its weight before being dried in an oven.

Table 1. Physicochemical Properties of Bagasse

list	value	unit
moisture content	50.23 ± 0.010	%
PH value	3.98	
total carbon	35.91	%
total nitrogen	0.62	%
C/N ratios	60	%
phosphorus (P)	0.2594 ± 0.020	g/kg^{-1}
potassium (K)	0.9212 ± 0.018	g/kg^{-1}
calcium (Ca)	2.6010 ± 0.021	g/kg^{-1}
iron (Fe)	0.5430 ± 0.001	g/kg^{-1}
aluminum (Al)	0.2060 ± 0.001	g/kg^{-1}
magnesium (Mg)	0.1031 ± 0.002	g/kg^{-1}
total Kjeldahl nitrogen (TKN)	1.6180 ± 0.013	g/kg^{-1}

2.2. Effects of Different Bagasse Pretreatments. The pretreatment of bagasse via various methods was investigated (Table 2). The liquid hot water (LHW) pretreatment condition was tested at 180 °C for 30 min with no catalyst. For LHW pretreatment, water is the main solvent that is maintained at high temperatures under pressurized conditions. Under these conditions, water is ionized to generate hydronium ions in situ. This releases acetic acid from hemicellulose, which in turn autocatalyzes hemicellulose solubilization in an acidic environment and degrades carbohydrates.[15] Autocatalysis during LHW pretreatment clearly enhanced the glucose yield and removed hemicellulose and lignin from the bagasse. LHW significantly improved the glucose yield by 50.2% compared with that of native bagasse (35.8%). The alkaline pretreatment of bagasse using 5% (w/v) NaOH at 90 °C for 30 min resulted in a higher cellulose content after delignification. The cellulose content of bagasse increased from 35.79% (native) to 67.61% (pretreated). Alkaline pretreatment involves the removal of lignin and part of hemicellulose to enhance the accessibility of enzymes to cellulose. Saccharification is significantly enhanced by alkaline pretreatment.[16] The hydrogen bonds between cellulose and hemicellulose and the ester bonds between saponified hemicellulose and lignin molecules are all weakened by OH during an alkaline treatment.[17] Alkaline pretreatment also causes raw wood fibers to swell, which damages the lignin structure, breaks chemical interactions between lignin and carbohydrates, increases the internal surface area, and decreases the degree of polymerization and crystallinity.[18] Xu et al.[19] treated beanstalks with 10% ammonia for 24 h, which decreased the lignin and hemicellulose yields by 30.61 and 41.45%, respectively. Zhao et al.[20] subjected *Ageratina adenophora* stems to various pretreatment methods for enzymatic hydrolysis. The glucose yield from enzymatic hydrolysis following a NaOH pretreatment was higher than the glucose yield obtained from H_2SO_4. Compared with LHW, alkaline pretreatments destroy more ester bonds in lignin, hemicellulose, and cellulose and avoid breaking hemicellulose polymers.[21] Previous studies have shown that using an alkaline pretreatment on a lignocellulosic biomass mainly depends on the lignin and hemicellulose contents in the raw material.[22]

2.3. Physiochemical Characterization of the Solid Fraction. Morphological and surface characterizations were performed to assess the changes induced by the LHW pretreatment of bagasse. The morphological characteristics of the local and pretreated samples are shown in Figure 1. The untreated bagasse exhibited a smooth, compact, and fibrous appearance due to the presence of recalcitrant structures (Figure 1A). In contrast, the pretreated bagasse exhibited noticeable surface disruptions that exposed the internal structures (Figure 1B). Scanning electron microscopy (SEM0) confirmed the deteriorated cell walls of the pretreated bagasse, indicated by loose fibrous networks and irregular, rough, microporous, and cracked surfaces.[23] This could be attributed to the hemicellulose and lignin removal as well as the effects of LHW and alkaline pretreatments. The structural

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Table 2. Bagasse Composition under Various LHW Pretreatment Conditions

sample	cellulose	hemicellulose	AIL	ASL	Ash	other (extractive)	pulp yield
raw bagasse	35.79	24.23	23.72	4.77	5.25	6.24	
remaining solid after liquid hot water pretreatment 180 °C 30 min	50.18	7.83	25.21	4.57	6.69	5.53	58.91
remaining solid after alkaline pretreatment (5% w/v NaOH) 90 °C 30 min	67.61	22.36	4.43	2.04	2.35	1.21	48.04

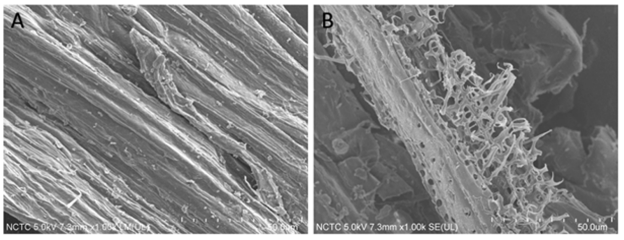

Figure 1. Scanning electron micrographs of (A) native and (B) pretreated bagasse under optimized conditions.

changes were further examined by X-ray diffraction (XRD), as shown in Figure 2. XRD demonstrated that the LHW and

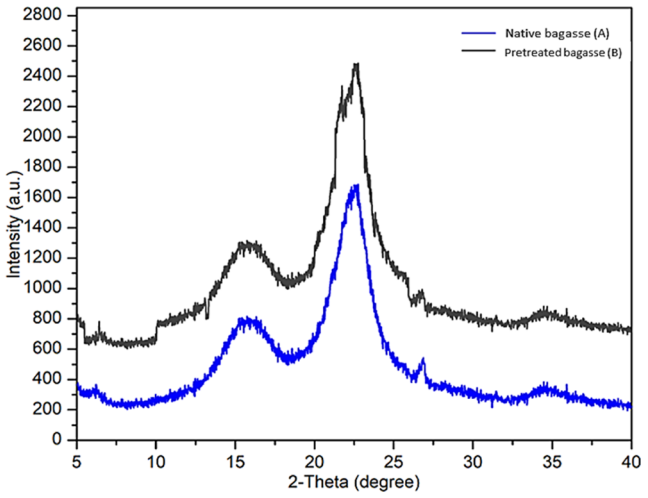

Figure 2. X-ray diffraction profiles of (A) native and (B) pretreated bagasse under optimized conditions.

alkaline pretreatments increased the crystallinity index from 48.3 to 54.2% and 55.4%, respectively. This result revealed that amorphous structures such as hemicellulose and lignin were removed from the cellulose content as the main remaining solid fraction.[24] Furthermore, the physiochemical changes in bagasse during the pretreatment processes in terms of surface area are summarized in Table 3. The table provides an overview of the correlation between the surface areas and pore volumes of native and pretreated bagasse under optimized conditions. Pretreatment increased the bagasse surface area by 2.4-fold from 5.7 to 13.6 m^2/g. Additionally, the pore volume increased from 0.07 to 0.21 cm^3/g. The significant changes in surface area caused by LHW and alkaline pretreatments were

Table 3. Surface Areas and Pore Volumes of Native and Pretreated Bagasse under Optimized Conditions

order	native bagasse	pretreated bagasse
pore volume (cm^3/g)	0.07	0.21
surface area (m^2/g)	5.7	13.6

determined by SEM analysis. The increased crystallinity index of the pretreated bagasse was caused by the elimination of the amorphous hemicellulose and lignin structures; nevertheless, the crystalline structure of cellulose was relatively unaffected. Previous studies have found increased crystallinity indexes in various lignocelluloses pretreated with LHW, for example, rice straw pretreated with LHW,[25] as well as other pretreatment methods such as dilute acid[26] and alkaline pretreatment.[27] However, certain pretreatment techniques such as those involving ionic liquids are known to completely destroy the crystalline cellulose structure.[28] This evidence supports the concept that subjecting bagasse to either LHW or alkaline pretreatment would facilitate its further applications.

2.4. Enzymatic Hydrolysis of Pretreated Bagasse. The effect of the enzyme loading on bagasse hydrolysis was studied. A maximum glucose yield of 1.53 g/g cellulose was obtained following the hydrolysis of LHW-pretreated sugarcane bagasse for 48 h at an enzyme loading of 25 FPU/g cellulose, whereas the glucose yield from an alkaline-pretreated hydrolysate was clearly lower (0.99 g/g cellulose) under the same conditions (Figure 3).

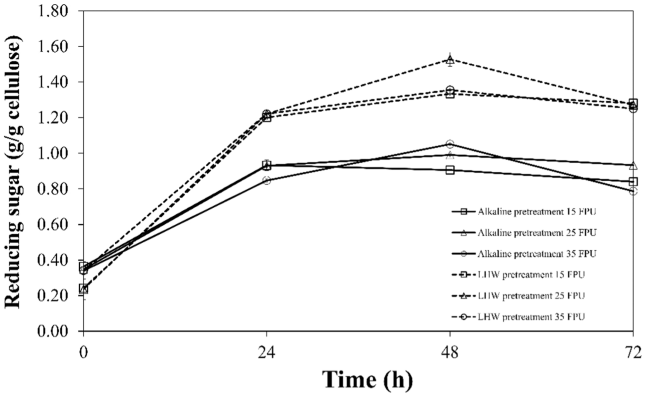

Figure 3. Effect of enzyme dosages at 15 (□), 25 (Δ), and 35 FPU/g cellulose (○) on hydrolysis of alkaline (solid line)- and LHW (dash line)-pretreated bagasse.

2.5. Screening and Identification of Actinobacteria for IAA Production and Medium Selection. The 34 actinobacteria isolates from rhizosphere soil samples produced IAA in the range of 3.32−13.08 μg/mL. These isolates were grown in three different media (International Streptomyces Project medium 2 (ISP2), yeast malt (YM), and glucose yeast extract peptone (GYP)) supplemented with 5 mg/mL L-tryptophan. Isolate BS50-1 yielded the most IAA (37.49 μg/mL) in ISP2 medium supplemented with L-tryptophan. This isolate was selected for further studies.

BS50-1 was identified based on the 16S rRNA gene sequence, which comprises 1,420 nucleotides and showed that isolate BS50-1 was closely related to *S. lavenduligriseus*. The 16S rRNA gene sequence BS50-1 shared 99.93% similarity with that of *S. lavenduligriseus*. The BS50-1 isolates were

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therefore identified as *S. lavenduligriseus*, and their 16S rRNA gene sequences were deposited to the GenBank database (accession number OQ135193). *S. lavenduligriseus* is renowned for its generation of potent antifungal compounds and was reported by Yang et al.[29] The researchers successfully extracted filipin III and three novel polyene macrolides that were designated as compounds 2, 3, and 4.[29]

2.6. Optimization of IAA Production. The effects of two independent variables, glucose (X_1) and L-tryptophan (X_2), on IAA production were determined by using an RSM based on a CCD with 11 experimental runs, as shown in Table 4.

Table 4. Effects of Process Factors, Including Glucose and L-Tryptophan, on IAA Production Obtained Experimentally

	level		actual level		IAA (μg/mL)	
run no.	X_1	X_2	X_1 (glucose, g/L)	X_2 (L-tryptophan, g/L)	observed	predicted
1	−1	−1	2	1	18.31	13.35
2	1	−1	6	1	46.01	48.83
3	−1	1	2	5	139.54	138.46
4	1	1	6	5	100.42	107.11
5	−1.68	0	1.17	3	42.49	47.04
6	1.68	0	6.83	3	56.41	49.97
7	0	−1.68	4	0.17	38.58	40.40
8	0	1.68	4	5.83	173.74	170.15
9	0	0	4	3	115.18	118.43
10	0	0	4	3	110.63	118.43
11	0	0	4	3	129.48	118.43

The regression-based coefficient of determination R^2 was evaluated to test the fit of the model equation. An R^2 value of 0.986 indicated a 98.6% fit between the observed and predicted values, with the remaining 1.4% being affected by other variables. Fisher's F value of 71.09 and a very low probability (p-model = 0.0001), which were indicative of the fit of the model, demonstrated the statistical significance of the model (Table 5). A lack of fit F value of 0.70 meant that the statistical value relative to the pure error was not significant, which was good for the model. This result indicated that the response equation suitably modeled the relationship between the independent variables and the response. IAA production could therefore be predicted by eq 1

Table 5. Analysis of Variance to Optimize IAA Production

source	sum of squares	DF	mean square	F-value	p-value
model	24913.21	5	4982.64	71.09	0.0001[a]
X_1	8.54	1	8.54	0.12	0.7412
X_2	16816.41	1	16816.41	239.95	<0.0001[a]
X_1X_2	1116.23	1	1116.23	15.93	0.0104[a]
X_1^2	6887.24	1	6887.24	98.27	0.0002[a]
X_2^2	243.61	1	243.61	3.48	0.1213
residual	350.42	5	70.08		
lack of fit	157.02	3	52.34	0.54	0.7001
cor total	25263.63	10			

[a]Significance leve l = 95%, R^2 = 0.9861, Adjusted-R^2 = 0.9723, C.V. = 9.49%

$$Y = -156.99511 + 82.89134X_1 + 49.48105X_2 - 4.17625X_1X_2 \pm 8.73075X_1^2 - 1.642X_2^2 \quad (1)$$

where Y, X_1, and X_2 are the concentrations of IAA (g/L), glucose (g/L), and L-tryptophan (g/L), respectively.

The regression model revealed that only L-tryptophan (X_2) significantly affected IAA production, whereas glucose showed no statistically significant effects. However, the interaction between glucose and L-tryptophan was significant. The quadratic terms of glucose (X_1^2) were significant, while those of L-tryptophan (X_2^2) were not.

The interactions and optimal values of the variables were determined by using response surface plots (Figure 4). Figures 4A and 4B show a significant interaction between glucose and L-tryptophan during IAA production, as indicated by the low p-value (0.0104 < 0.05) in Table 5. IAA production increased with increasing L-tryptophan and glucose concentrations. While an increase in the glucose concentration from 2.0 to 3.5 g/L increased the IAA production, an excessive increase in glucose produced no further increase in the IAA yield. IAA production also increased when L-tryptophan concentrations increased from 1.0 to 5.0 g/L. The highest IAA concentration was obtained for glucose concentrations of 3.40−3.60 g/L and an L-tryptophan concentration of 5.0 g/L.

The variables were optimized based on the regression equation and the response surface contour plots using Design Expert. The model predicted that a maximum IAA concentration of 159.47 μg/mL could be obtained with 3.55 and 5.0 g/L glucose and L-tryptophan, respectively. The experimental model was validated three times using the optimum conditions. The observed value of 159.25 μg/mL was close to the predicted value of 159.47 μg/mL. A yield of 0.07 g/g and productivity of 0.055 g/(L h) were achieved with an IAA concentration of 159.25 μg/mL, representing an approximately 4.25-fold increase compared with the control medium.

The optimum conditions obtained from the preliminary experiment were used to examine different time intervals (24, 48, 72, 96, 120, 144, and 168 h) to determine the effect of the incubation time on IAA production. The optimum time for the maximum IAA concentration of 159.25 μg/mL was 168 h. Actinobacteria directly stimulate plant growth by generating PGRs such as IAA, gibberellins, and cytokinins.[8,30] IAA is a particularly vital phytohormone that is essential for diverse plant growth and developmental processes, which includes cell division, cellular expansion, axial tissue elongation, and spore germination.[31] Herein, we focused on improving the efficiency of IAA production from actinobacteria using affordable medium. We emphasized the use of agricultural waste materials, such as bagasse from sugarcane, to replace commercial glucose through enzymatic hydrolysis. 34 potential IAA-producing actinobacteria were screened using ISP2 medium. Among the 34 screened actinobacteria, the *S. lavenduligriseus* BS50-1 strain yielded the highest concentration of IAA on ISP2 compared with other strains. This was consistent with the previous results. Benadjila et al.[32] optimized the biotechnological fermentation of IAA using actinobacterial strains from agricultural waste (roots and leaves of wheat) using the RSM. *Saccharothrix texasensis* MB15 produced the most IAA on medium that was based on the roots and leaves of wheat only. Factors including L-tryptophan, leaf extracts, and inoculum quantities significantly affected IAA production. The IAA concentration after 96 h of incubation was 148 μg/mL, which was consistent with the predicted value and 2.65-fold higher than in basal medium.[29]

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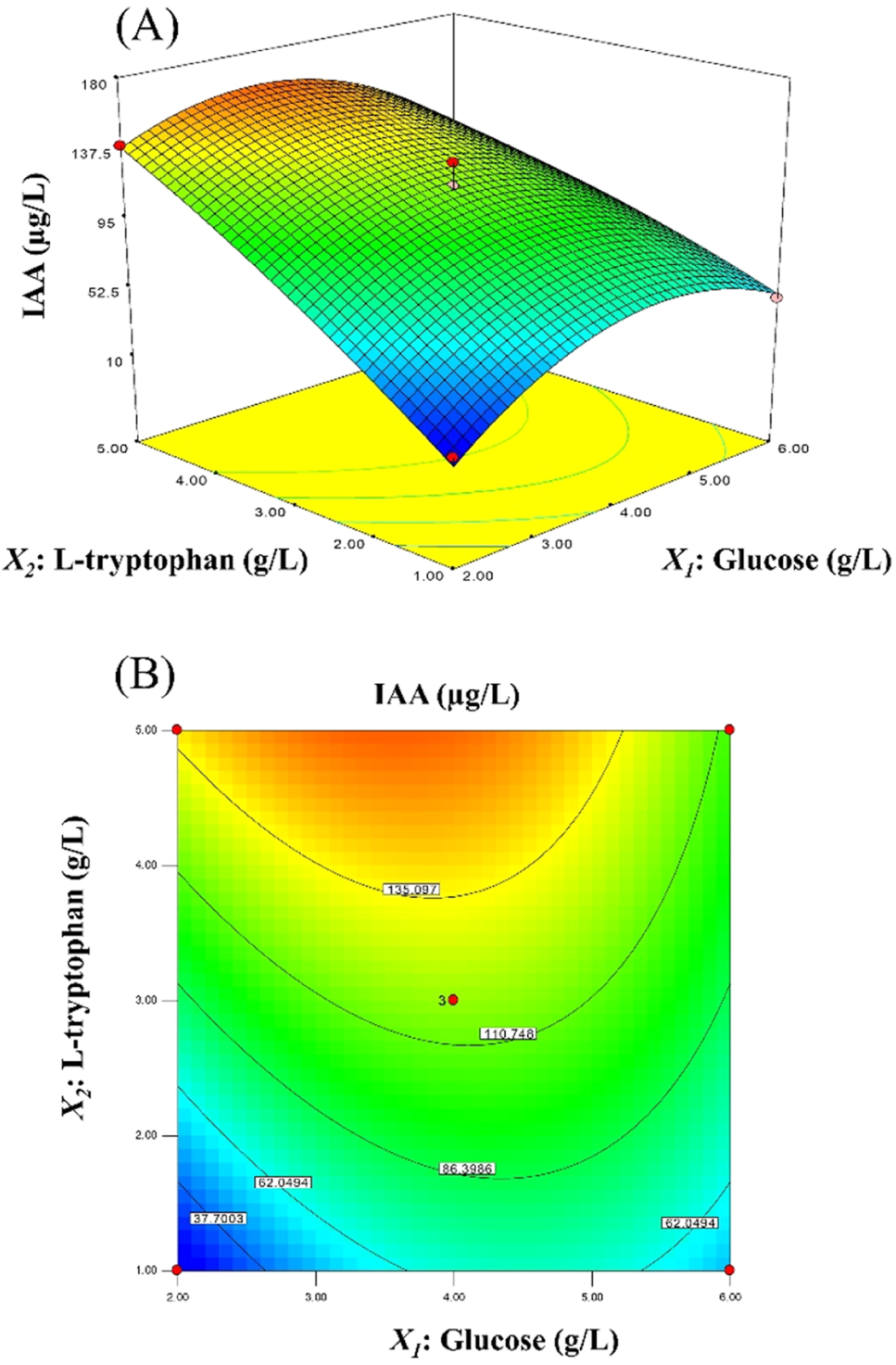

Figure 4. Response surface (a) and contour plots (b) of the combined effects between glucose and L-tryptophan concentrations on IAA production by isolate BS50-1.

This study investigated the optimization of IAA production from rhizospheric actinobacteria using low-cost medium based on pretreated bagasse. 34 actinobacteria capable of efficiently synthesizing IAA were screened using different media. Among these actinobacteria, *S. lavenduligriseus* BS50-1 yielded the highest IAA concentration on ISP2 supplemented with L-tryptophan. The concentration of IAA produced depends on the actinobacteria strain and the fermentation conditions. Several actinobacteria genera, including *Actinomadura*, *Actinoplanes*, *Frankia*, *Microbispora*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nonomurea*, *Saccharopolyspora*, *Streptomyces*, and *Verrucosispora*, produce PGRs in various plants such as beans, peas, rice, tomato, and wheat.[33−35] Statistical analysis of the significant model factors suggested that L-tryptophan significantly influenced IAA production by *S. lavenduligriseus* BS50-1, which was consistent with previous studies on IAA production using actinobacterial strains.[32,36] This suggests that L-tryptophan could be a primary precursor for IAA biosyn-

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thesis by microorganisms.[37,38] No studies so far have reported the use of bagasse for biosynthesizing IAA from *S. lavenduligriseus* BS50-1. A statistical RSM based on the CCD approach was used to optimize the fermentation conditions of *S. lavenduligriseus* BS50-1. The most IAA obtained in this study was higher that in other studies involving other plant growth-promoting microorganisms such as *S, texasensis* MB15 (148 μg/mL),[32] *Streptosporangium becharense* MB29 (141.00 μg/mL),[39] *Streptomyces* sp. PT2 (127 μg/mL),[40] *Setaria viridis* CMU-H009 (143.95 μg/mL),[41] *Streptomyces* sp. VSMGT1014 (26.63 μg/mL),[42] *Enterobacter ludwigii* BNM 0357 (30 μg/mL),[43] and *Pseudomonas aeruginosa* (32 μg/mL).[44] This study highlights the significance of *S. lavenduligriseus* BS50-1 for producing IAA using glucose hydrolysate derived from sugarcane bagasse and offers a viable commercial alternative. Moreover, this approach exhibits promise for wider applications across various agricultural waste materials, thereby contributing to their beneficial utilization in agriculture.

3. CONCLUSIONS

This study showed that actinobacterial strains from rhizosphere soil samples are interesting sources of PGRs, including IAA. *S. lavenduligriseus* BS50-1 effectively maximized IAA production in agricultural waste medium based on bagasse. A statistical CCD-based RSM approach was used to optimize the medium composition for maximum IAA production. The results revealed that a statistical approach is an effective tool for improving IAA production when low-cost agricultural waste is used as the substrate.

4. FUNDAMENTALS OF THE METHOD

4.1. Biomass Pretreatment. Bagasse was collected from a local field in the Phayao province in Thailand. The biomass was physically processed using a cutting mill (Retsch SM 2000, Hann, Germany) and sieved to collect the 250−420 μm particles (0.21−0.35 mesh). The processed biomass was then used as the starting material for subsequent experiments. The chemical compositions of the pretreated solids (% lignin, cellulose, hemicelluloses, ash, byproducts, and degradation products) were determined using the standard method from the National Renewable Energy Laboratory (NREL).[45]

4.2. LHW Pretreatment. The pretreatment was performed in stainless-steel reactors with a diameter of 2.5 cm, length of 37.5 cm, wall thickness of 2 mm, and total volume of 50 mL.[46] Thermocouples were installed in each reactor to measure the actual internal temperature. The standard reaction contained 1.5 g of bagasse and 15 mL of distilled water either without an alkali (noncatalyzed control) or catalyzed by 0.25−1% (w/v) of an alkali (NaOH). Nitrogen was passed through each reactor to purge it, and the initial pressure was adjusted to 20 bar. The reactors were placed in furnace slots in a reactor system consisting of six 50 mL reactors in a temperature-controlled jacket equipped with a vertical shaking system to provide optimal mixing. The reaction was heated to 180 °C for 30 min and then quenched in a water bath after heating under the desired conditions. The solid fraction was separated by filtration through filter paper in a Büchner funnel, washed with distilled water, and dried at 60 °C. The liquid fraction was collected to analyze sugar and inhibitory byproducts by high-performance liquid chromatography (HPLC).

4.3. Alkaline Pretreatment. Alkaline pretreatment was performed using milled bagasse at 5% w/v dry solids loaded in distilled water. The reactor was heated to 90 °C in a larger aluminum block heater with a 20 min heat ramp and maintained at that temperature for 30 min. The reactor was then removed and placed in an ice bath to quench the reaction. The pretreated biomass was recovered by filtration and washed with 500 mL of distilled water to remove excess alkali and dissolved byproducts. All experiments were performed in triplicate.

4.4. Compositional Analysis. The sugar and inhibitory byproduct (5-hydroxymethyl furfural and furfural) profiles in the enzymatic hydrolysates and liquid fractions from the pretreatment were analyzed by HPLC using a Waters e2695 chromatograph equipped with a differential refractometer and an Aminex HPX-87H column (Bio-Rad, Hercules, CA) operating at 65 °C with a 5 mM H_2SO_4 mobile phase and 0.5 mL/min flow rate. The liquid fraction from the LHW pretreatment was hydrolyzed using a modified NREL standard method.[15] The sugar yields were calculated as percentages of glucose and pentoses, which were based on the cellulose (1.11%) and hemicellulose (1.13%) contents in the native bagasse on a dry weight basis, respectively. Additionally, the elemental compositions of bagasse were determined using an elemental analyzer (CHNS-628, LECO, Saint Joseph, MI). The total carbon and nitrogen contents of bagasse were also determined by elemental analysis. The C/N ratios were then calculated from these values. The pH was determined by mixing water and bagasse (1:1 v/v).

4.5. Characterization of the Solid Residue. The total surface areas of the native and pretreated bagasse were determined by using the Brunauer−Emmett−Teller (BET) method. The surface areas and pore volumes of the samples were determined using nitrogen adsorption/desorption isotherms in a surface area analyzer (TriStar II 3020, Micromeritics Co.). The native and pretreated bagasse microstructures were analyzed by SEM using a JSM-6301F scanning electron microscope operating at 5 kV (JEOL, Japan). The samples were dried and coated with gold for the SEM analysis. The crystallinities of the native and pretreated bagasse were determined by XRD using an X'Pert PRO diffractometer (PANalytical, Almelo, Netherlands). The materials were scanned in the range of 2θ = 10−30° with a step size of 0.02° at 500 kV and 30 mA. The crystallinity was calculated using eq 2

$$\mathrm{CrI} = \frac{I_{002} - I_{\mathrm{amorphous}}}{I_{002}} \times 100 \quad (2)$$

Here, I_{002} is the scattered intensity at the main peak of the crystalline portion that typically lies near the 002 plane at 2θ = 22.4° and $I_{\mathrm{amorphous}}$ is the scattered intensity of the amorphous portion evaluated as secondary planes at 2θ = 18.0°.

4.6. Enzymatic Hydrolysis of Pretreated Bagasse. Each raw material (5% w/v) was autoclaved at 121 °C for 15 min and enzymatically saccharified using a Cellic CTec 2 system (185 FPU/mL, Novozyme A/S, Basgsværd, Denmark) in a citrate buffer (50 mM, pH 4.8)[47] at 50 °C and 150 rpm. The enzyme loading was 15, 25, or 35 FPU/g cellulose. Aliquots of the samples were extracted every 24 h to determine the reduction in the sugar concentration. The reduction of sugar was quantified using the Nelson−Somogyi method.[48] The enzymatic hydrolysate was further used for IAA production in a 250 mL Erlenmeyer flask supplemented with a starter culture and the selected medium with the enzymatic

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hydrolysate used in place of glucose. Fermentation occurred under constant agitation at 28 °C for 7 days.

4.7. Screening and Identification of Actinobacteria for IAA Production. 34 actinobacteria isolated from rhizosphere soil samples from the Rayong province in Thailand were screened for IAA production using the method described by Khamna et al.[49] The strains were grown on ISP2 agar medium and incubated at 28 °C for 7 days. Two full loops of spores were transferred to an ISP2 broth, incubated at 28 °C, and used as an inoculum. After 2 days, 10% v/v of the inoculum culture was transferred to an ISP2 broth supplemented with 2 mg/mL L-tryptophan and incubated for 7 days at 28 °C while shaking at 150 rpm. The supernatant was harvested by centrifugation at 11,000*g* for 15 min. Next, 1 mL of the supernatant was mixed with 2 mL of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$). The optical density (OD) was measured at 530 nm after incubating the mixture in the dark for 30 min. The amount of IAA produced was estimated by comparing the OD with an IAA standard calibration curve. The isolate that produced the most IAA was selected for identification based on the 16S rRNA (rRNA) gene sequence. Genomic DNA was extracted using a standard method.[50] The 16S rRNA was amplified by the polymerase chain reaction (PCR) with the universal bacterial primers 20F (5′-GAG TTT GAT CCT GGC TCA G-3′, 16S rRNA positions 9−27 in the *Escherichia coli* numbering system[51]) and 1500R (5′-GTT ACC TTG TTA CGA CTT-3′, 16S rRNA positions 1509−1492 in the *E. coli* numbering system). Amplification was performed using a DNA Engine Dyad thermal cycler (Bio-Rad Laboratories) using the following protocol: initial denaturation at 94 °C for 3 min; 25 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; and the final extension at 72 °C for 3 min. The PCR products were purified using a GenepHlow Gel/PCR kit (Geneaid Biotech Ltd., Taiwan) and sequenced using universal primers. The resultant 16S rRNA gene sequences were aligned against the corresponding sequences of the actinobacterial species retrieved from the European Molecular Biology Laboratory/GenBank database using ClustalX in BioEdit.

4.8. Medium Selection. Selected isolates were studied in three different media: yeast extract−malt extract agar (ISP2:4.0 g/L yeast extract, 10.0 g/L malt extract, and 4.0 g/L glucose, pH 7.0), YM agar (10.0 g/L glucose, 3.0 g/L malt extract, 5.0 g/L peptone, and 3.0 g/L yeast extract, pH 7.0), and GYP (20.0 g/L glucose, 10.0 g/L peptone, and 10.0 g/L yeast extract, pH 7.0) supplemented with 5 g/L L-tryptophan.

4.9. Optimization of IAA Production. The factors affecting IAA production were optimized using an RSM based on a CCD. The experimental factors were determined using a two-level factorial design that included glucose derived from pretreated sugarcane bagasse (1.17−6.83 g/L) and L-tryptophan (0.17−5.83 g/L), using a 2^2 full factorial design experiment. The CCD consisted of four star points ($\alpha = \pm 1.41$) and three replicates at the center. Eq 3 was fitted to evaluate the effect of each independent variable on the response,

$$Y = a_0 + a_1X_1 + a_2X_2 + a_{12}X_1X_2 + a_{11}{X_2}^1 + a_{22}{X_2}^2 \quad (3)$$

where Y is the predicted response (IAA concentration, μg/mL), a_0 is a constant term, a_1 and a_2 are linear terms, a_{11} and a_{22} are quadratic terms, a_{12} is an interaction term, and X_1 and X_2 are the test variables.

All experiments were performed in triplicate. The factors affecting IAA production were evaluated to verify the accuracy of the model that was predicted using software Design Expert (ver. 7.0, Stat Ease, Minneapolis, MN). Subsequently, a validation experiment was performed to verify the predicted values obtained from the software analysis.

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Notes

The authors declare no competing financial interest.

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