Biological conversion of agricultural wastes into indole-3-acetic acid by S. lavenduligriseus BS50-1 using response surface methodology (RSM)

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Article

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Abstract

Agricultural waste can be used as an alternative source for plant growth regulator (PRR) biosynthesis by microorganisms. Actinomycetes are an important group of soil microbes that play a significant role in the soil as plant growth-promoting rhizobacteria (PGPR) as well as biofertilizers. This study focused on the development of a low-cost medium based on bagasse to improve indole-3-acetic acid (IAA) production by *S. lavenduligriseus* BS50-1 using response surface methodology (RSM). Among thirty-four actinomycete strains, *S. lavenduligriseus* BS50-1 exhibited the highest IAA level under the selected medium. RSM based on central composite design (CCD) was used to optimize the appropriate nutrients for IAA production. Thus, glucose hydrolysate and L-tryptophan concentrations of 3.55 g/L and 5.0 g/L were optimal factors improving IAA production from 37.50 µg/mL to 159.47 µg/mL within 168 h. This study is the first to report the potential application of leftover bagasse as raw material for the cultivation of actinomycetes as an efficient IAA producer to promote plant growth.

Introduction

Currently, bagasse is a major agricultural crop cultivated in Thailand and other tropical countries. Bagasse is cultivated on a large scale; however, at the end of each cultivation, bagasse is openly burned, causing air pollution issues. In recent years, the replacement of chemical inputs with biotechnological products has gathered much attention in many research fields and is a rising alternative to the use of chemicals that irreversibly deplete the environment and cause health concerns for both producers and consumers. Accordingly, bagasse residue is selected as raw material to produce biochemical products such as bioethanol, PHA biosynthesis, biomethane, and xylitol\(^1\).

Accepting an alternate plan for soil biofertilization is urgently needed to accomplish sustainable agriculture under limited nutrient supplies. The rhizosphere is a rich source of microorganisms with a variety of advantageous processes promoting plant growth\(^2\). Several microorganisms, including bacteria, fungi, and algae, are capable of producing plant growth regulators (such as auxin, gibberellin, and ethylene), siderophores, HCN, and antibiotics, which may exert prominent effects on plant growth and development\(^3,4,5,6,7\). The category actinomycetes is the most widely distributed group of microorganisms in nature. The genus *Streptomyces* is an important group of soil bacteria, and 60% of this genus is a source of the most biologically bioactive compounds, such as antimicrobial, antibiotic, or plant growth-promoting compounds\(^8\). *Streptomyces* can 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 well-known plant growth regulator (PRR) that can be synthesized by microorganisms. IAA can be produced by several *Streptomyces* species, such as *Streptomyces olivaceoviridis*, *Streptomyces rimosus* and *Streptomyces viridis*\(^11\). IAA has been found to be the most effective metabolite in terms of promoting plant development in a variety of approaches. Additionally, it controls physiological processes in plants, 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 (PR) proteins. A secondary bacterial metabolite that regulates a variety of biological processes, including cell division, growth, sprouting, gene regulation, elongation of roots and shoots, increasing plant weight, and inhibition or promotion of flowering and fruiting, was previously reported. The price of IAA, a metabolite with a rising demand in the worldwide agricultural industry, might reach US$ 150/kg due to obstacles in creating a PGPR that is affordable. In the rhizosphere, bacterial populations compose a great organic plant-specific microecosystem. In the rhizosphere, microbes develop a complex connection with plants to increase productivity through the synthesis of a variety of metabolites, including phytohormones. Due to their significant mechanisms of IAA generation and other aspects of soil fertilization, the use of microorganisms that produce phytohormones, such as Streptomyces, has already been discussed. Streptomyces sp. was used to evaluate plant growth promotion in chickpea under in vitro and in vivo conditions. At the field application level, Streptomyces sp. exhibited an increase in nodule number, shoot weight, and yield by producing siderophores, cellulase, lipase, protease, chitinase, hydrocyanic acid, IAA, and β-1,3-glucanase. IAA production from biological technology may be a clean technology that can be selected to replace chemical IAA synthesis. The advantages of chemical technology are that it is expensive, unstable, and carried out under harsh conditions that generate highly toxic substances. Although IAA production from chemical and biotechnology has been reported over the last decade, IAA production from biomass pretreated with hydrothermal processes is still lacking. In the present study, we aimed to optimize IAA production from pretreated bagasse by S. lavenduligriseus BS50-1 using a statistical method. We believe this study provides a reasonable process development approach for realizing the production of bioproducts to replace toxic substances for use in the agricultural sector.

**Results and discussions**

**Composition of corn stover.** The composition of native bagasse used in this study was determined to be 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 accounted for 60.0% (as cellulose and hemicellulose). The other components that are chemically bound are water- and ethanol-soluble materials and proteins.

**Effects of different pretreatment of bagasse.** The pretreatment of bagasse with various methods was first investigated (Table 1). The LHW pretreatment condition was tested at 180°C for 30 min without catalyst. In LHW, water is the main solvent at high temperatures under pressurized conditions. Under these conditions, hydronium ions are generated in situ by the ionization of water, leading to the release of acetic acid from hemicelluloses, which in turn autocatalyses the solubilization of hemicelluloses in an acidic environment and leads to the degradation of carbohydrates. The presence of autocatalysts in LHW pretreatment could clearly enhance the glucose yield and remove hemicellulose and lignin from bagasse. The use of liquid hot water significantly improved the glucose yield by 50.2% compared to native bagasse (35.8%). Alkaline pretreatment of bagasse under conditions of 90°C for 30 min at a NaOH concentration...
of 5% w/v resulted in a higher cellulose content after delignification. The cellulose content in native bagasse increased from 35.79% (native) to 67.61% (pretreated). Alkaline pretreatment refers to the removal of lignin and part of hemicellulose so that it can enhance the accessibility of enzymes to cellulose. The saccharification effect can be significantly enhanced by alkaline pretreatment\textsuperscript{16}. The hydrogen link between cellulose and hemicellulose and the ester bond between saponified hemicellulose and lignin molecules can both be weakened by OH during alkali treatment\textsuperscript{17}. NaOH pretreatment can also cause raw wood fibers to swell, which damages the structure of the lignin and results in broken chemical connections between the lignin and the carbohydrates as well as increased internal surface area and decreased degree of polymerization and crystallinity\textsuperscript{18}. Xu et al.\textsuperscript{19} used beanstalk as a raw material and subjected it to a 24-hour period with 10% ammonia. The results showed that the lignin and hemicellulose yields decreased by 30.61% and 41.45%, respectively. Zhao et al.\textsuperscript{20} used Crofton weed stem as a raw material and treated it with various pretreatment methods for enzymatic hydrolysis. The results showed that the glucose yield from enzymatic hydrolysis from NaOH pretreatment was higher than the glucose yield obtained from H$_2$SO$_4$. Compared with LHW, alkaline pretreatment is more destructive to the ester bonds among lignin, hemicellulose, and cellulose and can avoid the breakage of hemicellulose polymers\textsuperscript{21}. Previous studies have shown that using alkaline pretreatment on lignocellulosic biomass mainly depends on the lignin and hemicellulose contents in the raw materials\textsuperscript{22}.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>AIL</th>
<th>ASL</th>
<th>Ash</th>
<th>Other (Extractive)</th>
<th>Pulp yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw bagasse</td>
<td>35.79</td>
<td>24.23</td>
<td>23.72</td>
<td>4.77</td>
<td>5.25</td>
<td>6.24</td>
<td>-</td>
</tr>
<tr>
<td>Remaining solid after liquid hot water pretreatment 180ºC 30 min</td>
<td>50.18</td>
<td>7.83</td>
<td>25.21</td>
<td>4.57</td>
<td>6.69</td>
<td>5.53</td>
<td>58.91</td>
</tr>
<tr>
<td>Remaining solid after alkaline pretreatment (5%w/v NaOH) 90ºC 30 min</td>
<td>67.61</td>
<td>22.36</td>
<td>4.43</td>
<td>2.04</td>
<td>2.35</td>
<td>1.21</td>
<td>48.04</td>
</tr>
</tbody>
</table>

**Physiochemical characterization of solid fraction.** The morphology and surfaces were characterized to comprehend the change in LHW pretreatment of bagasse. The morphological characteristics of the local and pretreated samples are depicted in Fig. 1. The untreated bagasse exhibited a smooth, compact, and fibrous appearance, attributed to the presence of recalcitrant structures (Fig. 1A). In contrast, the pretreated bagasse displayed noticeable surface disruption, leading to the exposure of internal structures (Fig. 1B). SEM micrographs confirmed that the cell wall of pretreated bagasse was broken down, which exhibited loosened fibrous networks and irregular, rough, microporous, and cracked surfaces\textsuperscript{23}. These observations can be attributed to the removal of hemicellulose and lignin, as well as the effects of LHW and alkaline pretreatment. Further insight into the structural changes was gained through X-ray
diffraction (XRD) analysis, as shown in Fig. 2. The results demonstrated that the crystallinity index increased during the LHW pretreatment from 48.3–54.2%, as during the alkaline pretreatment from 48.3–55.4%. This result revealed that amorphous structures (e.g., hemicellulose and lignin) were removed from the cellulose content as the main remaining solid fraction\textsuperscript{24}. Furthermore, the physiochemical changes in bagasse during the pretreatment processes are summarized in Table 2 in terms of surface area. The table provides an overview of the correlation between the surface area and pore volume of native and pretreated bagasse under optimized conditions. The surface area of the pretreated bagasse increased from 5.7 to 13.6 m\textsuperscript{2}/g, which was 2.4-fold greater than that of the native bagasse. Additionally, the pore volume increased from 0.07 cm\textsuperscript{3}/g to 0.21 cm\textsuperscript{3}/g. A significant surface area corresponded to the surface change caused by LHW and alkaline pretreatment, as determined by SEM analysis. The crystallinity index of pretreated bagasse was observed due to the elimination of the amorphous structure of hemicellulose and lignin; nevertheless, the crystalline structure of cellulose was relatively unaffected. Previously, increases in lignocellulose crystallinity were observed in various lignocelluloses pretreated with LHW, for instance, rice straw pretreated with LHW\textsuperscript{25} and other pretreatment methods, e.g., dilute acid pretreatment\textsuperscript{26} and alkaline pretreatment\textsuperscript{27}. On the other hand, certain pretreatment techniques, such as ionic liquids, have been reported to cause complete destruction of the crystalline cellulose structure\textsuperscript{28}. This evidence supports the notion that the pretreatment of bagasse with either LHW or alkaline methodologies could facilitate further applications.

<table>
<thead>
<tr>
<th>order</th>
<th>Native Bagasse</th>
<th>Pretreated Bagasse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pore volume (cm\textsuperscript{3}/g)</td>
<td>0.07</td>
<td>0.21</td>
</tr>
<tr>
<td>Surface area (m\textsuperscript{2}/g)</td>
<td>5.7</td>
<td>13.6</td>
</tr>
</tbody>
</table>

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

**Screening and identification of actinomycete for IAA production and medium selection.** Thirty-four actinomycete isolates obtained from rhizosphere soil samples showed valuable IAA production in the range of 3.32–13.08 µg/ml. These isolates were grown in three different media (ISP No. 2, YM and GMP) supplemented with 5 mg/mL L-tryptophan. It was found that isolate BS50-1 produced the highest IAA (37.49 µg/mL) from ISP No. 2 medium supplemented with L-tryptophan. This isolate was selected for further study.
The isolate BS50-1 was identified based on the 16 s rRNA gene sequence, consisting of 1,420 nucleotides, and showed that isolate BS50-1 was closely related to *Streptomyces lavenduligriseus* species. The 16S rRNA gene sequences of strain BS50-1 shared 99.93% similarity with *S. lavenduligriseus* species. BS50-1 isolates were identified as *S. lavenduligriseus*, and their 16S rRNA gene sequences were deposited in the GenBank database with accession number OQ135193.

**Optimization of IAA production.** The effect of two independent variables, glucose (X1) and L-tryptophan (X2) on IAA production was determined using response surface methodology (RSM) based on central composite design (CCD) with 11 experimental runs, as shown in Table 3.

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Level</th>
<th>Actual level</th>
<th>IAA (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Observed</td>
</tr>
<tr>
<td>X1</td>
<td>X2</td>
<td>X1 (Glucose, g/L)</td>
<td>X2 (L-tryptophan, g/L)</td>
</tr>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>-1</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>-1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>-1.68</td>
<td>0</td>
<td>1.17</td>
</tr>
<tr>
<td>6</td>
<td>1.68</td>
<td>0</td>
<td>6.83</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>-1.68</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>1.68</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

The regression-based determination coefficient, $R^2$, was evaluated to test the model equation's fit. The value of the determination coefficient ($R^2 = 0.986$) explained 98.6% of the fitness between the observed and predicted values, and the other 1.4% was affected by other variables. Fisher's F test (71.09) and a very low probability value ($p$-model = 0.0001), which was indicative of the fit of the model demonstrated that the model was statistically significant (Table 4). The 0.70 lack-of-fit F value means that the statistical value relative to the pure error is not significant, which is good for the model. This result indicated that the response equation provided a suitable model of the relationship between the independent variables and the response. IAA production can be predicted by Eq. 2,
Table 4
Analysis of variance (ANOVA) for optimization of IAA production

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>DF</th>
<th>Mean Square</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>24913.21</td>
<td>5</td>
<td>4982.64</td>
<td>71.09</td>
<td>0.0001*</td>
</tr>
<tr>
<td>$X_1$</td>
<td>8.54</td>
<td>1</td>
<td>8.54</td>
<td>0.12</td>
<td>0.7412</td>
</tr>
<tr>
<td>$X_2$</td>
<td>16816.41</td>
<td>1</td>
<td>16816.41</td>
<td>239.95</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>$X_1X_2$</td>
<td>1116.23</td>
<td>1</td>
<td>1116.23</td>
<td>15.93</td>
<td>0.0104*</td>
</tr>
<tr>
<td>$X_1^2$</td>
<td>6887.24</td>
<td>1</td>
<td>6887.24</td>
<td>98.27</td>
<td>0.0002*</td>
</tr>
<tr>
<td>$X_2^2$</td>
<td>243.61</td>
<td>1</td>
<td>243.61</td>
<td>3.48</td>
<td>0.1213</td>
</tr>
<tr>
<td>Residual</td>
<td>350.42</td>
<td>5</td>
<td>70.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>157.02</td>
<td>3</td>
<td>52.34</td>
<td>0.54</td>
<td>0.7001</td>
</tr>
<tr>
<td>Cor Total</td>
<td>25263.63</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significance level = 95%, $R^2 = 0.9861$, Adjusted-$R^2 = 0.9723$, C.V. % = 9.49

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

where $Y$ is IAA concentration (g/L), $X_1$ is glucose (g/L), and $X_2$ is L-tryptophan (g/L),

The results of the regression model revealed that only L-tryptophan ($X_2$) had a significant effect on IAA production, whereas the effect of glucose concentration was not statistically significant. The interaction between glucose and L-tryptophan was significant. The quadratic terms of glucose ($X_1^2$) were significant, while those of L-tryptophan concentration ($X_2^2$) were not significant.

The interaction relationship and the optimal values of the variables were determined by the response surface plots (Fig. 4). Figures 4A and 4B indicate that there was a significant interaction between glucose and L-tryptophan for IAA production, as shown by the low $p$-value (0.0104 < 0.05) in Table 4. IAA production increased with increasing L-tryptophan and glucose concentrations. It was evident that when glucose increased from 2.0 g/L to 3.5 g/L, IAA production increased, but an excessive increase in glucose did not increase the yield of IAA any further. When L-tryptophan concentrations were increased from 1.0 g/L to 5.0 g/L, IAA production increased. In this case, the highest IAA concentration was obtained with glucose within the range of 3.40 to 3.60 g/L and concentrations of L-tryptophan at 5.0 g/L.

The optimized values of the variables were obtained from the regression equation and from the response surface contour plots using Design Expert software. The model predicted that the maximum IAA
concentration of 159.47 µg/mL occurred at 3.55 g/L glucose and 5.0 g/L L-tryptophan. Validation of the experimental model, repeated three times under the optimum conditions, showed that the observed value of 159.25 µg/mL was close to the predicted value of 159.47 µg/mL. A yield and productivity of 0.07 g/g and 0.055 g/L/h, respectively, were obtained with an IAA concentration of 159.25 µg/mL, approximately 4.25-fold higher than that from the control medium.

The optimum conditions obtained from the preliminary experiment were further studied for different time intervals (24, 48, 72, 96, 120, 144, and 168 h) to determine the effect of incubation time on IAA production. The optimum time with the maximum IAA concentration at 159.25 was observed after 168 h of incubation. The obtained results are consistent with this work. Benadjila et al.29 optimized the biotechnological fermentation of IAA by strains of actinomycetes from agricultural waste (roots and leaves of wheat) using RSM. The results showed that Saccharothrix texensis MB15 is the best IAA-producing strain on a medium based on the roots and leaves of wheat only. Factors including L-tryptophan, leaf extract, and inoculum quantity showed a significant effect on IAA production. After 96 h of incubation, the test result of 148 µg/mL IAA production agreed with the prediction and increased by 2.65-fold compared to the basal medium29.

In this study, the optimization of IAA production from rhizospheric actinomycetes using a low-cost medium based on pretreated bagasse was investigated. Thirty-four actinomycete strains with IAA biosynthesis efficiency were screened using different media. The results indicated that among these actinomycetes, the strain S. lavenduligriseus BS50-1 showed the highest IAA production on ISP No. 2 medium supplemented with L-tryptophan. The concentration of IAA production depends on the actinomycete strains and fermentation conditions. Several actinomycete genera, including Actinomadura, Actinoplanes, Frankia, Microbispora, Micromonospora, Mycobacterium, Nocardia, Nonomurea, Saccharopolyspora, Streptomyces, and Verrucosispora, can produce PGRs in various plants, e.g., beans, peas, rice, tomato, and wheat30,31,32. Statistical analysis of the significant model factors suggested that L-tryptophan had a significant effect on IAA production by S. lavenduligriseus BS50-1, which is consistent with previous works on IAA production by actinobacterial strains29,33. This finding suggests that L-tryptophan is believed to be a primary precursor for IAA biosynthesis by microorganisms34,35. According to the literature, there is no research on the use of bagasse waste for the biosynthesis of IAA from S. lavenduligriseus BS50-1. The statistical RSM based on the CCD approach was used to optimize the fermentation conditions of S. lavenduligriseus BS50-1. The highest amount of IAA found in this study was higher than that found in other studies for some plant growth-promoting microorganisms, such as Saccharothrix texensis MB15 (148 µg/mL)29, Streptosporangium becharense MB29 (141.00 µg/ml)36, Streptomyces sp. PT2 (127 µg/ml)37, S. viridis CMU-H009 (143.95 µg/mL)38, Streptomyces sp VSMGT1014 (26.63 µg/mL)39, Enterobacter ludwigi BNM 0357 (30 µg/ml)40 and Pseudomonas aeruginosa (32 µg/ml)41.

Conclusion
The present study indicates that actinomycete strains from rhizosphere soil samples could provide interesting sources of PGRs, including IAA. *S. lavenduligriseus* BS50-1 was an effective strain for maximum IAA production on agricultural waste medium based on bagasse. The statistical RSM-CCD approach was applied to find the ideal medium composition for maximum IAA production. This result revealed that a statistical design methodology is an effective tool for improving IAA production using low-cost agricultural waste as substrate.

**Materials and methods**

**Biomass pretreatment.** Bagasse was collected from a local field in Phayao Province, Thailand. The biomass was physically processed using a cutting mill (Retsch SM 2000, Hann, Germany) and sieved to particles 250–420 µm (0.21–0.35 mesh). The processed biomass was then used as a starting material for experimental studies. The chemical compositions of the pretreated solids (% lignin, cellulose, hemicelluloses, ash, byproducts and degradation products) were analyzed using the standard NREL method.

**Liquid hot water pretreatment (LHW).** The pretreatment was performed in a stainless-steel reactor (2.5 cm diameter and 37.5 cm in length with a wall thickness of 2 mm and a total volume of 50 mL). The individual reactor vessel was installed with a thermocouple to measure the actual temperature inside the reactor. The standard reaction contained 1.5 g of rice straw and 15 mL of distilled water alone in the absence of alkali (noncatalyzed control) or supplemented with 0.25–1% (w/v) alkali (NaOH) as a catalyst. The effect of flowing nitrogen into the reactor resulted in purging and then adjusting the initial pressure to 20 bar. The reactors were placed in furnace slots in a reactor system consisting of a 6×50 mL reactor 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. The reaction was quenched in a water bath after heating under the desired conditions. The solid fraction was separated by filtration on filter paper using a Büchner funnel, washed with distilled water and dried at 60°C. The liquid fraction was collected for analysis of sugar and inhibitory byproducts by HPLC.

**Alkaline pretreatment.** Alkaline pretreatment was carried out with the milled rice straw at 5% w/v dry solids loading in distilled water. The reactor was heated in a larger aluminum block heater to 90°C with a 20 min heat ramp and held at temperature for 30 min. At the end of the experimental run, the reactor was removed and placed in an ice bath to quench the reaction. Pretreated biomass was recovered by filtration and washed with 500 mL of distilled water to remove excess alkali and dissolved byproducts. All experiments were conducted in triplicate.

**Compositional analysis.** The profiles of sugars and inhibitory byproducts (5-hydroxymethyl furfural and furfural) in the enzymatic hydrolysates and liquid fractions from pretreatment were analyzed on a Waters e2695 high-performance liquid chromatograph equipped with a differential refractometer using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) operating at 65°C with 5 mM H₂SO₄ as the mobile phase at a flow rate of 0.5 mL/min. The liquid fraction from LHW pretreatment was prehydrolyzed
according to a modified NREL standard method\textsuperscript{15}. The sugar yields were calculated as the percentages of glucose and pentoses obtained based on the percent cellulose (1.11) and percent hemicellulose (1.13) in the native rice straw on a dry weight basis, respectively.

**Characterization of solid residue.** The total surface areas of the native and pretreated bagasse were determined using the Brunauer–Emmett–Teller (BET) method. The surface area and pore volume of the samples were determined using nitrogen adsorption/desorption isotherms in a surface area analyzer (TriStar II 3020, Micromeritics Co., USA). The microstructures of the native and pretreated bagasse were analyzed using scanning electron microscopy (SEM) with a JSM-6301F scanning electron microscope (JEOL, Japan). The samples were dried and coated with gold for analysis. An electron beam energy of 5 kV was used for analysis. The crystallinity of the native and pretreated bagasse was determined using X-ray diffraction (XRD) with an X’Pert PRO diffractometer (PANalytical, Almelo, Netherlands). The materials were scanned in the range $2\theta = 10^\circ$–$30^\circ$ with a step size of 0.02$^\circ$ at 500 kV and 30 mA. The crystallinity was calculated according to Eq. (5).

$$C_{\text{rI}} = \frac{I_{002} - I_{\text{amorphous}}}{I_{002}} \times 100$$

Here, $I_{002}$ is the scattered intensity at the main peak of the crystalline portion that typically lies around the 002 plane at $2\theta = 22.4^\circ$ and $I_{\text{amorphous}}$ is the scattered intensity of the amorphous portion evaluated as the secondary planes at $2\theta = 18.0^\circ$.

**Enzymatic hydrolysis of pretreated bagasse.** Each raw material was enzymatically saccharified using a Cellic CTec 2 (185FPU/mL, Novozyme A/S, Basgsværd, Denmark) in citrate buffer (50 mM, pH of 4.8)\textsuperscript{44} at 50°C and 150 rpm. The enzyme loading was varied at 15, 25 and 35 FPU/g cellulose. Aliquots of the samples were taken every 24 h for determining sugar concentration reduction. The released sugar concentration was quantitatively determined using the Nelson-Somogyi method\textsuperscript{45}. The enzymatic hydrolysate was further used for IAA production in a 250-mL Erlenmeyer flask supplemented with the starter culture and the selected medium, except that the enzymatic hydrolysate was used in place of glucose. Fermentation was performed under shaking conditions at 28°C for 7 days.

**Screening and identification of actinomycete for IAA production.** Thirty-four actinomycetes isolated from rhizosphere soil samples from Rayong Province, Thailand, were screened for the production of IAA by following the method of Khamna et al\textsuperscript{46}. The strains were grown on ISP2 agar medium and incubated at 28°C for 7 days. Two full loops of spores were transferred to ISP2 broth, incubated at 28°C and used as inoculum. After 2 days, 10% v/v inoculum culture was transferred to ISP2 broth supplemented with 2 mg/ml L-tryptophan and incubated at 28°C with shaking at 150 rpm for 7 days. The supernatant was harvested by centrifugation at 11,000xg for 15 min. One mL of the supernatant was mixed with 2 mL of Salkowski reagent (2% 0.5 FeCl\textsubscript{3} in 35% HClO\textsubscript{4} solution). Optical density (OD) was measured at 530 nm after incubation in the dark for 30 min. The level of IAA produced was estimated by comparison with the calibration curve of the IAA standard. The isolate producing maximum IAA was selected for identification.
based on the 16S rRNA gene sequence. Genomic DNA was extracted using a standard method\textsuperscript{47}. The 16S rRNA was amplified by PCR with the universal bacterial primer 20F (5’-GAG TTT GAT CCT GGC TCA G-3’, positions 9–27 on 16S rRNA by the E. coli numbering system\textsuperscript{48}) and 1500R (5’-GTT ACC TTG TTA CGA CTT-3’, position 1509 – 1492 on 16S rRNA by the E. coli numbering system). Amplification was performed using a DNA Engine Dyad® Thermal Cycler (Bio-Rad Laboratories) according to the following profile: initial denaturation at 94°C for 3 min followed by 25 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min; these cycles were followed by a final extension at 72°C for 3 min. The PCR products were purified with a GenepHlowTM Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan) and sequenced using universal primers. The resultant 16S rRNA gene sequences were aligned against corresponding sequences of the type strains of actinomycete species retrieved from the EMBL/GenBank database using CLUSTAL_X in the BioEdit Program.

Medium selection. Selected isolates were studied in three different media, yeast extract-malt extract agar (ISP No. 2: g/L, yeast extract 4.0, malt extract 10.0, glucose 4.0, pH 7.0), yeast malt agar (YM: g/L, glucose 10.0, malt extract 3.0, peptone 5.0, yeast extract 3.0, pH 7.0) and glucose yeast extract peptone medium (GYP: g/L, glucose 20.0, peptone 10.0, yeast extract 10.0, pH 7.0) supplemented with 5 g/L L-tryptophan.

Optimization of IAA production. A suitable tool to optimize factors affecting IAA production was response surface methodology (RSM) based on central composite design (CCD) The experimental factors were determined by employing a two-level factorial design that included glucose derived from pretreated sugarcane bagasse (1.17 g/L to 6.83 g/L) and L-tryptophan (0.17 g/L to 5.83 g/L), using a 2\textsuperscript{2} full factorial design experiment. The CCD consisted of four star points (α = ± 1.41) and three replicates at the center point. Eq. 1 was fitted to evaluate the effect of each independent variable on the response,

\[
Y = a_0 + a_1X_1 + a_2X_2 + a_{12}X_1X_2 + a_{11}X_1^2 + a_{22}X_2^2 (1)
\]

where Y is the predicted response (IAA concentration, µg/ml), a\textsubscript{0} is a constant term, a\textsubscript{1} and a\textsubscript{2} are linear terms, a\textsubscript{11} and a\textsubscript{22} are quadratic terms, a\textsubscript{12} is an interaction term, and X\textsubscript{1} and X\textsubscript{2} are the test variables studied.

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

**Declarations**

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Author contributions

All authors contributed to the study conception and design. Methodology, investigation, visualization, and visualization were performed by A.B., S.W., N.S., S.I., P.V., T.K., and W.A. The first draft of the manuscript was written by S.I. and reviewed and edited by A.B. and S.I. Project administration and funding acquisition were carried out by S.I. and A.B. All authors reviewed and approved the final manuscript.

Data availability

The data generated during and/or analyzed during the current study are available from the corresponding author S.I. on reasonable request.

Competing Interests

The authors declare no competing interests.

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References


**Figures**
Figure 1

Scanning electron micrographs of (A) native bagasse and (B) pretreated bagasse under optimized conditions.
Figure 2

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

![Graph showing reducing sugar production over time for different enzyme dosages and pretreatments.]

Figure 3

Effect of enzyme dosages at 15 FPU/g cellulose ( ), 25 FPU/g cellulose (Δ), and 35 FPU/g cellulose (●) on hydrolysis of alkaline (solid line) and LHW (dash line) pretreated bagasse.
Figure 4

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

Supplementary Files

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