ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Biotechnology

ISSN 1682-296X
DOI: 10.3923/biotech.2021.22.30

Research Article
Amplification and Sequence Analysis of Indole-3-Pyruvic Acid (IPyA) Pathway Related Genes from *Bacillus* spp.

[1]Karen B. Alviar, [2]Krizzia Mae R. Lumangaya, [2]Joan Christine O. Adajar and [3]Mannix S. Pedro

[1]Institute of Weed Science, Entomology and Plant Pathology, University of the Philippines Los Banos, Los Banos, Laguna Philippines
[2]Institute of Biological Sciences, University of the Philippines Los Banos, Los Banos, Laguna Philippines
[3]National Institute of Molecular Biology and Biotechnology, Los Banos, Laguna Philippines

Abstract

Background and Objective: Several species of *Bacillus* are plant growth-promoting rhizobacteria that can produce the auxin phytohormone Indole-3 acetic acid (IAA) which regulates plant growth and development. This study aimed to amplify key genes, patB and dhaS, two of the three component enzymes found in the IPyA biosynthetic pathway for IAA synthesis and conduct 16S rRNA sequence analysis of two *Bacillus* isolates from Fermented Plant Juice (FPJ) extracted from Madre de Cacao, *Gliricidia sepium*. **Materials and Methods:** Genomic DNA extraction was performed using the CTAB method followed by the amplification of IPyA pathway genes through a polymerase chain reaction. Sequence similarity search was performed using NCBI BLASTn and the protein structure was predicted using Phyre2. **Results:** The dhaS gene from isolate 1 (*B. amyloliquefaciens*) had a top nucleotide BLAST hit with *B. velezensis* strain FJAT-45028 and a translated BLAST hit with an aldehyde dehydrogenase protein. The resulting rank three models in protein structure prediction revealed dhaS which encodes for an enzyme responsible for the conversion of indole-3-acetaldehyde (IAAld) to IAA. Furthermore, phylogenetic trees generated using the Maximum likelihood method revealed the relationship of each isolate with its top five BLAST hits. **Conclusion:** These amplified IAA-related genes may be further utilized in optimizing phytohormone production that has the potential to improve the capability of *Bacillus* isolates as biofertilizers.

Key words: Auxin, dhaS, IAA, IPyA, patB

Citation: Alviar, K.B., K.M.R. Lumangaya, J.C.O. Adajar and M.S. Pedro, 2021. Amplification and sequence analysis of indole-3-pyruvic acid (IPyA) pathway related genes from *Bacillus* spp. Biotechnology, 20: 22-30.

Corresponding Author: Karen B. Alviar, Institute of Weed Science, Entomology and Plant Pathology, University of the Philippines Los Banos, Los Banos, Laguna Philippines

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The genus *Bacillus* refers to ubiquitous, endospore-forming, rod-shaped bacteria which may be further classified as either gram-positive or gram-variable and aerobic or facultatively anaerobic[1]. Their ability to produce endospores allow these species to thrive in various environmental conditions[2]. Colonization of *Bacillus* spp. in plant roots improves plant functions under various stress conditions such as soil salinity, drought, presence of heavy metals, pests and other pathogenic microorganisms[3]. Furthermore, metabolites secreted by these species facilitate the nutrient acquisition and contribute to plant hormone production[3].

Recent studies on *Bacillus* spp. were conducted proving its beneficial effects on plant growth and development. In a study by Yuan *et al.*[4], root colonization of *B. amyloliquefaciens* strain NJN-6 and detection of phytohormone compounds such as Indole-3 acetic acid (IAA) and gibberellin A3 were observed along with the improved growth of banana plants against fusarium wilt. In another study by Reetha *et al.*[5] application of *Bacillus subtilis* and *Pseudomonas fluorescens* improved the growth of the onion plant (*Allium cepa*) and both microorganisms were found out to be capable of producing IAA. With its potential for improvement in crop production, commercialization of *Bacillus* species, specifically *Bacillus ellenbachensis* was evident since the late 19th century in a fertilizer named Alinit® as a "bacteriological fertilizer for inoculation of cereals" by Borriss[6]. Indole-3 acetic acid (IAA) is a naturally occurring auxin, a collection of plant hormones that controls plant growth and development[7] through induction of cell division, differentiation and expression. Pathways for IAA biosynthesis has been documented in *Arabidopsis thaliana*[8], ectomycorrhizal fungi[9] and plant-beneficial bacteria such as *Arthrobacter pascens* ZZ21[10] and *Bacillus amyloliquefaciens* SQR9[11]. Multiple IAA biosynthesis pathways particularly indole-3-acetonitrile (IAN), tryptamine (TAM) and indole-3-pyruvic acid (IPyA) pathways were suggested to be present in *Bacillus amyloliquefaciens* strain SQR9[11]. The IPyA pathway is thought to function in both plants and bacteria, including Bradyrhizobium, Enterobacter, Azospirillum and Rhizobium[12]. The first step of this pathway involves tryptophan which is converted to IPyA by an aminotransferase1[13,14]. The rate-limiting step in this process is the conversion of IPyA to indole-3-acetaldehyde (IAAld) by indole-3-pyruvate decarboxylase (IPDC) (encoded by ipdC)[15,16]. The IAAld is then oxidized to IAA by aldehyde dehydrogenase, mutase or oxidase enzymes[17]. In Bacillus amyloliquefaciens, dhaS is thought to encode indole-3-acetaldehyde dehydrogenase[18]. The IPyA pathway in this bacterium comprises the genes patB, which codes for the enzyme tryptophan transaminase, yclC, which codes for indole-3 pyruvate decarboxylase and dhaS, which codes for indole-3 acetaldehyde[11]. In this study, two *Bacillus* spp. selected from a biofertilizer derived from fermented plant juice of Madre de Cacao, *Gliricidia sepium* to identify one possible biosynthetic pathway for IAA synthesis. Two genes from the IPyA pathway, dhaS and patB, were amplified and deduced to be highly similar with other organisms' genes encoding for enzyme tryptophan transaminase and indole-3-acetaldehyde respectively (dhaS and patB).

MATERIALS AND METHODS

Species collection: Two *Bacillus* isolates from the University of the Philippines Los Baños National Institute of Molecular Biology and Biotechnology (BIOTECH) were grown in LB broth (10 g Peptone, 10 g Yeast Extract and 5 g NaCl per litre of distilled water) using stab culture technique. These *Bacillus* isolates were incubated overnight with shaking (250 rpm, 37°C) before subjecting to genomic DNA extraction.

Study area: The experiments were carried out at the Insect Physiology laboratory from January, 2019 to February, 2020 at the Institute of Weed Science, Entomology and Plant Pathology, College of Agriculture and Food Science, University of the Philippines Los Banos.

Genomic DNA extraction: The protocol for cetyltrimethylammonium bromide (CTAB) DNA extraction was based on Raymundo and Opulencia[19] with some modifications. Ten stab cultures of *Bacillus* isolates, 5 each from putative *B. amyloliquefaciens* and *B. subtilis* were transferred to 1.5 mL Eppendorf tubes and centrifuged at 12000 rpm for 45 sec. The resulting pellet in each tube was resuspended and dispersed in 20 µL Tris-EDTA buffer. The lipid component of the bacterial cell wall was degraded by adding 25 µL of 2% SDS which was followed by incubation at 37°C with shaking (250 rpm) for 1 hr. The addition of 45 µL of 5 M NaCl and 45 µL of 10% CTAB were done for the dissolution and precipitation of proteins and polysaccharides, respectively. After this, the tubes were immersed in a hot water bath (65°C) for 20 min. An equal volume of chloroform: isoamyl alcohol was added for the removal of lipids and separation of aqueous and organic phases. After 30 min, centrifugation was done at 15000 rpm for 10 min. The resulting top layer was pipetted out and an equal volume of isopropanol and 1 mL of 70% ethanol were mixed in the solution. Another centrifugation was

Table 1: Primer sequences for the amplification of IAA-related genes in two *Bacillus* isolates

Gene	*Bacillus* isolate	Primer sequence	Expected product size (bp)*
patB	1 and 2	Forward ATGAACTTTGATMWWCGAGAA	1,164
		Reverse TTACGATAATGCGGCTTTGAT	
dhaS	1	Forward TTTCGGGAGGTTTGTTACGA	1,530
		Reverse AGGCAGCAGTTTCTTGTTTGA	
	2	Forward TGGAGGTTTGTTACGGATGAGT	1,192
		Reverse CTGGCAGGCAGCAGTTTTT	

*bp: Base pairs

Table 2: PCR reaction components for the amplification of patB and dhaS genes from two *Bacillus* isolates

Reagent	Initial concentration*	Final concentration	Amount per tube (µL)**
2x Taq master mix: Taq DNA	2.00 X	1.00 X	25
Polymerase		1.25 unit	
-ViBuffer		1.00 X	
-dNTPs		0.20 mM	
-$MgCl_2$		1.50 mM	
Forward primer	10.00	0.20 µM	1
Reverse primer	10.00	0.20 µM	1
Nuclease-free water	-	-	Adjust to a final volume of 50
Total			50

*Micro molar, **Microliter

done at 13000 rpm for 5 min followed by the removal of supernatant and drying of the pellet. The extracted genomic DNA was resuspended in 100 µL TE buffer and stored at 4°C.

To determine the concentration and purity of the extracted genomic DNA, spectrophotometric analysis was performed using Epoch™ Microplate Spectrophotometer. The extracts were also subjected to agarose gel electrophoresis for visual examination of their quality.

Amplification of IAA-related genes and sequence analysis: The IAA-related genes, patB and dhaS were amplified from specific primers which were designed using the primer designing tool from the National Center for Biotechnology Information (NCBI) based on the sequence of *Bacillus amyloliquefaciens* (Accession: CP000560) for isolate 1 and *Bacillus subtilis* (Accession: NC_000964.3) for isolate 2. The identity of the isolates was determined using 16s rRNA as described below. The generated primer sequences given in Table 1 were sent for DIAMED Enterprise for synthesis.

As presented in Table 2, Vivantis 2X Taq Master mix/Promega GoTaq® Colorless Master Mix DNA Amplification product, extracted genomic DNA, generated primers and nuclease-free water was the components utilized for amplification. Combining these components, PCR was then performed with the reaction conditions indicated in Table 3 as suggested in Vivantis DNA Amplification Product 2X Taq Master Mix. The annealing temperature was also varied based on the indicated melting temperature of the primers from Tm calculator tool of Thermo Fisher. The resulting amplicons were subjected to agarose gel electrophoresis to visualize and confirm the presence of the gene in the expected molecular weight.

Table 3: PCR reaction conditions for the amplification of IAA-related genes from two *Bacillus* isolates as recommended in Vivantis DNA Amplification Product 2x Taq master mix

Step	Temperature (°C)	Time (s)*
Initial denaturation	94	120
Denaturation	94	2
Annealing	Varied	30
Extension/1 kb	72	30
Final extension	72	420

*Seconds

Table 4: Primers used for the amplification of 16S rRNA genes from two *Bacillus* isolates

Primer	Sequence (5'-3')
8FFAM	AGAGTTTGATCMTGGCTCAG
1512R	ACGGYTACCTTGTTACGACTT

The obtained forward and reverse sequences of amplified IAA-related genes were aligned using Bio-Edit sequence alignment editor and the graphic format of the alignment were exported from CLC Sequence Viewer 8. Similar sequences from databases were also acquired using the Basic Local Alignment Search Tool (BLAST) from NCBI. To further utilize the sequences, the protein structure was predicted using Protein Homology/Analogy Recognition Engine Version 2.0 Phyre 2.

16S rRNA sequence analysis: Amplification of 16S rRNA Sequences from two *Bacillus* isolates were done using 8F and 1512R primers given in Table 4. The obtained sequences were compared with an online sequence database utilizing BLAST from NCBI. The 16S rRNA sequences

from each *Bacillus* isolate, along with its top BLAST hits were used to generate a phylogenetic tree applying Maximum Likelihood in MEGAX.

RESULTS AND DISCUSSION

Genomic DNA extraction: Assessment of the extracted genomic DNA using spectrophotometry shows that all ten samples having an absorbance ratio of 1.7-2 are purely given in Table 5. This classification was based on the study of Sainz *et al.*[20] in which the resulting absorbance ratio between 1.7-2.0 may be characterized as pure DNA. An increase in RNA absorbance ratio can also be observed in a solution with adjusted pH between 7.5-8.5[21].

The extracted samples subjected to agarose gel electrophoresis showed intact bands above 12000 bp as observed in Fig. 1. The observed band size above the 1 kb ladder coincides with the previous analysis by Zhang *et al.*[22] that the genome size of *B. subtilis* is 4.05 Mb while *B. amyloliquefaciens* is 4.01 Mb. Some of the lanes had a faint or almost unobserved band which may be due to a few or absence of the extracted genomic DNA. Furthermore, the extracts having an intact band and characterized as pure were then used as a template for amplification.

Table 5: Assessment of purity and concentration (ng μL^{-1}) of the extracted genomic DNA of two *Bacillus* isolates using spectrophotometry

Bacillus isolate	Tube number	ng μL^{-1}	260/280	Indication
1	1	86.383	1.994	Pure
	2	27.983	1.792	Pure
	3	136.383	2.057	Pure
	4	22.883	1.799	Pure
	5	32.783	1.997	Pure
2	1	36.483	1.949	Pure
	2	48.783	1.875	Pure
	3	34.983	2.009	Pure
	4	54.983	1.853	Pure
	5	34.083	1.737	Pure

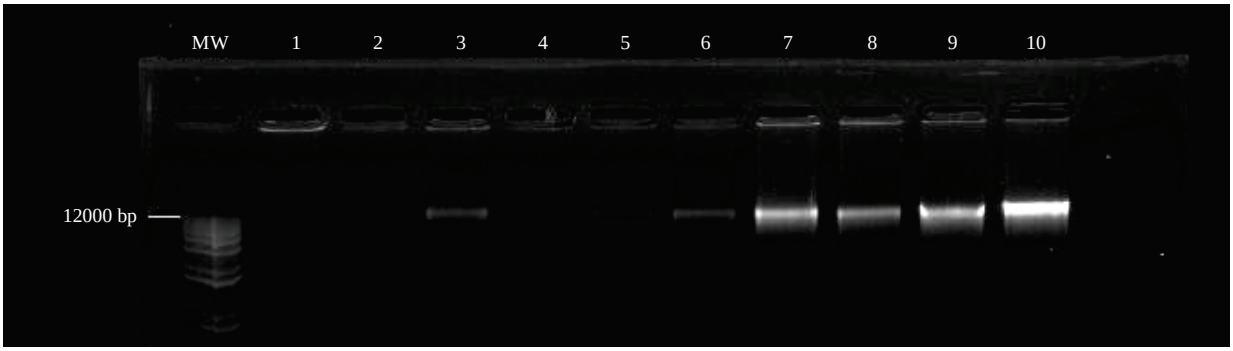

Fig. 1: Electrophoretogram of the genomic DNA extracts from *Bacillus* isolates

Lanes 1-5 are genomic DNA from five different stab cultures of isolate 1 (*B. amyloliquefaciens*), Lanes 6-10 are genomic DNA from five different stabs cultured of isolate 2 (*B. subtilis*)

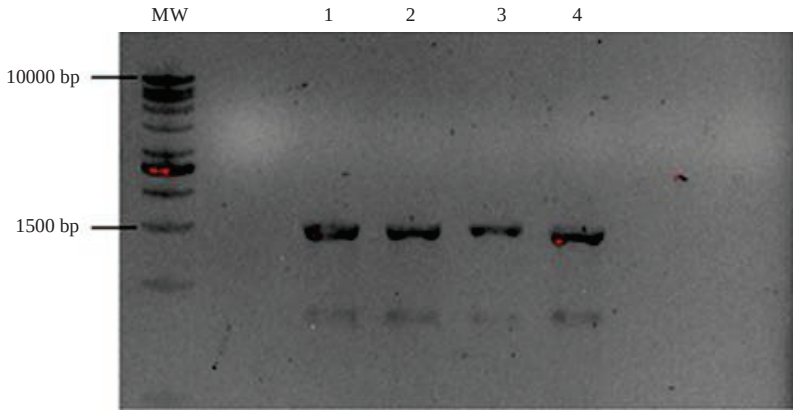

Fig. 2: Electrophoretogram of the amplified dhaS gene using the extracted genomic DNA from isolate 1 (*B. amyloliquefaciens*) as a template at 60°C annealing temperature

Lanes 1-4 are PCR products from the genomic DNA template isolated from 4 different stab cultures

Table 6: Standard nucleotide BLAST (BLASTN) result for the consensus sequence of the amplified dhaS gene from isolate 1 (*Bacillus amyloliquefaciens*)

Species	Identity of gene in sequence	Accession number	Max score	Query cover (%)	E-value	Percent identity (%)
Bacillus velezensis strain FJAT-45028	GRT15_09865	CP047157.1	2591	99.00	0.0	98.28
Bacillus velezensis strain OSY-GA1	D0U0_09770	CP031880.1	2591	99.00	0.0	98.28
Bacillus amyloliquefaciens strain LM2303	BSF20_16290	CP018152.1	2591	99.00	0.0	98.35
Bacillus subtilis strain B-1	MA22_13570	CP009684.1	2591	99.00	0.0	98.28

Table 7: Translated BLAST (BLASTX) result for the consensus sequence of the amplified dhaS gene from isolate 1 (*B. amyloliquefaciens*)

Protein	Accession number	Max score	Query cover (%)	E-value	Percent identity (%)
Aldehyde dehydrogenase family protein (*Bacillus velezensis*)	WP_031379034.1	884	98.00	0.0	96.03
Multispecies: Aldehyde dehydrogenase family protein (*Bacillus*)	WP_007611960.1	884	98.00	0.0	96.03
Multispecies: Aldehyde dehydrogenase family protein (*Bacillus*)	WP_003153776.1	883	98.00	0.0	96.03
Multispecies: Aldehyde dehydrogenase family protein (*Bacillus*)	WP_024085472.1	884	98.00	0.0	96.24
Multispecies: Aldehyde dehydrogenase family protein (*Bacillus*)	WP_063636660.1	884	98.00	0.0	96.03

Amplification of IAA-related genes and bioinformatic analysis of sequences: Amplification of *dhaS* from isolate 1 at an annealing temperature of 60°C resulted in a distinct band as observed in Fig. 2. The molecular weight of the amplicon lies at approximately 1500 bp mark in the molecular ladder. In contrast with the dhaS gene from *Bacillus* isolate 2, application of varying annealing temperature ranging from 56-58°C resulted in an amplicon observed at approximately 300 bp in Fig. 3.

On the other hand, degenerate primers were used for the amplification of patB gene for the two isolates. Varying the annealing temperature between 43-45°C resulted in an amplicon having a molecular weight of approximately above 1500 bp in Fig. 4.

As shown in Table 6, the amplified dhaS gene from isolate 1 (*B. amyloliquefaciens*) had a top BLAST hit with *B. amyloliquefaciens* strain *LM2303* (Accession no. CP018152.1) with percent identity of 98.35% and an E-value of 0.0 for a 99% coverage with a max score of 2591. An E-value of 0.0 was also gathered for *B. velezensis* strain FJAT-45028 and *B. velezensis* strain OSY-GA1 suggesting a very similar sequence identity with the isolate being studied. A study by Fan *et al.*[23] suggests that both *B. velezensis* and *B. amyloliquefaciens* are included under Clade II of *Bacillus subtilis* complex having B. *velezensis* as the taxonomic synonym of *B. amyloliquefaciens*. Aside from this, the identity of the gene in all of the top BLAST hits had a reference sequence that pertains to 3-hydroxypropionaldehyde dehydrogenase/aldehyde dehydrogenase DhaS which further implies that the aligned sequence shares similarity with the expected gene product of dhaS gene.

Further analysis of the sequence of the amplified dhaS gene in isolate 1 revealed that the top hit in the translated BLAST corresponds to an aldehyde dehydrogenase family protein having a percent identity of 96% given in Table 7. This result also supports the expected enzyme that is coded by dhaS gene which is an aldehyde dehydrogenase.

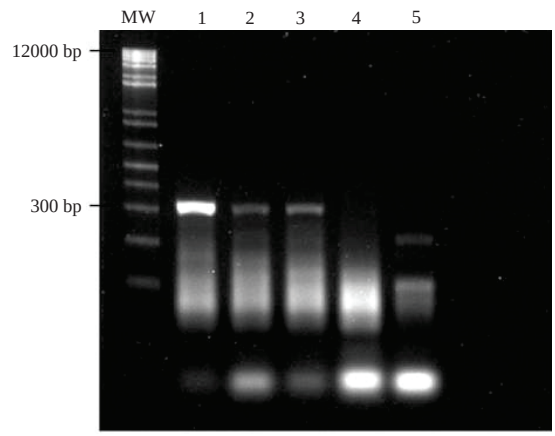

Fig. 3: Electrophoretogram of the amplified dhaS gene using the extracted genomic DNA from isolate 2 (*B. subtilis*) with varying annealing temperature
Lane 1: 56°C, Lane 2: 57°C, Lane 3: 58°C, Lane 4: 58°C, Lane 5: Negative control, genomic DNA templates used were from the five different stab cultures

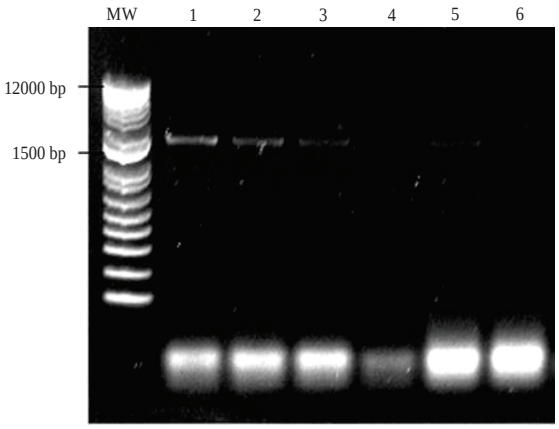

Fig. 4: Electrophoretogram of the amplified patB gene using the extracted genomic DNA from two *Bacillus* isolates
Lane 1: Isolate 2, 43°C; Lane 2: Isolate 2, 44°C; Lane 3: Isolate 2, 45°C; Lane 4: Negative control, 45°C; Lane 5: Isolate 1, 44°C; Lane 6: Isolate 2, 45°C

Table 8: Overview of the BLAST result using the 16S rRNA sequence from *Bacillus* isolate 1 with organisms limited to *Bacillus* (taxid: 1386)

Species	Accession number	Max score	Query cover (%)	E-value	Percent identity (%)
Bacillus sp. strain 1CY1 16S ribosomal RNA gene, partial sequence	MG062819.1	2246	98.00	0.0	99.67
Bacillus sp. W4(2008) 16S ribosomal RNA gene, partial sequence	EU596423.1	2246	98.00	0.0	99.51
Bacillus badius strain KSI 1261 16S rRNA gene	KC113129.1	2161	99.00	0.0	98.07
Bacillus subtilis partial 16S rRNA	HE612877.1	2158	98.00	0.0	98.22
Bacillus badius partial 16S rRNA gene	HE612879.1	2154	98.00	0.0	98.22

Table 9: Overview of the BLAST result using the 16S rRNA sequence from *Bacillus* isolates 2 with organisms limited to *Bacillus* (taxid: 1386)

Species	Accession number	Max score	Query cover (%)	E-value	Percent identity (%)
Bacillus subtilis strain GX S-11 16S ribosomal RNA gene, partial sequence	KU904283.1	1853	94.00	0.0	95.65
Bacillus subtilis strain IISI-6 16S ribosomal RNA gene, partial sequence	MK367790.1	1847	91.00	0.0	96.32
Bacillus subtilis strain FX4 16S rRNA gene	MG241307.1	1847	91.00	0.0	96.32
Bacillus sp. 531-12 16S ribosomal RNA gene, partial sequence	KU361226.1	1842	94.00	0.0	95.34
Bacillus subtilis strain BIL-BS-168 16S rRNA gene, partial sequence	MH298834.1	1840	93.00	0.0	95.70

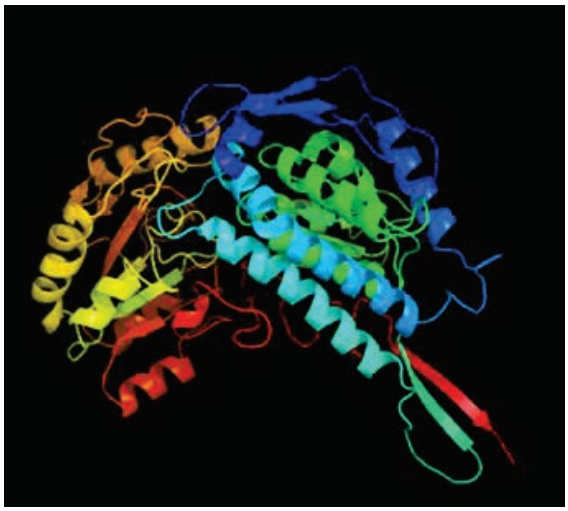

Fig. 5: Rank three model of the predicted secondary structure of the amino acid translated from the amplified dhaS gene from *Bacillus* isolate 1 (*B. amyloliquefaciens*) generated using Phyre2

Red portion indicates the N-terminus while the Indigo depicts the C-terminus

Prediction of secondary structure using Phyre2 was also done resulting in a top model of a proline dehydrogenase/ delta-1-pyrroline-5-carboxylate[24]. Aside from this, the rank three models in the structures generated was an aldehyde dehydrogenase with 100% confidence and 25% identity in Fig. 5. The resulting PDB title was described as the structure of a bacterial aldh16 complexed with Nadh[24]. According to Vasilou *et al.*[25] ALDH16 is a member of the aldehyde dehydrogenase superfamily. This protein has a distinguishing feature of the presence of two protein domains[25,26].

A previous study on dhaS gene was conducted by Shao *et al.*[11] in which the gene from *B. amyloliquefaciens* strain SQR9 was found out to be upregulated in the presence of tryptophan and possibly be involved in the IPyA pathway of IAA biosynthesis. The IPyA pathway is one of the various tryptophan dependent pathways for IAA biosynthesis[15]. This pathway has been widely observed in bacteria[27], such as in *Azospirillium brasilense* sp.7[28], *Pseudomonas putida* GR12-2[15] and *Enterobacter cloacae* UW5[29]. Other than the IPyA pathway, dhaS gene has also been suggested to play a role in catalyzing the conversion of Indole-3 acetaldehyde to Indole-3 acetic acid in the terminal step of Tryptamine pathway[11]. Contrastingly, a study by Idris *et al.*[18] showed that mutation in dhaS gene of *B. amyloliquefaciens* FZB42 did not affect the IAA production implying that this gene is not involved in IAA biosynthesis. The involvement of dhaS gene in the abovementioned two pathways gives its importance towards IAA biosynthesis. However, it also needs to be taken into consideration that there are differences in the IAA biosynthesis pathway across different strains of the same species which further implies that isolation of the dhaS gene may not be enough to deduce its involvement in the IAA biosynthesis pathway and thus needs further confirmation through in vitro assays and gene knock-out mutation.

16S rRNA sequence analysis: Standard nucleotide BLAST (BLASTN) of 16S rRNA sequence in isolate* 1 showed that the top nucleotide BLAST hit is *Bacillus* sp. strain 1CY1 with a percent identity of 99.67% in Table 8. Whereas isolate 2 had a top nucleotide BLAST hit with *Bacillus subtilis* strain GX S-11 having the highest percent identity of 95.65% given in Table 9.

To infer the relationships between 16S rRNA sequences from the two isolates and its top five nucleotide BLAST hits, phylogenetic trees were generated using Maximum Likelihood and Tamura-Nei model with 1000 replicates from MEGA X. In isolate 1, the phylogenetic tree with a maximum log-likelihood

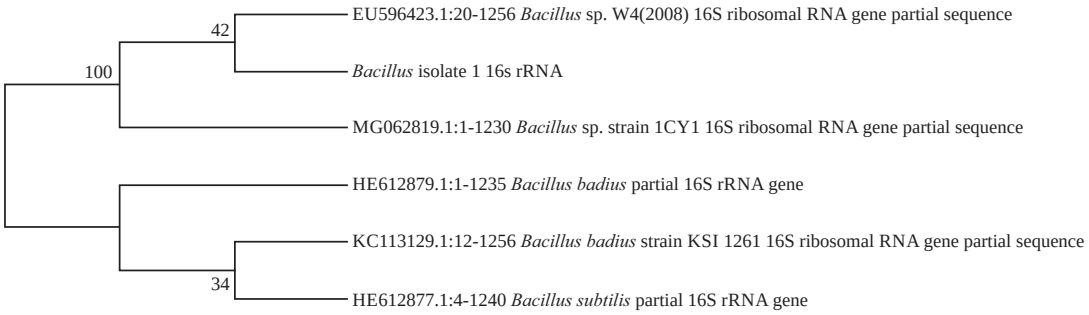

Fig. 6: Phylogenetic tree of *Bacillus* isolate 1 and its resulting top five BLAST hits based on 16S rRNA gene sequence generated
Using maximum likelihood and Tamura-Nei model from MEGA X with 1000 replicates

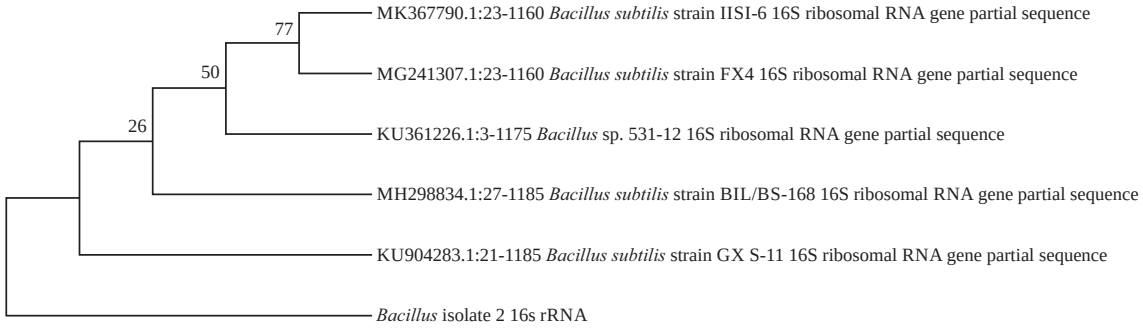

Fig. 7: Phylogenetic tree of *Bacillus* isolate 2 and its resulting top five BLAST hits based on 16S rRNA gene sequence generated
Using maximum likelihood and Tamura-Nei model from MEGA X with 1000 replicates

of -1812.42 implies that *Bacillus* isolate 1, *Bacillus* sp. W4 and *Bacillus* sp. strain 1CY1 are more closely related to each other than *Bacillus badius, Bacillus badius* strain KSI 1261 and *Bacillus subtilis*[30]. As presented in Fig. 6, the observed highest value (100) in the node indicates that the three species (*Bacillus* isolate 1, *Bacillus* sp. W4 and *Bacillus* sp. strain 1CY1) appeared in the same clade in all of the trees generated whereas a lower value of 42 in the internal node pertains to a less occurrence of the node in the trees replicated.

In contrast with isolate 2, the phylogenetic tree having the highest log-likelihood of -1751.33 suggests that *Bacillus* isolate 2 lies outside the clade comprising its top five BLAST hits in Fig. 7. The result is also evident in the multiple sequence alignment, in which the sequence from the isolate had several mismatched bases with its top five BLAST hits. Aside from this, the probability of base substitutions and conditional likelihoods affects the tree topology generated using maximum likelihood[31].

CONCLUSION

In this study, the indole-3-pyruvic acid pathway is one plausible pathway that may be used by the selected *Bacillus* species for IAA synthesis. Successful amplification of patB and dhaS from the isolates and the bioinformatics data presented for these two IPyA component genes provides a baseline understanding of the *Bacillus* spp. isolates found in FPJ biofertilizer. Altogether, the data suggest that IPyA may be the route taken by these species for IAA synthesis which may be further utilized in optimizing phytohormone production that has the potential to improve the capability of *Bacillus* isolates as biofertilizers.

SIGNIFICANCE STATEMENT

There are reports on IAA biosynthesis pathways in various organisms but only a few studies are available regarding the analysis of each gene involved in these pathways. The bioinformatics data presented here for two-component genes of the IPyA biosynthesis pathway for IAA production provide us with a baseline understanding of the Bacillus spp. isolates found in FPJ biofertilizer. Further identification of the presence of IAA-related genes in several species of Bacillus may pave the way for further understanding of bacterial IAA biosynthesis which has the potential to improve crop production.

REFERENCES

1. Fekete, T., 2014. *Bacillus* Species and Related Genera Other Than *Bacillus anthracis*. In: Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases, Bennett, J.E., R. Dolin and M.J. Blaser (Eds.)., Elsevier, New York, pp: 2410-2414.
2. Moat, A.G., J.W. Foster and M.P. Spector, 2002. Microbial Physiology, 4th Edn., Wiley, United States, Pages: 715.
3. Radhakrishnan, R., A. Hashem and E.F. Abd_Allah, 2017. *Bacillus*: A biological tool for crop improvement through bio-molecular changes in adverse environments. Front. Physiol., Vol. 8. 10.3389/fphys.2017.00667.
4. Yuan, J., Y. Ruan, B. Wang, J. Zhang, R. Waseem, Q. Huang and Q. Shen, 2013. Plant growth-promoting rhizobacteria strain *Bacillus amyloliquefaciens* NJN-6-enriched bio-organic fertilizer suppressed Fusarium wilt and promoted the growth of banana plants. J. Agric. Food Chem., 61: 3774-3780.
5. Reetha, S., G. Bhuvaneswari, P. Thamizhiniyan and T.R. Mycin, 2014. Isolation of Indole Acetic Acid (IAA) producing rhizobacteria of *Pseudomonas fluorescens* and *Bacillus subtilis* and enhance growth of onion (*Allim cepa* L.). Int. J. Curr. Microbiol. Applied Sci., 3: 568-574.
6. Borriss, R., 2017. Plant growth promoting bacteria – Early investigations, present state and future prospects. Vegetos Int. J. Plant Res., 30: 211-215.
7. Khalid, A., S. Tahir, M. Arshad and Z.A. Zahir, 2004. Relative efficiency of rhizobacteria for auxin biosynthesis in rhizosphere and non-rhizosphere soils. Aust. J. Soil Res., 42: 921-926.
8. Mashiguchi, K., K. Tanaka, T. Sakai, S. Sugawara and H. Kawaide *et al*., 2011. The main auxin biosynthesis pathway in Arabidopsis. Proc. Natl. Acad. Sci., 108: 18512-18517.
9. Kumla, J., N. Suwannarach, K. Matsui and S. Lumyong, 2020. Biosynthetic pathway of indole-3-acetic acid in ectomycorrhizal fungi collected from northern Thailand. PLoS ONE, Vol. 15. 10.1371/journal.pone.0227478.
10. Vullings, H.G.B., J.H.B. Diederen, D. Veelaert and D.J. Van Der Horst, 1999. Multifactorial control of the release of hormones from the locust retrocerebral complex. Microsc. Res. Tech., 45: 142-153.
11. Shao, J., S. Li, N. Zhang, X. Cui and X. Zhou *et al*., 2015. Analysis and cloning of the synthetic pathway of the phytohormone indole-3-acetic acid in the plant-beneficial *Bacillus* amyloliquefaciens SQR9. Microb. Cell Fact., Vol. 14. 10.1186/s12934-015-0323-4.
12. Spaepen, S., J. Vanderleyden and R. Remans, 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol. Rev., 31: 425-448.
13. Kittell, B.L., D.R. Helinski and G.S. Ditta, 1989. Aromatic aminotransferase activity and indoleacetic acid production in *Rhizobium meliloti*. J. Bacteriol., 171: 5458-5466.
14. Koga, J., K. Syōono, T. Ichikawa and T. Adachi, 1994. Involvement of l-tryptophan aminotransferase in indole-3-acetic acid biosynthesis in *Enterobacter cloacae*. Biochim. Biophys. Acta-Protein Struct. Mol. Enzymol., 1209: 241-247.
15. Patten, C.L. and B.R. Glick, 2002. Regulation of indoleacetic acid production in *Pseudomonas putida* GR12-2 by tryptophan and the stationary-phase sigma factor RpoS. Can. J. Microbiol., 48: 635-642.
16. Brandl, M.T. and S.E. Lindow, 1996. Cloning and characterization of a locus encoding an indolepyruvate decarboxylase involved in indole-3-acetic acid synthesis in *Erwinia herbicola*. Applied Environ. Microbiol., 62: 4121-4128.
17. Pollmann, S., A. Müller and E.W. Weiler, 2006. Many roads lead to "auxin": of nitrilases, synthases and amidases. Plant Biol., 8: 326-333.
18. Idris, E.E., D.J. Iglesias, M. Talon and R. Borriss, 2007. Tryptophan-dependent production of Indole-3-Acetic Acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. Mol. Plant-Microbe Interact., 20: 619-626.
19. Atashpaz, S., S. Khani, A. Barzegari, J. Barar, S.Z. Vahed, R. Azarbaijani and Y. Omidi, 2010. A robust universal method for extraction of genomic DNA from bacterial species. Microbiology, 79: 538-542.
20. Sainz, M.J., J.M. González-Jartín, O. Aguín, J.P. Mansilla and L.M. Botana, 2018. Isolation, Characterization and Identification of Mycotoxin-Producing Fungi. In: Environmental Toxicology, Botana, L.M. (Ed.)., De Gruyter, Berlin Heidelberg, pp: 202-245,.
21. Wilfinger, W.W., K. Mackey and P. Chomczynski, 1997. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques, 22: 474-481.
22. Zhang, N., D. Yang, J.R.A. Kendall, R. Borriss and I.S. Druzhinina *et al*., 2016. Comparative genomic analysis of *Bacillus amyloliquefaciens* and *Bacillus subtilis* reveals evolutional traits for adaptation to plant-associated habitats. Front. Microbiol., Vol. 7. 10.3389/fmicb.2016.02039.
23. Fan, B., J. Blom, H.P. Klenk and R. Borriss, 2017. *Bacillus amyloliquefaciens*, *Bacillus velezensis* and *Bacillus siamensis* form an "operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex. Front. Microbiol., Vol. 8. 10.3389/fmicb.2017.00022.
24. Kelley, L.A., S. Mezulis, C.M. Yates, M.N. Wass and M.J. Sternberg, 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protocols, 10: 845-858.
25. Vasiliou, V., M. Sandoval, D.S. Backos, B.C. Jackson and Y. Chen *et al*., 2013. ALDH16A1 is a novel non-catalytic enzyme that may be involved in the etiology of gout via protein–protein interactions with HPRT1. Chem. Biol. Interact., 202: 22-31.
26. Pavlopoulos, G.A., T.G. Soldatos, A. Barbosa-Silva and R. Schneider, 2010. A reference guide for tree analysis and visualization. Biodata Min., Vol. 3. 10.1186/1756-0381-3-1.

27. Zhang, P., T. Jin, S.K. Sahu, J. Xu, Q. Shi, H. Liu and Y. Wang, 2019. The distribution of tryptophan-dependent indole-3-acetic acid synthesis pathways in bacteria unraveled by large-scale genomic analysis. Molecules, Vol. 24. 10.3390/molecules24071411.
28. Castro-Guerrero, J., A. Romero, J.J. Aguilar, M.L. Xiqui, J.O. Sandoval and B.E. Baca, 2012. The hisC1 gene, encoding aromatic amino acid aminotransferase-1 in *Azospirillum brasilense* Sp7, expressed in wheat. Plant Soil, 356: 139-150.
29. Ryu, R.J. and C.L. Patten, 2008. Aromatic amino acid-dependent expression of indole-3-pyruvate decarboxylase is regulated by tyrr in *Enterobacter cloacae* UW5. J. Bacteriol., 190: 7200-7208.
30. Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura, 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol., 35: 1547-1549.
31. Cho, A., 2012. Constructing phylogenetic trees using maximum likelihood. Scripps Senio Theses. https://scholarship.claremont.edu/scripps_theses/46