

# Bioprospecting for extracellular enzymes from endophytic bacteria isolated from *Vigna radiata* and *Cajanus cajan*

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## ARTICLE INFO

### Article history:

Received on: January 08, 2021

Accepted on: February 05, 2021

Available online: May 10, 2021

### Key words:

Amylase,  
*Cajanus cajan*,  
Cellulase,  
Pectinase,  
Protease,  
*Vigna radiata*

## ABSTRACT

Endophytes are known to improve plant growth using a multitude of mechanisms. Bacteria use a variety of mechanisms to penetrate and reside in a host. Producing hydrolytic enzymes is one of these mechanisms which hydrolyses the cell wall components of plant cells helping bacteria to enter through root hairs and lateral roots. The present study focused on the production of hydrolytic enzyme by endophytic bacteria isolated from *Vigna radiata* and *Cajanus cajan*. Out of forty seven endophytic bacteria, nine isolates, namely, MJIR8, MJIN16, MJIN13, MJHN14, ARR4, MBR9, MHN3, MBN2, and MHN12 were found to produce all the four enzymes, namely, amylase, cellulase, protease, and pectinase. Five isolates ARR4, MBR9, MHN3, MJIN13, and MHN12 showed distinct restriction pattern on the basis of ARDRA profiling and further identified using 16S rDNA sequencing. The results revealed their identity toward the different species of *Bacillus* or *Bacillus* derived genera, that is, ARR4 (*Bacillus megaterium* NAP8), MBR9 (*Bacillus subtilis* NMP1), MHN3 (*Bacillus cereus* NMP2), MJIN3 (*Bacillus panacihumi* NMP3), and MHN12 (*Bacillus licheniformis* MHN12). The isolates also possessed different plant growth promoting (PGP) features and significantly enhanced all plant growth parameters except nodulation. These highly efficient strains with multiple enzyme production and PGP features can be harnessed to facilitate the close association between host plants and PGP bacteria providing better yield and reduced application of agro-chemicals.

## 1. INTRODUCTION

Endophytic bacteria, omnipresent in every plant species known, have been isolated from all plant parts, namely, leaves, stems, flowers, roots, and seeds [1]. These endophytes are sheltered inside the plant tissue from environmental stresses and microbial competition and, in turn, improve the health of host plant directly by procuring nutrients, indirectly by defeating soil borne pathogens or ameliorating the ill effects caused by environment [2-5]. The endophytic bacteria enter into plants interior by several mechanisms, cracks or wounds in primary and lateral roots being the most common mode, as the plant metabolites leakage through these sites attracts the bacteria [6]. Bacteria also enter through the root hairs and lateral roots by producing hydrolytic enzymes such as cellulases, pectinases, proteases, and lipases and hydrolyze the cell wall components of epidermis, hypodermis, and cortical cells [7,8]. Plant growth promoting (PGP) endophytic bacteria also synthesize other enzymes such as amylases, esterases, chitinases, and  $\beta$ -1, 3 glucanases [9]. There is always a strong interest in microbial enzymes because of commercial application in numerous fields such as textile industry, food processing, agriculture research, and pharmaceuticals.

Despite being playing a role in PGP, these endophytes can also be explored as a source of microbial enzymes.

Bacterial cellulases are capable of catalyzing cellulosic biomass, the principal component of all existing plant cell walls and the most abundantly present biopolymer in nature [10]. Thus, the enzyme cellulase has many useful applications in the paper industry, bioethanol generation, textile industries, detergent industry, and animal feed production [11]. Amylase hydrolyses the starch by catalyzing  $\alpha$ -1, 4 glycosidic linkage, and world widely used commercially for preparation of sugar syrups, starch liquefaction, paper, food, pharmaceuticals industries, etc. [12]. Production of peptide digesting microbial proteases gained attention due to their broad use in the detergent, food, chemical, pharmaceutical, and leather processing industries [13]. Pectinases are the enzymes that cause breakdown of pectin by depolymerizing the bonds. Many organisms produce pectinolytic enzymes but bacterial pectinases are predominately most advantageous over others. Therefore, these enzymes have immense application in juice and food industries, paper and pulp industries, decomposition, recycling industries, etc. [14].

Endophytes residing in leguminous crops fix atmospheric nitrogen, control soil-borne pathogens, produce useful metabolites such as auxins, siderophores, hydrogen cyanide (HCN), and play an important role in PGP [15-17]. Exploration of endophytic bacteria to produce number of exocellular enzymes allowing them to enter the host interior and their effect on PGP will be a great opportunity in industrial and

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agriculture field. Therefore, in the present study endophytic bacteria from roots and nodules of leguminous crops were screened for enzymes of industrial importance, namely, amylase, cellulase, protease, and pectinase. Furthermore, these endophytic bacterial strains with multiple enzyme activities were also investigated for the PGP potential.

## 2. MATERIALS AND METHODS

### 2.1. Microbial Cultures

A total of forty seven endophytic bacteria isolated from mungbean (*Vigna radiata*) and pigeon pea (*Cajanus cajan*) plants grown in different districts of Haryana (India) were obtained from our laboratory. Out of total, twenty isolates from roots and twenty seven isolates from nodules were used in the present study. The cultures were maintained on tryptone soy agar slants at 4°C and preserved in 50% glycerol at -20°C.

The study was carried out during the period of February 2019 to February 2020, in the Department of Microbiology of Maharshi Dayanand University, Rohtak, Haryana, India.

### 2.2. Exoenzyme Production by Endophytic Isolates

Amylase, cellulase, protease, and pectinase production were evaluated using specific indicator media supplemented with 1% starch, 0.5% carboxymethyl cellulose (CMC), 1% casein and 0.5% pectin, respectively. After an incubation of 48 h, enzymatic index (EI) was measured and expressed as Hankin and Anagnostakis [18].

$$\text{Enzymatic index} = \frac{\text{Colony diameter (cm)} + \text{Halozone diameter (cm)}}{\text{Colony diameter (cm)}}$$

For amylase production, Gram's iodine was flooded over the starch medium and perceived for appearance of colorless zone around the growth [19]. For cellulase production, the medium was flooded with Congo red solution (0.5%) for 15 min, discarded and then washed with sodium chloride (NaCl) solution (1 M). The formation of clear zone around the growth indicated the cellulase production [20]. Protease activity was determined by the presence of clear halo around the colonies on casein agar plates. For pectinase activity, surface of medium was flooded with 2% hexadecyl trimethyl ammonium bromide for 30 min, allowing the appearance of zone around bacterial growth [21].

### 2.3. Enzymatic Assay

The isolates were grown in nutrient broth supplemented with soluble starch (1%), CMC (1%), casein (1%), and polygalacturonic acid (0.5%) for amylase, cellulase, protease, and pectinase activity, respectively. One unit of enzyme activity is defined as the amount of enzyme required to catalyze 1  $\mu\text{mol}$  of the substrate in 1 min.

### 2.4. Calculation of Enzyme Activity

$$\text{Enzymatic activity (IU / ml)} = \frac{\left( \frac{\text{Amount of substrate consumed}(\mu\text{mol}) \times \text{dilution factor} \times 1000}{\text{Molecular weight of glucose}(\mu\text{mol})} \right)}{\times \text{time} \times \text{enzyme reaction volume (ml)}}$$

#### 2.4.1. Amylase activity

The crude enzyme was obtained by centrifuging the bacterial culture (10,000 rpm  $\times$  15 min) and to 0.5 ml of culture supernatant, 0.5 ml

of freshly prepared starch solution was added. After an incubation of 5 min at room temperature, amylolytic activity was stopped by adding 1 ml of 3,5-dinitrosalicylic acid (DNS) solution. The solution was then heated in boiling water bath for 5 min and allowed to cool down. The volume was made up to 10 ml by adding distilled water and absorbance was measured at 540 nm. The absorbance values were transformed into micromoles of reducing sugar produced during the reaction using standard curve of maltose.

#### 2.4.2. Cellulase activity

To the 1 ml of culture supernatant, added 1 ml of 1% CMC in 0.1 M sodium acetate buffer. Incubated the solution for 5 min at 65°C for cellulolytic activity and then the reaction was stopped by adding 2.5 ml of DNS. The mixture was heated at boiling water bath for 5 min and cooled down thereafter. Concentration of reducing sugar produced was detected by comparing optical densities (540 nm) with the standard curve of glucose.

#### 2.4.3. Protease activity

The bacterial broth was centrifuged at 5000 rpm for 6 min and 150  $\mu\text{l}$  of supernatant was mixed with 300  $\mu\text{l}$  of 1% casein dissolved in 20 mM Tris-HCl (pH=7.4). For protease activity, incubated the mixture at 30 $\pm$ 2°C for 30 min. To stop the reaction, 450  $\mu\text{l}$  of 10% tri-chloroacetate (w/v) was added to make the final concentration of 5% (w/v) and incubated for 1 h at room temperature. The mixture was again centrifuged at 12,000 rpm for 5 min and absorbance measured at 280 nm [22]. Protease concentration was determined by comparing the absorbance value with tyrosine standard curve.

#### 2.4.4. Pectinase activity

For pectinase activity, 0.5 ml of supernatant (6000 rpm for 10 min) was mixed with equal volume (0.5 ml) of 1% pectin solution prepared in 0.05 M citrate buffer (pH=5). It was then incubated for 30 min at 50°C for reaction to take place. The reaction was stopped with 1.5 ml of DNS and kept in boiling water bath for 5 min [19]. Absorbance was measured at 540 nm and compared with glucose standard curve.

For control, the crude enzyme was replaced by uninoculated media. All the results were analyzed statistically with at least three replicates of each sample and given as mean  $\pm$  SEM (Standard error of mean).

## 2.5. Molecular Identification of Isolates

### 2.5.1. Genomic DNA extraction and 16S rDNA amplification

Molecular identification was conducted using 16S rDNA gene sequencing. Modified CTAB method was used to extract genomic DNA of pure cultures [4]. Amplification of 16S rDNA gene was achieved thermocycler (BioRad, USA) using 8F (5'AGAGTTTGATCCTGGCTCAG 3') as forward primer and 1541R (3'AAGGAGGTGATCCAGCC 5') as reverse primer [23,24]. The reaction mixture of 30  $\mu\text{l}$  containing 0.25  $\mu\text{M}$  of each primers, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM of each dNTP, 1X Taq polymerase buffer, 1U of Taq polymerase (Promega, USA), and 50 ng DNA template was subjected to initial denaturation of for 3 min at 94°C, followed by 30 cycles of 3.5 min each including 45 sec at 94°C, 45 sec at 58°C, 2 min at 72°C and subsequent final extension for 7 min at 72°C. The amplification was confirmed by obtaining high reproducibility of the band patterns by agarose (1.2% w/v) gel electrophoresis (GeNei Pvt. Ltd., Bengaluru, India) at 60 V for 40 min and observed under gel documentation system (Azure Biosystems, Dublin, CA 94568 USA).

### 2.5.2. ARDRA (amplified ribosomal DNA restriction analysis)

Restriction endonuclease *MspI*, *Hinfi*, and *HaeIII* were used for restriction digestion of amplified 16S rDNA product. For this digestion, 5  $\mu\text{l}$  of amplified 16S rDNA product was treated with 5 units of restriction

enzyme and held at constant temperature of 37°C for 4 h in dry water bath. The digested product was resolved on 1.5% agarose gel. Two-dimensional binary matrices were prepared from the polymorphic patterns of restriction digestion profiles and combined for cluster analysis. Binary matrices were constructed with the help of SimQual coefficient and further analyzed by UPGMA cluster analysis using biostatistical analysis program NTSYS-PC program 2.1 of Exeter Software's USA [25].

### 2.5.3. 16S rRNA gene sequencing

The isolates were identified by 16S rRNA gene sequencing (AgriGenome Labs Pvt. Ltd., Kochi, India). The obtained sequences were compared with genome data available at National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) server. Similar sequences redeemed and multiple sequence alignment was performed. The phylogenetic tree was constructed by Mega X software using neighbor joining method with 1000 bootstrap data sets [26].

### 2.6. Plant growth promoting traits

The isolates were also screened for PGP properties, namely, organic acid, ammonia, HCN, IAA, siderophore production, and P- solubilization. Ammonia and organic acid production was tested by adding Nessler's reagent and methyl red to 5 days old culture grown in peptone water and MRVP broth, respectively [27]. HCN production was detected using Lorck's method [28]. For siderophore production and phosphate solubilization, inoculum was spotted on CAS (Chrome azurol S) agar [29] and Pikovskaya's agar [30] plates, respectively. Phytohormone IAA production was estimated by colorimetric assay using Salkowski's method [31].

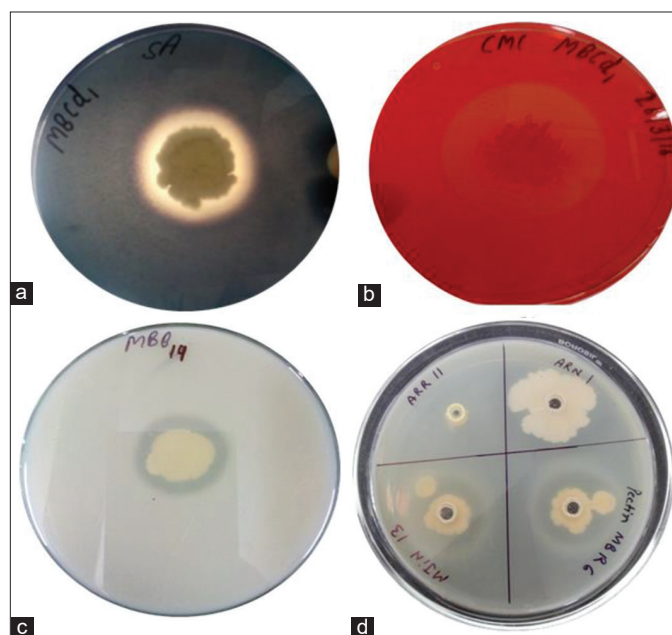
### 2.7. Pot Experiment

The ability of these endophytic isolates to promote plant growth was also assessed under pot culture conditions using *V. radiata* as test host. Seed bacterization was done by incubating the seeds overnight with endophytic bacterial isolates and sown in a set of five seeds per pot. Three pots of each isolate were used to minimize the data error. After germination, three plants in each pot were maintained by uprooting the extra plantlets and irrigated with Slogger's solution every day or as and when required. Plants were uprooted after 60 days of growth and observed for various parameters, namely, number of nodules, root and shoot length, fresh and dry weight of roots and shoots [32].

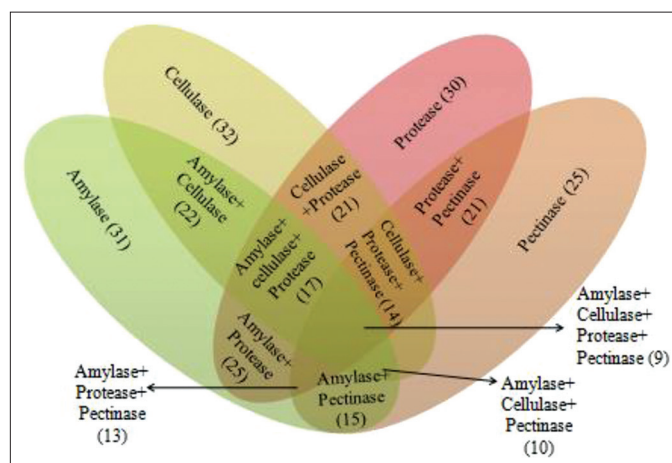
## 3. RESULTS AND DISCUSSION

### 3.1. Screening of Endophytic Bacteria for Exoenzyme Production

Endophytic bacteria are common inhabitants of every plant tissue and the recent studies on these bacteria mainly focused on improvement of economically important crops, biocontrol, and sustainable agriculture. Hydrolytic enzymes are secreted extracellularly by endophytic bacteria and help the plants to setup systemic resistance against attack by pathogens [33,34]. The endophytic bacteria obtained from our laboratory were screened for the presence of active exoenzymes, namely, amylase, cellulase, protease, and pectinase [Figure 1]. The enzymatic index of all the isolates was assessed and observed that all of them possessed ability to produce one or more exoenzyme except isolates MJiN3, MHN19 and MHN20 [Table 1]. Among them, nine isolates, that is, MJiR8, MJiN16, MJhN14, ARR4, MBR9, MHN3, MJiN13, MBN2, and MHN12 produced all the four enzymes while others produced combination of two or three enzymes [Figure 2]. Enzymatic index is the practical and rapid tool for estimating enzyme production by bacteria. Enzymatic index more than 1.0 indicates the enzyme secretion by isolates [35].



**Figure 1:** Zone of hydrolysis shown by endophytic bacterial isolates on nutrient agar plates supplemented with (a) 1 % starch; (b) 0.5 % carboxymethyl cellulose; (c) 1 % casein; and (d) 0.5 % pectin.



**Figure 2:** Venn diagram showing integrative exoenzyme production by bacterial isolates.

In the present study, the enzyme producing isolates produced  $EI \geq 1.0$  for at least one of the four evaluated enzymes, showing their potential for industrial applications. Highest amylase production was shown by isolate MJiR8 with EI of 2.1; cellulase and protease production by MBR9 with Enzymatic index of 2.79 and 2.38, respectively; pectinase production by isolate MJiN13 with EI of 2.6 [Table 1].

### 3.2. Enzyme Activity

On the basis of screening, all positive isolates were grown in specific liquid media and enzyme activity was measured, respectively. Amylase activity of 22 isolates was observed in the range of  $200.36 \pm 32.8$  to  $2473.29 \pm 16.3$  U/ml; cellulase activity of 23 isolates, from  $38.1 \pm 2.1$  to  $574.63 \pm 27.8$  U/ml; and pectinase activity of 17 isolates, from  $430 \pm 43.5$  to  $672.6 \pm 22.3$  U/ml while protease activity for 31 isolates, from  $1.8 \pm 0.09$  to  $34.95 \pm 0.15$  U/ml [Table 2]. Out of total 47 isolates,

**Table 1:** Exoenzymes production by endophytic bacterial isolates.

Isolates	Amylase		Cellulase		Protease		Pectinase	
	Production	EI	Production	EI	Production	EI	Production	EI
MRR 11	++	1.45	++	2	+	1.22	–	–
MRR 10	–	–	+	1.4	++	1.31	+++	3
MRR 14	–	–	+	1.4	+++	1.66	+++	2.3
MRN 3	–	–	–	–	++	1.12	++	1.7
ARR 5	+	1.2	–	–	+	1.2	–	–
ARR 8	+	1.21	–	–	+++	1.47	++	1.7
ARR 11	+	1.2	+	1.25	++	1.5	–	–
ARR 12	++	1.9	–	–	+++	1.83	+	1.6
ARR 15	+	1.31	++	1.58	+++	1.66	–	–
ARR 16	+	0.6	–	–	+++	1.60	–	–
MCDR 8	+++	1.9	+	1.14	+++	1.66	–	–
ARN 1	–	–	–	–	++	1.55	+	1.2
ARN 1a	+	1.2	+	1.25	–	–	–	–
ARN 17	+	1.18	++	1.7	–	–	–	–
MJIR 6	+	1.3	–	–	++	1.40	–	–
MJIR 7	–	–	++	2	+++	1.36	–	–
MJIR 8	+++	2.1	+++	2.67	+++	1.50	++	1.7
MJIR 9	+	0.5	++	2	–	–	–	–
MJIN 3	–	–	–	–	–	–	–	–
MJIN 16	++	1.8	+++	2.42	+++	1.47	+	1.2
MCDN 1	+	1.4	–	1.67	+	1.21	+	1.2
MCDN 2	+	1.1	–	–	–	–	+	1.1
MCDN 7	–	–	+	1.1	+	1.3	–	–
MJHN 1	+	1.6	–	–	+++	1.76	++	1.9
MJHN 4	++	1.43	–	–	+++	1.50	–	–
MJHN 5	–	–	+++	1.67	+	1.18	+	1.1
MJHN 10	–	–	–	–	+	1.4	–	–
MJHN 11	++	1.6	++	1.5	–	–	–	–
MJHN 14	+	1.4	++	1.1	++	1.72	++	2.1
ARR4	+	1.3	+	1.2	++	1.41	++	2.0
MBR 1a	++	1.47	+	1.1	++	1.69	–	–
MBR 4	–	–	+++	2.5	+++	2.0	++	2.0
MBR 6	–	–	–	–	++	1.43	++	1.9
MBR 9	+++	1.55	+++	2.79	+++	2.38	++	2.0
MBR 12	–	–	–	–	++	1.36	+	1.2
MHN 2	–	–	–	–	–	–	+	1.3
MHN 3	+	1.18	++	1.26	+	1.17	+	1.16
MHN 12	+	1.15	+++	1.76	+	1.2	+	1.3
MHN 19	–	–	–	–	–	–	–	–
MHN 20	–	–	–	–	–	–	–	–
MJiN13	++	1.4	+	1.12	+	1.27	+++	2.6
MBN 2	++	1.6	++	2.05	+++	1.23	++	1.6
MBN 3	–	–	+	1.29	+++	1.92	–	–
MBN 8	++	1.45	+++	2.8	–	–	+	1.1
MBN 17	++	1.2	+++	2.4	+++	1.57	–	–
MBN 17(i)	+++	1.77	++	2.3	++	1.69	–	–
MBN 19	+	1.1	+	1.1	+++	1.5	–	–

“EI” means enzymatic index, “–” means showed no production, “+” means showed low production, “++” means moderate production, “+++” means high production



**Table 2:** Exoenzymatic activity of endophytic bacterial isolates from *Vigna radiata* and *Cajanus cajan*.

Isolates	Amylase activity*	Cellulase activity *	Protease activity*	Pectinase activity*
MRR 11	2473.29±16.3	-	13.43±1.46	-
MRR 10	-	97.14±3.6	6.48±1.15	605.1±24.1
MRR 14	-	117.4±7.21	6.7±2.35	461.58±5.7
MRN 3	-	-	19.44±2.1	491.8±27.5
ARR 8	904.89±24.4	-	34.95±0.15	430±43.5
ARR 11	200.36±32.8	-	-	-
ARR 12	305.93±25.1	-	4.66±0.46	-
ARR 15	-	38.1±2.1	-	-
ARR 16	-	-	11±0.48	-
MCDR 8	581.7±42.4	473.71±25.7	1.8±0.09	-
ARN 17	452.43±52.9	-	-	-
MJIR 6	1443.47±33.2	-	18.65±1.8	-
MJIR 7	-	111.22±2.1	15.85±1.45	-
MJIR 8	1247.42±67.9	458.26±6.3	19.44±2.1	520.4±46.4
MJIR 9	1049.21±24.7	327.48±17.51	-	-
MJIN 16	773.44±24.3	359.74±10.82	8.56±0.22	521.4±3.3
MCDN 1	-	503.35±4.12	34.34±1.2	657.7±3.4
MJHN 1	-	-	28.86±0.26	633.7±48.4
MJHN 4	1139.7±20.8	-	24.68±1.1	-
MJHN 5	-	574.63±27.8	-	-
MJHN 10	-	-	32.39±1.9	-
MJHN 11	1021.2±64.9	492.59±20.1	-	-
MJHN 14	596.8±38.6	293.15±11.9	15.69±1.3	510.1±13.7
ARR4	533.2±18	379.7±25.9	21.1±0.59	466.6±19.5
MBR 1a	1405.77±81.9	-	-	-
MBR 4	-	423.25±30.9	18.9±0.71	672.6±22.3
MBR 6	-	-	24.58±0.76	597.1±10.5
MBR 9	598.9±35.7	484.01±13.39	10.94±1.5	621.1±21
MHN 3	441.66±3.7	13.04±2.6	11.8±0.6	503.23±29.3
MHN 12	482.6±51.8	398.5±3.1	16.19±1.12	468.9±31.5
MHN 20	-	-	27.59±0.22	-
MJiN13	404±19.4	17.51±8.23	15.8±0.34	504.4±65.5
MBN 2	1677.23±16.2	188.8±21.1	13.9±1.8	598.2±5.2
MBN 3	-	-	25.05±0.64	-
MBN 8	1496.26±34.04	461.35±19.57	26.42±1.4	-
MBN 17	-	453.8±26.26	25.9±3.9	-
MBN 17(i)	1477.9±19.5	485±8.23	29.35±2.4	-
MBN 19	-	64.9±1.03	16.4±0.23	-

\*activity measured in U/ml

29 isolates exhibited multiple (two or more) enzyme activities while ten isolates showed activity for only one enzyme. Nine isolates, namely, MJIR8, MJIN16, MJiN13, MJhN14, ARR4, MBR9, MHN3, MBN2, and MHN12 were found to produce all the four enzymes and showed significant enzyme activity as shown in Table 2. Recently, bioactive compounds and enzymes from microorganisms are attracting more attention as these are more active and stable than the enzymes sourced from animals and plants [34]. Production of hydrolytic exoenzymes by endophytic bacteria is important for their colonization in plant roots [36]. Variation in enzyme production by different isolates

is probably due to their colonization capability [37]. The isolates with no hydrolytic enzyme production ability might be using some other strategy for penetrating the plant tissue. In another study, bacteria lacking gene for plant cell wall degradation is also reported as a successful endophyte which confirms the existence of two or more strategies for entrance of endophytic bacteria [38].

### 3.3. Molecular Analysis and Identification

Nine isolates producing all the four hydrolytic enzymes were selected for identification using 16S rDNA. On the basis of ARDRA profiling,

five isolates ARR4, MBR9, MHN3, MJiN13, and MHN12 showed distinct restriction pattern. The amplified 16S rDNA products of these isolates were sequenced and BLAST analysis revealed their identity towards *Bacillus megaterium* NAP8 (MH744747), *Bacillus subtilis* NMP1 (MH744750), *Bacillus cereus* NMP2 (MH744751), *Bacillus panacihumi* NMP3 (MH744752), and *Bacillus licheniformis* (MG273753) [Table 3]. The sequences were submitted to GenBank and phylogenetic tree of closest related sequences obtained from NCBI was constructed using Neighbor-Joining method with 1000 bootstrap values [Figure 3]. The isolates which possessed all the four enzymes with significant enzyme activities belonged to *Bacillus* or its derived genera, the most abundant bacterial genera especially of the soil habitat. A variety of exoenzymes, namely, cellulases, proteases, pectinases, amylases, and lipases have been produced by genus *Bacillus* with significant enzyme activity due to its stability at varying pH and high temperature [11,39]. Several species of *Bacillus* genera, namely, *B. megaterium*, *Bacillus cereus*, *B. mojavensis*, *B. stercorophilus*, and *B. subtilis* have been involved in protease production and used as the source of commercially available proteolytic enzymes [40,41]. Cellulase production with strong enzymatic activity has been reported in many *Bacillus* sp. including *B. pumilis*, *B. cereus*, *B. licheniformis*, etc. [11,42]. Pectinase production is an important aspect of endophytic bacteria for hydrolyzing pectin of middle lamella and spreading intercellularly in plant hosts [43]. *Bacillus* sp. is predominately known

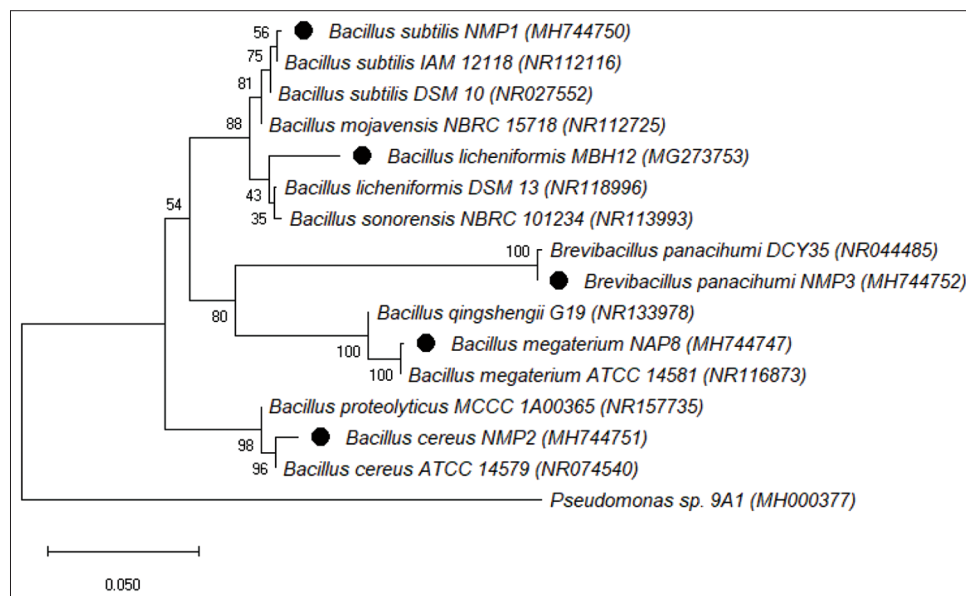
to produce alkaline pectinases which have importance in plant cell protoplast formation, retting and degumming of plant material and fruit waste treatment [44]. The extracellular amylase production by *Bacillus* sp., *B. megaterium*, *B. licheniformis*, and *B. pumilis* has also been reported in the previous studies [18,45]. Members of *Bacillus* sp. are considered as the important source of amylase production [46].

### 3.4. Plant growth promoting traits

The five isolates were able to produce ammonia as well as IAA ( $11.83 \pm 0.54$  to  $35.83 \pm 2.2$   $\mu\text{g/ml}$ ). All were producing HCN except MHN3; three isolates named MHN12, MJiN13, MHN3 were producing organic acid while isolate MBR9, MHN3, and MHN12 were producing siderophore. Only two isolates ARR4 and MBR9 were able to solubilize phosphate (Table 4). Endophytic bacteria are mostly recognized as potential plant growth promoters having various mechanisms to improve the plant growth [47]. Along with multiple enzyme production ability, these isolates also possessed four or more PGP traits. Endophytic bacteria influence the growth of plants directly by producing phytohormones, by solubilizing phosphate or fixing atmospheric nitrogen, and indirectly through producing secondary metabolites such as antibiotics, cyanide, and siderophores to inhibit pathogenic microorganisms. The endophytic actinobacteria associated with plant tissues are capable of promoting plant growth by making nutrients/substrates (e.g., phosphorous,

**Table 3:** Analysis of 16S rDNA gene sequences.

Isolate	Source plant	Source	Nearest National Center for Biotechnology Information match	Identity (%)	Query cover (%)	Endophytic bacterial strain	Accession number
ARR4	<i>Cajanus cajan</i>	Roots	<i>Bacillus megaterium</i> ATCC 14581 (NR116873)	100	99	<i>Bacillus megaterium</i> NAP8	MH744747
MBR9	<i>Vigna radiata</i>	Roots	<i>Bacillus subtilis</i> IAM 12118 (NR112116)	99	100	<i>Bacillus subtilis</i> NMP1	MH744750
MHN3	<i>Vigna radiata</i>	Nodules	<i>Bacillus cereus</i> ATCC 14579 (NR074540)	100	100	<i>Bacillus cereus</i> NMP2	MH744751
MJiN13	<i>Vigna radiata</i>	Nodules	<i>Brevibacillus panacihumi</i> DCY35 (NR044485)	99	100	<i>Bacillus panacihumi</i> NMP3	MH744752
MHN12	<i>Vigna radiata</i>	Nodules	<i>Bacillus licheniformis</i> (NR118996)	97	100	<i>Bacillus licheniformis</i> MHN12	MG273753

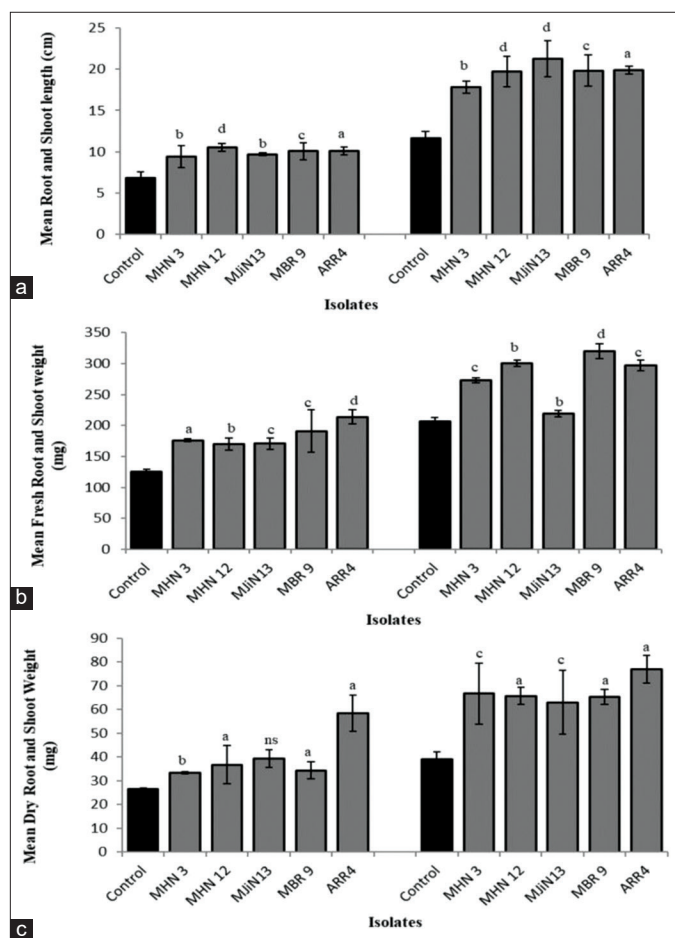


**Figure 3:** Phylogenetic tree based on 16S rRNA gene sequences of isolates with closest related sequences obtained from National Center for Biotechnology Information.

**Table 4:** Plant growth promoting traits of selected endophytic bacterial isolates.

Isolate	Ammonia production	Organic acid production	Hydrogen cyanide production	IAA production (µg/ml)	Phosphate solubilization	Siderophore production
ARR4	+++	—	+	24.9±0.51	+++	—
MBR9	+++	—	+	11.83±0.32	+	+
MHN3	++	+++	—	19.2±0.54	—	++
MJiN13	+++	+++	+++	35.83±2.2	—	—
MHN12	++	+++	++	27±0.7	—	+++

IAA values are mean of three replicates±SD. “—” means showed no production, “+” means showed low production, “++” means moderate production, “+++” means high production



**Figure 4:** Effect of endophytic bacterial isolates on various growth parameters of *Vigna radiata* plants after 60 days of sowing (a) root and shoot length (b) fresh root and shoot weight (c) dry root and shoot weight. Values are presented as mean ± SD ( $n = 4-6$ ). Significantly different values ( $P \leq 0.05$ ) are marked by different letters (a-d). “ns” represents  $P > 0.05$ , “d” represents  $P \leq 0.05$ ; “c” represents  $P \leq 0.01$ ; “b” represents  $P \leq 0.001$ ; “a” represents  $P \leq 0.0001$ .

nitrogen, and iron) accessible to the host plant, producing various plant hormones and protection from phytopathogens [48].

### 3.5. Pot Experiment

The *V. radiata* seedlings bacterized with the five selected isolates (MHN3, MHN12, MJiN13, MBR9, and ARR4) significantly enhanced all the parameters including the root (up to 1.5 folds) and shoot (up to 1.8 folds) lengths, fresh root (up to 1.7 folds), shoot weights (up to 1.6 folds), dry root (up to 2.2 folds), and shoot (up to 2.0 folds) weights as compared

with the control plants [Figure 4]. The statistical data showed that the inoculation with isolate ARR4 (*Bacillus megaterium* NAP8) resulted in significant increase in all the parameters ( $P < 0.05$ ). None of the isolate resulted in nodulation in plant roots upon inoculation indicating non-rhizobial behavior. The PGP ability of these endophytes was also reflected in pot experiment performed, resulting in significant increase in all the parameters of plant growth as compared to uninoculated control. The results were in agreement with various previous reports of endophytic bacteria [49,50]. The hydrolytic enzymes produced by endophytic bacteria have also been found able to degrade cell wall of various pathogens and thus reported to possess the biocontrol activity [51]. Therefore, the endophytic bacteria with PGP as well as hydrolytic enzymes producing abilities are excellent alternative to chemical fertilizers and pesticides [52]. All the four enzymes simultaneously produced by one or more endophytic bacteria might have better agronomic and industrial applicability than that producing single enzyme activity.

### 4. CONCLUSION

Leguminous crops provide an exclusive niche harboring diverse group of microorganisms including endophytic bacteria. The present study explored the endophytic bacteria from *V. radiata* and *C. cajan* for their economic and ecological roles in sustainable agriculture and industrial productions. On the basis of hydrolytic enzymes production and PGP traits, it can be assessed that these endophytic bacteria lyse the cell wall, loosen plant cell contacts, and enter inside the plant tissue. The bacteria multiply there and facilitate the close association with the host plant providing better yield and reduced application of agro-chemicals. Moreover, these highly efficient strains with multiple enzyme production can be harnessed as potentially favorable candidates for use in industrial field.

### 5. CONFLICTS OF INTEREST

The author declares that there is no conflict of interest.

### 6. ACKNOWLEDGEMENT

This research work was supported by financial grant (SB/YS/LS-273/2013) sanctioned by Department of Science and Technology, Government of India, New Delhi, India. NB received university research scholarship. Authors are also thankful to the Department of Microbiology, MD University, Rohtak, for providing infrastructure support.

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#### How to cite this article:

Bhutani N, Maheshwari R, Kumar P, Dahiya R, Suneja P. Bioprospecting for extracellular enzymes from endophytic bacteria isolated from *Vigna radiata* and *Cajanus cajan*. *J App Biol Biotech.* 2021;9(3):26-34.  
DOI: 10.7324/JABB.2021.9304