Optimization of indole-3-acetic acid production by *Bacillus subtilis* strain IA3

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ABSTRACT

Indole-3-Acetic Acid (IAA) is a crucial plant growth-promoting hormone produced as a secondary metabolite by various microorganisms. The present study aims to isolate and screen IAA-producing endospore-forming bacteria from soil samples collected in Vietnam. A total of ten bacteria were isolated, and they were shown to have the ability to produce IAA, with strain IA3 exhibiting the highest production. Strain IA3 was identified as *Bacillus subtilis* based on the 16S rRNA sequencing and subsequently used to optimize conditions for IAA production. To maximize IAA yield, key factors influencing production were analyzed using Box-Behnken design. The optimized conditions led to a maximum IAA production of 47.67 ± 0.78 μg/mL under the following conditions: 15.65 g/L molasses, 1.06 g/L $(NH_4)_2SO_4$, and 0.52 g/L tryptophan. Therefore, *Bacillus subtilis* IA3 has potential applications for stimulating and enhancing plant growth or crop production.

1. Introduction

Soil is a crucial factor in determining the growth and development of plants; however, it is gradually degrading, and natural ecosystems are being threatened (Sherpa et al., 2021). There are many reasons why soil becomes degraded, but the main reason is the overuse of chemical fertilizers. The excessive use of chemical fertilizers causes the soil to become acidic, leading to soil poisoning and stunted plant growth, which in turn results in poor productivity. A non-hazardous, sustainable approach, such as organic fertilizer, is considered a potential solution for replenishing soil nutrients and increasing crop productivity (Wang et al., 2019).

It is reported that soil microbes play a crucial role in enhancing soil fertility by converting insoluble nutrients into soluble forms, decomposing organic matter, releasing minerals, and transforming nutrients (Kaur & Kaur, 2021). Additionally, the enhancement of plant growth by plant growth-promoting bacteria is observed. In which Indole-3-Acetic Acid (IAA) was reported to play a key role in the cell enlargement and the initiation of root cell division. The increase in IAA on the root surface area could improve the access of soil nutrients through the root (Wang et al., 2019). Various species of plant-associated bacteria, including those from the genera Alcaligenes, Arthrobacter, Azospirillum, and Bacillus, have been reported to enhance plant growth (Habibi et al., 2019; Li et al., 2017; Muthukumarasamy et al., 2017; Nguyen et al., 2022). Culture conditions were also optimized for high IAA production, revealing that tryptophan, carbon source, and nitrogen source are key factors (Khianngam et al., 2023; Lebrazi et al., 2020; Wang et al., 2019). However, a study of native microbes for biofertilizer application is still lacking in Vietnam. This study aimed to isolate

bacterial strains capable of forming endospores, as well as those capable of producing IAA, from soil samples collected in Vietnam for application in soil improvement. Optimization of IAA production was also carried out in a selected bacterial strain.

2. Materials and methods

2.1. Chemicals and media

Corn, mung bean sprouts, rice, potato, and soybean were purchased from a local market. Salkowski's reagent was prepared by mixing 1.5mL of 0.5M FeCl3·6H2O, 30mL of concentrated H2SO4, and 50mL of distilled water. Nutrient Broth (NB) medium was prepared by mixing 10 g of peptone, 10g of NaCl, and 5g of yeast extract, and then dissolved in 1L of distilled water. To prepare Nutrient Agar (NA) medium, 20g of agar was added to 1L of NB medium. A plant-based extract was prepared by boiling 20% (w/v) of the plant in distilled water for one hour. The extract was then filtered and restored to its original volume by adding distilled water. The extract was used as a broth medium. The culture medium's pH was adjusted to 7.0 using 1N NaOH or 1N HCl. All media were sterilized at 121°C for 20min. The culture conditions were maintained at 30°C and 180rpm for 03 days.

2.2. Sampling, isolation, and characterization of IAA-producing endospore-forming bacteria

Soil samples were collected in June 2024 from Tra Su forest (10°34'55"N, 105°3'33''E), Tinh Bien, An Giang Province; Con Son islet (10°05'11"N, 105°44'49"E), Can Tho City; and Nam Cat Tien forest (11°24'25"N, 107°26'37"E), Dong Nai Province in Vietnam and stored in Zip-lock plastic bag. A 1g soil sample was serially diluted in 9mL of sterile distilled water up to a $10^{\wedge -5}$ dilution. The diluted suspension was treated at 80°C for 10 minutes to screen endospore-forming bacterial strains. The diluted suspension was then used to spread on NA medium separately (Nguyen, 2018; Nguyen et al., 2021). Morphologically different colonies were selected and used for evaluating IAA production. For identifying bacteria at the species level, total genomic DNA was extracted to amplify the partial 16S rRNA gene for sequence analysis as previously described (Nguyen, 2018; Nguyen et al., 2021). The IAA produced was estimated by using the Salkowski colorimetric assay (Khianngam et al., 2023; Lebrazi et al., 2020). Briefly, 1.5mL of the culture was centrifuged at 10,000rpm for 10 minutes. The culture supernatant was then collected and mixed with Salkowski reagent (in a 1:1 ratio) for 30 minutes in the dark. IAA production was detected at 530nm using a spectrophotometer. ANOVA was applied using Minitab 16.2.4 software. A p-value of < 0.05 was considered statistically significant.

2.3. Screening of plant-based extract medium on IAA production

To select the plant-based medium, five different plant-based media (Corn Extract Medium: CEM; Mung bean sprouts Extract Medium: MEM; Potato Extract Medium: PEM; Rice Extract Medium: REM; Soybean Extract Medium: SEM) with a concentration of 10% were evaluated in the presence of 0.1 g/L tryptophan.

2.4. Screening of carbon, nitrogen, and tryptophan sources on IAA production

To evaluate the effect of carbon and nitrogen sources on IAA production, cultures were conducted in test tubes filled with 5mL of the plant-based medium. The bacterial pre-culture medium (5%, v/v) was then inoculated into test tubes with 5mL of plant-based medium supplemented with different carbon sources (fructose, glucose, mannitol, sucrose, sugarcane molasses, or soluble starch) at a concentration of 10 g/L in the presence of 0.1 g/L tryptophan. Then, based on the optimal carbon source, 06 concentrations of 0.1, 0.5, 1.0, 1.5, 3.0, and 5.0% were used to estimate the optimal concentration. For nitrogen source screening, different

nitrogen sources (NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, or urea) at a concentration of 1 g/L were added to a plant-based medium containing 1.5% sugarcane molasses and 0.01% tryptophan. Based on the optimal nitrogen source, six concentrations - 0.01%, 0.05%, 0.1%, 0.5%, 1.0%, and 2.0% - were used to determine the optimal concentration. The effect of tryptophan concentration on IAA production was observed on the optimal medium supplemented with L-tryptophan at different concentrations: 0.001%, 0.005%, 0.01%, 0.03%, 0.05%, and 0.1%.

2.5. Optimize culture medium on IAA production by response surface methodology

According to the results of the single-factor experiments, the optimal combination of the best carbon source (1.5% sugarcane molasses), nitrogen source (0.1% $(NH_4)_2SO_4$), and 0.05% tryptophan was further examined by using the Box-Behnken Design (BBD) of RSM. Each factor was studied at 03 levels (-1, 0, +1) and the model had 15 treatments. Minitab 16.2.4 software was used to design response surface experiments and perform the regression and graphic analysis.

3. Result

3.1. Isolation and characterization of IAA-producing endospore-forming bacteria

Table 1

Information on IAA-Producing Endospore-Forming Bacterial Strains Isolated from Soil Samples in An Giang, Can Tho, and Dong Nai Provinces

Isolate source	Latitude, longitude	Strain	IAA (µg/mL)	Colony morphology	Gram staining
Soil, Tra Su forest, Tinh Bien, An Giang Province	10°34'55"N, 105°3'33"E	IA1	$5.5^{f} \pm 0.4$	White, wrinkled, elevated, and irregular	Positive
		IA2	$13.4^{d,e} \pm 1.2$	Translucent tan color, wrinkled, elevated, and irregular	Positive
		IA3	$34.8^{a} \pm 0.7$	Milky, wrinkled, elevated, and irregular	Positive
Soil, Con Son islet, Can Tho city	10°05'11"N, 105°44'49"E	IA4	$7.8^{f} \pm 0.7$	Grey, wrinkled, flated, and irregular	Positive
		IA5	$21.6^{c} \pm 1.6$	Milky, wrinkled, elevated, and irregular	Positive
		IA6	$31.1^{b} \pm 0.8$	Grey, wrinkled, flated, and irregular	Positive
Soil, Nam Cat Tien forest, Dong Nai Province	11°24'25"N, 107°26'37"E	IA7	$5.4^{f} \pm 0.9$	Grey, wrinkled, flated, and irregular	Positive
		IA8	$16.1^{d} \pm 1.0$	White, wrinkled, elevated, and irregular	Positive
		IA9	$21.7^{c} \pm 1.6$	Milky, wrinkled, elevated, and irregular	Positive
		IA10	$12.4^{e} \pm 1.0$	Translucent tan color, wrinkled, elevated, and irregular	Positive

Note. IAA: Indole-3-Acetic Acid. Experiments were performed in triplicate. Nutrient medium was used. $^{a-f}$: Different letters indicate significant statistical differences ($p < 0.05$, Tukey's test) by Results derived from survey data processing

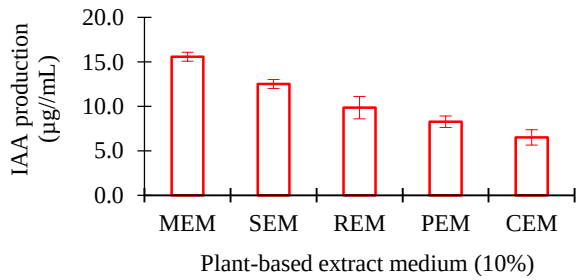
In this study, ten IAA-producing spore-forming bacteria were isolated from soil samples in three different provinces (**Table 1**). Strain IA3, isolated from An Giang Province, revealed the highest IAA production of 34.8 ± 0.7 µg/mL on Nutrient medium. The one-way ANOVA test has shown a significant difference in IAA production for strain IA3 compared to all other isolates (p-value < 0.05). Colonies of strain IA3 on nutrient agar medium were milky, wrinkled, elevated, and irregular. To clarify the taxonomic status of the isolates, the 16S rRNA gene sequence of strain IA3 was analyzed using BLASTN analysis through NCBI GenBank. The results revealed that the 16S rRNA gene sequence of strain IA3 (1415 nucleotides) has the closest relative (100%) to the sequence of *Bacillus subtilis* DSM 23521 (accession number CP120613).

3.2. Effect of plant-based extract medium on IAA production in B. subtilis IA3

In this study, the 05 different plant-based media were tested in the presence of 0.01% tryptophan. The results revealed that bacterial growth on MEM produced the highest amount of IAA (15.57 ± 0.51 µg/mL), while growth on CEM yielded the lowest (6.5 ± 0.87 µg/mL) (**Figure 1**).

Figure 1

Screening of Different Plant-Based Extract Media



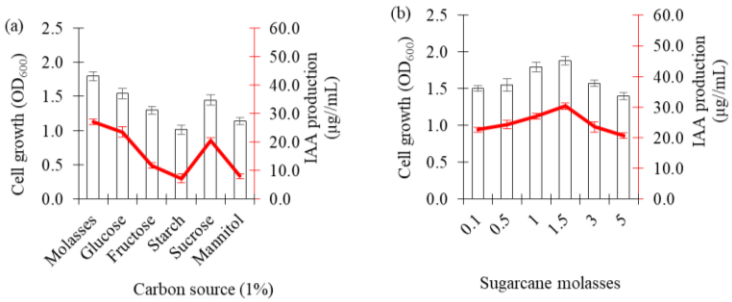
Note. MEM, Mung bean sprouts extract medium; SEM, Soybean Extract Medium; REM, Rice Extract Medium; PEM, Potato Extract Medium; CEM, Corn Extract Medium by Results derived from survey data processing

3.3. Effect of carbon and nitrogen source and their optimal concentration on IAA production in B. subtilis IA3

To test the effect of carbon source on IAA production in strain IA3, six different carbon sources were added to the mung bean sprouts extract medium, supplemented with 0.01% tryptophan. The total cell density of sugarcane molasses was the highest, followed by that of glucose and sucrose. In terms of IAA biosynthesis, sugarcane molasses and starch were the highest and lowest (**Figure 2a**). Different concentrations of sugarcane molasses were tested, and the result revealed that at a concentration of 15 g/L of sugarcane molasses, the total cell density and IAA production were 1.88 ± 0.06 and 30.37 ± 1.06 µg/mL, respectively (**Figure 2b**).

Figure 2

Screening of Different Carbon Sources (a, b)

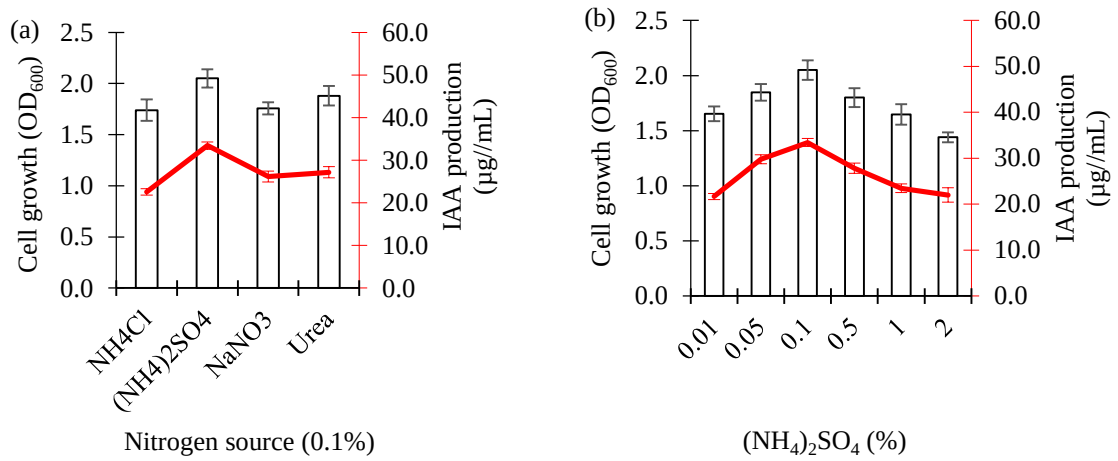


Note. Results derived from survey data processing

As shown in **Figure 3a**, among the four different nitrogen sources with a concentration of 1 g/L, the total cell density and IAA production of (NH4)2SO4 were the highest, followed by those of urea and $NaNO_3$. Then, different concentrations of $(NH_4)_2SO_4$ were used as the only nitrogen source to analyze IAA production. The maximum value appeared at 0.1% $(NH_4)_2SO_4$, at which the total cell density and IAA production were 2.05 ± 0.09 and 33.43 ± 0.86 μg/mL, respectively (**Figure 3b**).

Figure 3

Screening of Different Nitrogen Sources (a, b)

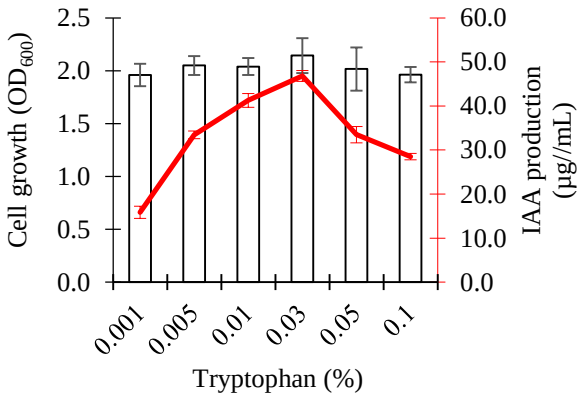


Note. Results derived from survey data processing

The different concentrations of tryptophan were used to analyze IAA production. At 0.03% tryptophan, the total cell density and IAA production were the highest of 2.14 ± 0.17 and 46.8 ± 1.25 μg/mL, respectively (**Figure 4**).

Figure 4

Screening of Different Tryptophan



Note. Results derived from survey data processing

3.4. Optimization of the selected medium components using the BBD

The sugarcane molasses, $(NH_4)_2SO_4$, and tryptophan were studied at three levels (-1, 0, +1). The experimental design and the experimental responses of IAA production were reported (**Table 2**). The fitting polynomial equation was then obtained after data fitting, and the observed and predicted IAA production are shown in **Table 2**.

Table 2

Box-Behnken Design for Optimization on IAA Production with B. Subtilis IA3

StdOrder	Factor (%)			IAA production (μg/mL)	
	Sugarcane molasses	$(NH_4)_2SO_4$	Tryptophan	Actual value	Predicted value
1	1	0.05	0.05	38.4	38.98
2	2	0.05	0.05	40.4	40.85
3	1	0.15	0.05	41.5	41.05
4	2	0.15	0.05	42.6	42.03
5	1	0.1	0.03	36.1	36.06
6	2	0.1	0.03	37.4	37.49
7	1	0.1	0.07	38.2	38.11
8	2	0.1	0.07	39.5	39.54
9	1.5	0.05	0.03	36.1	35.56
10	1.5	0.15	0.03	36.5	36.99
11	1.5	0.05	0.07	37.9	37.41
12	1.5	0.15	0.07	38.7	39.24
13	1.5	0.1	0.05	47.3	47.13
14	1.5	0.1	0.05	47.6	47.13
15	1.5	0.1	0.05	46.5	47.13

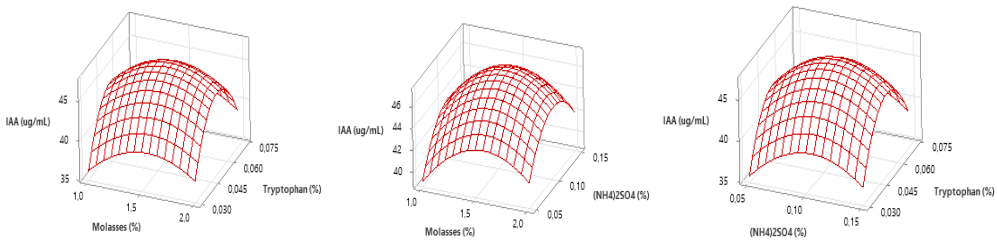
Note. Results derived from survey data processing

$$Y = 47.133 + 0.712X_1 + 0.813X_2 + 1.025X_3 - 2.954X_1^2 - 3.454X_2^2 - 6.379X_3^2 - 0.225X_1X_2 + 0.1X_2X_3 \quad (1)$$

The analysis of variance revealed that the fitted model was statistically significant, with a model F-value of 43.86 (p = 0). The coefficient values indicate that individual or double interaction factors significantly affect IAA production in strain IA3. Tryptophan dosage (p = 0.012) and the sugarcane molasses dosage (p = 0.043) had the strongest and weakest influence on the IAA production, respectively. The highest IAA production of 47.26 μg/mL was predicted based on the quadratic model under the following conditions: sugarcane molasses (X_1) = 1.556%; $(NH_4)_2SO_4$ (X_2) = 0.106%; tryptophan (X_3) = 0.052%. The observed IAA production by the optimized condition was 47.67 ± 0.78 μg/mL. The three-dimensional response surface plot is presented in **Figure 5**.

Figure 5

Response Surface Plots for IAA Production by B. Subtilis IA3 Versus Sugarcane Molasses, $(NH_4)_2SO_4$, and Tryptophan Concentration



Note. Results derived from survey data processing

4. Discussion

In this study, ten IAA-producing endospore-forming bacteria were isolated. Strain IA3 exhibited the highest IAA production compared to all other isolates (p-value < 0.05) and was identified as *Bacillus subtilis* based on 16S rRNA sequence analysis. *B. subtilis* is generally recognized as safe, which has enabled its widespread use in various applications (Zhang et al., 2020). To enhance the potential application in the fertilizer industry, a low-cost plant-based medium is recommended as a replacement for commercial media. In this study, the five different plant-based media were tested. The results revealed that the bacterial growth on MEM produced the highest IAA compared to growth on SEM, REM, PEM, and CEM (**Figure 1**). It is reported that mung bean sprouts are highly nutritious, containing approximately 60% carbohydrates and 30% protein in their dry weight (Mehta et al., 2021). Due to its high nutritional value and low price, the mung bean sprout has been used as an alternative medium for growing microorganisms (Mehta et al., 2021). The composition of the culture medium plays a crucial role in IAA production by *Bacillus* species (Chen et al., 2010). To test the effect of carbon source on IAA production, six different carbon sources were added to a medium containing mung bean sprouts extract. The total cell density and IAA production of sugarcane molasses were the highest. Molasses, an economical source, is considered a suitable candidate for *Bacillus* growth (Shasaltaneh et al., 2013; Wu et al., 2017). For the nitrogen source, the best source for IAA production in B. subtilis IA3 was (NH4)2SO4 at a concentration of 1.0 g/L. Numerous studies have confirmed that tryptophan can influence IAA production (Khianngam et al., 2023; Lebrazi et al., 2020; Wang et al., 2019). In this study, the addition of different concentrations of tryptophan had no significant effect on total cell density. However, it led to a noticeable improvement in IAA production (**Figure 4**), which is in agreement with previous studies (Khianngam et al., 2023; Lebrazi et al., 2020; Wang et al., 2019). The observed IAA production that was determined by the optimized condition sugarcane molasses (X_1) = 1.556%; $(NH_4)_2SO_4$ (X_2) = 0.106%; tryptophan (X_3) = 0.052%) was 47.67 ± 0.78 μg/mL, which agrees with the predicted model. Considering the results, the model is reliable for evaluating the effects of sugarcane molasses, $(NH_4)_2SO_4$, and tryptophan dosages on the IAA production in *B. subtilis* IA3.

5. Conclusions

In this study, ten IAA-producing endospore-forming strains were isolated. The strain IA3 showed the highest IAA production. Strain IA3 was identified as *Bacillus subtilis* based on the 16S rRNA sequencing and was used to optimize conditions for IAA production. We employed a response surface methodology approach to optimize IAA production by *B. subtilis* IA3. A suitable medium for improved IAA production was successfully established.

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NO CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflict of interest.

References

Chen, Z. M., Li, Q., Liu, H. M., Yu, N., Xie, T. J., Yang, M. Y., Shen, P., & Chen, X. D. (2010). Greater enhancement of Bacillus subtilis spore yields in submerged cultures by optimization of medium composition through statistical experimental designs. *Applied Microbiology and Biotechnology, 85*(5), 1353-1360. https://doi.org/10.1007/s00253-009-2162-x

Habibi, S., Djedidi, S., Ohkama-Ohtsu, N., Sarhadi, W. A., Kojima, K., Rallos, R. V., Ramirez, M. D. A., Yamaya, H., Sekimoto, H., & Yokoyama, T. (2019). Isolation and screening of indigenous plant growth-promoting rhizobacteria from different rice cultivars in Afghanistan soils. *Microbes and Environments, 34*(4), 347-355. https://doi.org/10.1264/jsme2.ME18168

Kaur, R., & Kaur, S. (2021). Plant growth-promoting potential of 'Myroides gitamensis' isolated from virgin soils of Punjab. *Archives Microbiology, 203*(5), 2551-2561. https://doi.org/10.1007/s00203-021-02231-8

Khianngam, S., Meetum, P., Chiangmai, P. N., & Tanasupawat, S. (2023). Identification and Optimisation of indole-3-acetic acid production of endophytic bacteria and their effects on plant growth. *Tropical Life Sciences Research, 34*(1), 219-239. https://doi.org/10.21315/tlsr2023.34.1.12

Lebrazi, S., Niehaus, K., Bednarz, H., Fadil, M., Chraibi, M., & Fikri-Benbrahim, K. (2020). Screening and optimization of indole-3-acetic acid production and phosphate solubilization by rhizobacterial strains isolated from Acacia cyanophylla root nodules and their effects on its plant growth. *Journal of Genetic Engineering and Biotechnology, 18*(1), 71-82. https://doi.org/10.1186/s43141-020-00090-2

Li, Y., Liu, X., Hao, T., & Chen, S. (2017). Colonization and maize growth promotion induced by phosphate solubilizing bacterial isolates. *International Journal of Molecular Sciences, 18*(7), 1253-1268. https://doi.org/10.3390/ijms18071253

Mehta, N., Rao, P., & Saini, R. (2021). A review on metabolites and pharmaceutical potential of food legume crop mung bean (Vigna radiata L. Wilczek). *BioTechnologia (Pozn), 102*(4), 425-435. https://doi.org/10.5114/bta.2021.111107

Muthukumarasamy, R., Revathi, G., Vadivelu, M., & Arun, K. (2017). Isolation of bacterial strains possessing nitrogen-fixation, phosphate and potassium-solubilization and their inoculation effects on sugarcane. *Indian Journal of Experimental Biology, 55*(3), 161-170.

Nguyen, T. N. (2018). Acinetobacter soli SP2 capable of high-efficiency degradation of food emulsifier polysorbate 80. *Current Microbiology, 75*(7), 896-900. https://doi.org/10.1007/s00284-018-1463-7

Nguyen, T. N., Vo, T. V., Nguyen, P. T. H., & Kiefer, R. (2022). Isolation and optimization of a glyphosate-degrading Rhodococcus soli G41 for bioremediation. *Archive Microbiology, 204*(5), 252-261. https://doi.org/10.1007/s00203-022-02875-0

Nguyen, T. N., Pham, T. T. T., Tran, N. T., Kumar, V. B., Saikia, S., & Kiefer, R. (2021). Identification and biodegradation potential of a novel strain of kosakonia oryzae isolated from a Polyoxyethylene tallow amine paddy soil. *Current Microbiology, 78*(8), 3173-3180. https://doi.org/10.1007/s00284-021-02592-9

Shasaltaneh, M. D., Moosavi-Nejad, Z., Gharavi, S., & Fooladi, J. (2013). Cane molasses as a source of precursors in the bioproduction of tryptophan by Bacillus subtilis. *Iranian Journal Microbiology*, *5*(3), 285-292.

Sherpa, M. T., Sharma, L., Bag, N., & Das, S. (2021). Isolation, characterization, and evaluation of native rhizobacterial consortia developed from the rhizosphere of rice grown in organic state Sikkim, India, and their effect on plant growth. *Frontiers in Microbiology, 12*, 1-15. https://doi.org/10.3389/fmicb.2021.713660

Wang, X., Li, Q., Sui, J., Zhang, J., Liu, Z., Du, J., Xu, R., Zhou, Y., & Liu, X. (2019). Isolation and characterization of antagonistic bacteria paenibacillus jamilae HS-26 and their effects on plant growth. *Biomed Research International*, 1-13. https://doi.org/10.1155/2019/3638926

Wu, L., Wu, S., Qiu, J., Xu, C., Li, S., & Xu, H. (2017). Green synthesis of isomaltulose from cane molasses by Bacillus subtilis WB800-pHA01-palI in a biologic membrane reactor. *Food Chemistry, 229*, 761-768. https://doi.org/10.1016/j.foodchem.2017.03.001

Zhang, X., Al-Dossary, A., Hussain, M., Setlow, P., & Li, J. (2020). Applications of Bacillus subtilis spores in biotechnology and advanced materials. *Applied and Environmental Microbiology, 86*(17), 1-13. https://doi.org/10.1128/AEM.01096-20