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Production of Potential Enzymes from Endophytic Bacteria Are Sustainable Alternative for Pharma Industries

KasimMuhtari[1], Inampudi Sailaja[2,*], Beena Kanwar Shekhawat[3], Sonia Kaura[3], Jonwal Harshita[3]

[1]Department of Biotechnology, Parul Institute of Applied Sciences, Parul University, India
[2]Department of Biotechnology, Shree Swaminarayan Science College, Swaminarayan University, India
[3]Department of Biotechnology, Institute of Medical Science and Research, Dr B. Lal Institute of Biotechnology, India

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Abstract Endophytic microbes harbored by medicinal plants cooperatively synthesize significant pharmaceutical metabolites in their host plant. The study is aimed to delve into bacterial endophytes of *Mangifera indica, Psidium guajava, Cassia occidentalis, Calotropis procera, and Hibiscus roasa-sinensa,* by identifying the important industrial enzymes they produce. Surface sterilization was achieved using ethanol, sodium hypochlorite and mercuric chloride as decontaminants. The isolates were identified based on the physiological and biochemical features they express. Then, various screening methods like Carboxymethyl cellulose, tributyrin, Carboxymethyl cellulopectinse, starch, and skim milk agar were used to screen out the strains with the ability to form a halo zone surrounding the grown culture on the agar plate as amylase, cellulase, protease, lipase, and pectinase enzymes producers. And finally, the qualitative assay of the screen isolates was performed according to the enzymes needed to be assayed, e.g., DNS method using starch as substrate for amylase, DNS using glucose as substrate cellulase, caseinase assay method for protease, DNS method for pectinase and titrimetric determination of the free fatty acids for lipase. 30 bacterial strains were isolated, more than 50% of the isolates have shown presence of cellulase, protease, pectinase and lipase as indicated by the halo zone and the whole isolates were expressed to be amylase producers except one. The Quantitative assay of the positive amylase and cellulase isolates detailed low activity while lipase, protease and pectinase positive isolates showed high activity. The study remarks that bacterial endophytes residing in five medicinal plants in this work are producers of essential metabolites applicable in pharmaceutics, medicine and agriculture.

Keywords Endophytes, Enzymes, Pharmaceutics, Industry, Medicinal Plants

1. Introduction

Endophytes are microorganisms that live internally within living plant tissues causing no substantial harm to their host. Endophytic bacteria could produce antimicrobial, antiviral plant-growth regulatory as well as insecticidal substances for enhancement of the host growth [1]. Intensification of the competitiveness possessed by the host plant Is due to the substances produced by the endophytes [2]. It's assured that endophytes are good producers of active biological products applicable for medicinal processing and agricultural purposes. Several reports concretely support the potentiality of the endophytes as good producers of anticancer composites, biologically active metabolites, immune-suppressants and biocontrol

agents. The metabolites from endophytes have great potentiality and they could be applied in several industrial processes pharmaceutically, agriculturally, and medicinally [3,4]. Endophytes are best to be recognised as a depot for unique metabolites of attainable pharmaceutical, industrial and pharmaceutical importance. Thus, endophytes are newly prospective sources of functionalized molecules significantly as vehicles for biotechnological operations [1]. As a consequence of these characteristics, endophytes are paramount in the perspective of bioprospection achievable by biotechnological means with the possibility of developing highly economical products from them [5].

Enzymes are catalysts synthesized naturally by creations mainly living to improve chemical reactions immensely and multiply as needed by life. Endophytes produce enzymes that promote plant growth [6]. They are, however, involved in whole processes crucial for sustaining a life like transcription and DNA replication, metabolism, protein synthesis, and signal transduction. Their efficiency for a specified chemical transformation has made them fundamentally unique, increasing their usage for industrial purposes. Enzymatic complexes, specifically those that can grant initiation of economically riveting energy derivation are said to be the focus of several researches [7]. An enormous repertory of commercial microbes producing enzymes has been getting lots of attention in industrial areas, payable to their exceptional functionality noticeable in lenient situations to catalysts chemically traditional [8,9]. Naturally advanced durability of enzymes produced by endophytes may be regarded as an asset in applications such as biocatalysis, and also in variable industrial aspects like cosmetics, pharmaceuticals, or detergent [10]. Among the specified enzymes derived from endophytes are pectinase by *Sclerotinia libertine* &*A. niger,* alkaline & acid enzymes by *Aspergillus oryzae* &*A. niger*, cellulase by *Trichoderma koningi,* lipase by *Rhizopus spp,* lig-ninase by *Phanerochaete chrysosporium,* diastase by *Aspergillus oryzae*, glucoamylase by *Aspergillus oryzae* &*A. niger,* proteases by *A. sojae*, & *Aflavus* [11,12].

Naturally, microorganisms are enriched with superb potentials, evidently producing a bunch of enzymes that were used commercially and agriculturally several decades ago. The technology of enzymes nowadays is microbes dependent like actinomycetes and bacteria. These likable microorganisms are greatly impressionable to manipulations genetically with generous provision for improving strain as well as for future investigation. Based on modern society, the processes of biotechnologically eco-friendly prioritize microbial enzyme recognition as a compotent tool. There is a high need for discovering novel antimicrobials receivable to the growing resistance of human and plant pathogens to the extant chemicals and drugs [13]. Many reports affirm the production of sensing compounds of antiquorum like baicalein, catechin, naringenin, phytolapicidin, and quercetin by certain endophytic species: *Bacillus, Pseudomonas, Enterobacter asburiae, Variovorax, Rhodococcus, Stenotrophomonas, Alternaria and Penicillium* which impacted positively against gram positive and gram-negative bacteria [14,15]. More so, endophytes were evidently highlighted having bioactive compounds which are currently being used in curing cancer as a remarkable replacement for chemotherapy aimed at bypassing its side effect [16]. Endophyte essentialities are expressed as good producers of insecticidal secondary metabolites [17]. A lot of endophytes have insecticidal features for numerous vital pharmacological therapeutics, and some were discovered to have potent bioactive compounds of insecticidal against agriculturally known pest and insects applicably as bioinsecticides [18].

This work is aimed at exploring the highest possible enzymes being able to be produced by endophytic bacteria isolated from different medicinal plants, which are most hopefully applicable in pharmaceutical industries. A screening of strains showing great synthetization of amylase, cellulase, pectinase, lipase, and protease were made via a generally known system "qualitative assay" and those selected were assayed quantitatively.

2. Material and Methods

2.1. Collection of the Explant

Psidium guajava, Mangifera indica, Cassia occidentalis, Calotropis procera and *Hibiscus rosa-sinensa* healthy parts, namely leaves, roots, stems, bark and flowers were selected and randomly collected at a different location from Parul Institute of applied science's biological/medicinal garden. The samples collected were carried separately inside clean plastics and quickly brought to the laboratory to maintain the freshness.

2.2. Purification and Surface Sterilisation

A sequential sterilisation technique was applied to achieve a successful isolation of the bacterial strains. Initially, freshly collected bark, flowers, leaves, roots and stems are washed under tap water for 10-15 minutes and washing in Tween 20 (a drop in 200 mL sterilised distilled water) comes after 1 minute. The plant tissues were transferred into a laminar air flow cabinet, and rinsed three times with sterilised distilled water. Commonly used sterilizing agents are ethanol 70-95% for 30 seconds to 4 minutes hydrogen peroxide 0.05-0.2%, chloride 0.02 to 0.2% for 30 seconds and sodium hypochlorite 1 to 5% for 2 to 10 minutes [19]. All of which were used differently in the treatment of the explants.

2.3. The Media Used for Bacterial Endophyte Isolation

Medium is what determines the type and number of endophytic microorganisms to be isolated from different

plant tissues, so the choice of the medium is very important. The media that goes well with bacterial endophyte isolation is nutrient agar (NA). Among other media that can be used to isolate bacterial endophytes are TSA (tryptic soya agar) and R2A [20].

2.4. Isolation of Bacterial Endophytes

Following the final rinsing of the sterilized plant tissue surfaces in the laminar air flow, Anjum and Chandra [19] steps were taken. Aseptically the surface of the stems was removed using a sterile scalpel, leaves were cut into pieces, so also the flower and the roots. The pieces were dried properly after which the pieces were implanted upon the nutrient agar plate. Each plate was independently inoculated with 2-3 small pieces of the plant organ and was incubated at 37°C to redeem the possible bacterial endophyte colonies maximally. The plates were observed between the period of 24 to 48 hrs. Morphologically, the colonies of the different bacteria observed were sub-cultured and streaked many times on nutrient agar plates to attain pure bacterial isolates. Lastly, the clarified endophytes were kept at 4°C for use.

2.5. Characterisation and Identification of Bacterial Strains

The strains of the bacteria isolated were experimentally treated in different ways such as Gram's staining for morphological characterisation and biochemical tests like Indole production test, Methyl red and Voges–Proskauer test, Citrate utilization test, Triple sugar iron test, Oxidase test, Catalase test was achieved for accurate identification of the isolates [21].

2.6. Zone Production and Screening of Endophytic Bacteria

Screening of Lipase production was done using tributyrin by the method of Mobarak-Qamsari [22]; Screening of Cellulase production was done using Carboxymethyl cellulose by the method of Teather and Wood [23]; Screening of Amylase production was done using starch by the method of Jacob & Gerstein [24]; Screening of Pectinase production was done using Carboxymethyl cellulopectinse by the method of Beg [25]; Screening of Protease production was done using skim milk by the method of Jani [26].

2.7. Quantitative Assay

Quantitative assay of amylase, cellulase, protease, lipase and pectinase production was carried out respectively by selecting broth media which were used as the production media. A loopful of selected strains were inoculated in broth media and incubated for 24 h at 37 °C. The aliquot of production medium of 25 mL was inoculated with 2% of inoculum in 100 mL Erlen-Meyer flasks. The inoculated flasks were incubated for 72 h at 37 °C. The centrifugation of the content after incubation was done at 10000 g for 10 minutes at 4 °C. At last, the supernatant was collected and used for enzyme assay [27].

2.8. Amylase Assay

Amylase was investigated by dinitrosalicylic acid (DNS) method, leaning on the estimation of reducing sugars at 560 nm [28] and using maltose as the standard [29]. One amylase unit was interpreted as the amount of enzyme per millilitre culture permeate that released one microgram of reducing sugar per minute.

2.9. Cellulase Assay

The activity of the cellulase was measured based on the method of Denison and Kohen [30] taking 1% CMC in citrate buffer (0.1 M, pH 5.0) as substrate. The reducing sugar (glucose) produced from the CMC substrate because cellulolytic activity was measured at 540 nm by DNS method with the standard known to be glucose. One cellulase unit was interpreted as the amount of enzyme per millilitre culture permeate that released 1mg glucose per minute.

2.10. Protease Assay

The activity of protease was measured by caseinase assay method [31], using tyrosine as standard. One unit of enzyme activity serves as the amount of enzyme required to liberate 1 mg of tyrosine per millilitre culture seep under standard assay conditions.

2.11. Pectinase Assay

The activity of the pectinase was measured based on the method of Denison and Kohen [30] taking 1% CMC in citrate buffer (0.1 M, pH 5.0) as substrate. The reducing sugar (glucose) produced from CMC substrate because cellulolytic activity was measured at 540 nm by DNS method with the standard known to be glucose. One cellulase unit was interpreted as the amount of enzyme per millilitre culture permeate that released 1mg glucose per minute.2.12. Lipase Assay

Lipase activity was determined based on the titrimetric determination of the free fatty acids liberated from triacylglycerols by lipase catalysed hydrolysation. The skills applied involve sample incubation of the aliquot portion of the culture Erlen-Meyer flasks after 48 h in the incubator with a loopful of the strains, centrifugation and end-point alkaline titration of the released acids or continual titration of the generated products in a pH-stat titrator. Results are reliant upon the lipase activity. The known used titrant is NaOH. Phenolphthalein was used as an indicator marking the end point of the process [32].

Olive oil was used as the substrate.

3. Results

3.1. Isolation Procedure

3.1.1. Sample Collection

The medicinal plants collected were authenticated in the Department of Botany Parul institute of applied science, Parul university.

3.1.2. Surface Sterilization

The plant's organs were treated individually with different combinations of chemical disinfectants. 75% ethanol, and 4% sodium hypochlorite were used to sterilise *Psidium guajava, Cassia occidentalis*, *Calotropis procera* and *Hibiscus rosa-sinensa*, while *Mangifera indica* was treated with75% ethanol, 4% sodium hypochlorite and 0.1% mercuric chloride followed by rinsing with sterilised distilled water three times.

3.1.3. Sterility Check

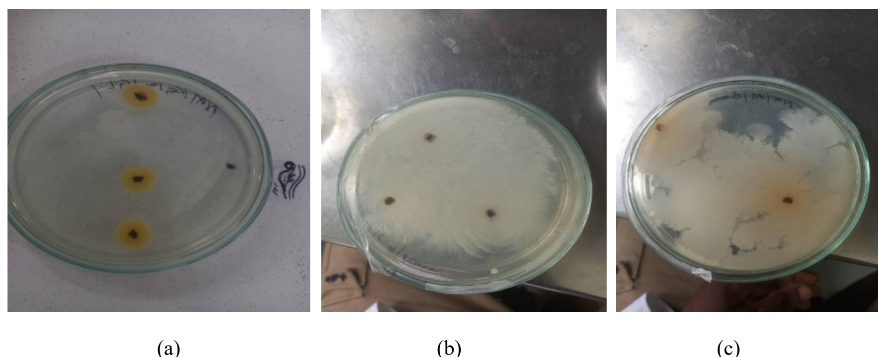
There was no appearance of microbial growth on the control plate upon growing the aliquot of water from the final rinsing on nutrient media as one of the methods of checking sterility.

3.1.4. Purification and Isolation

Nutrient Agar (NA) was the media used in isolating the bacterial endophytes in this work. After optimal incubation, variable bacteria were isolated. A total number of 30 bacteria had been purely isolated from different parts of the plants (Table 1). The purified growth of *Pseudomonas putida* COR-1 (a), *Bacillus pumilus* HBL-2 (b), and *Bacillus cereus* GL-4 (c) is shown in (Fig. 1). Streaked pure culture of the latter mentioned bacterial endophytes from the root of *C. occidentalis* (d), leaves of *H. rosa-sinensis* (e), *and* leaves of *P. guajava* (f) is shown in (Fig. 2).

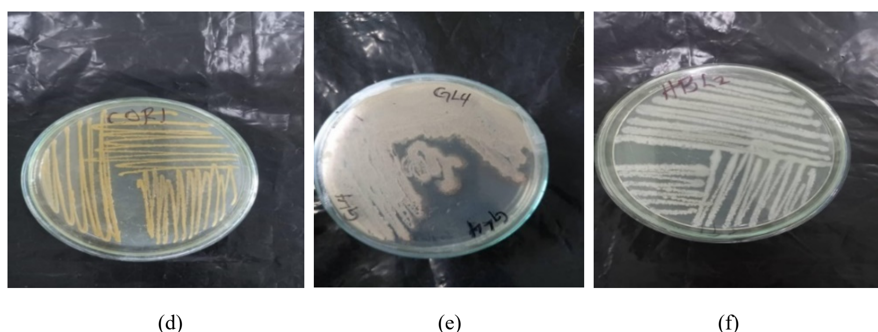
Table 1. Isolated bacterial strains (colonies) from different regions of five medicinal plants

Plants/isolates	Leaves	Stem	Bark	Root	Flower
Psidium guajava	5	1	3		
Mangifera indica	2		3		
Cassia occidentalis	3		2	2	
Calotropis procera	1	2			2
Hibiscus rosasinensa	3	1			



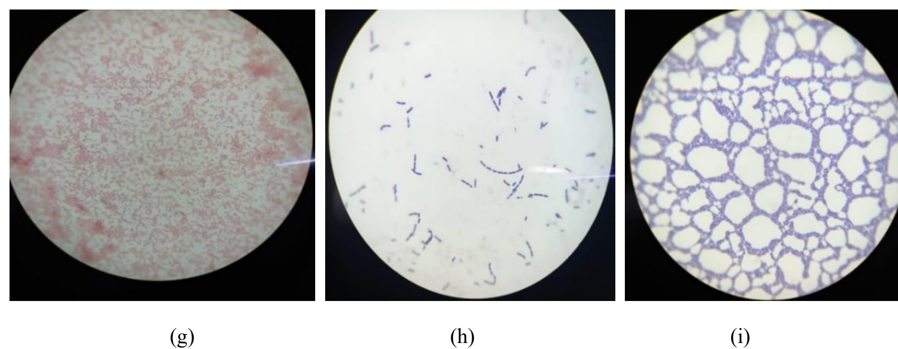
(a) (b) (c)

Figure 1. Showing pure growth of *Pseudomonas putida* (a), *Bacillus pumilus* (b), and *Bacillus cereus* (c) colonies successfully isolated from the root of *Cassia occidentalis*, *Hibiscus rosa-sinensis* leaf and Guava leaf (*Psidium guajava*), which indicated that surface sterilisation is achieved



(d) (e) (f)

Figure 2. Streaked colonies of *Pseudomonas putida* (d), *Bacillus pumilus* (e), and *Bacillus cereus* (f) after isolation from the root of *Cassia occidentalis*, *Hibiscus rosa-sinensis* leaf and Guava leaf (*Psidium guajava*) affirming the pure culture of the isolates are attained



(g) (h) (i)

Figure 3. Microscopic view of *Pseudomonas putida* (g) indicating gram negative with rod shape isolated from the root of *Cassia occidentalis, Bacillus pumilus* (h) indicating gram positive with rod shape isolated from leaf of *Hibiscus rosa-sinensis,* and *Bacillus cereus* (i) indicating gram positive with rod shape as well but different arrangement isolated from leaf of *Psidium guajava*

3.2. Morphological and Physiological Characteristics

The morphological characteristics shown by the bacteria isolated varied in coloration and spreading on the plates. Among which are white (feather-like spread), clump yellow, wide spread slightly reddish, clump white, clump dark yellow, clump off White, and white flat ascending colonies. Microscopic views of COR-1 *Pseudomonas putida* (g), HBL-2 *Bacillus pumilus* (h), and GL-4 *Bacillus cereus* (i) are shown in Fig. 3.

3.3. Gram Staining, Biochemical Test and Identification

The results of the staining of the endophytic bacteria revealed that 19 (63%) of the strains are Gram positive and 11 (36%) are Gram negative (Table 2). Biochemical tests of endophytic bacteria showed both positive and negative results of Indole test, Methyl red test, Voges prokauer test and Citrate test. 26 are catalase producers, 25 are oxidase producers, 5 are lactose fermenters while 6 are not. 18 are seen with yellow slant and 12 with red slant, while 27 are seen with yellow butt and 3 are seen with red butt, whereas none of the endophytic bacteria indicate the presence of H_2, but 3 produced gas. The identified endophytic bacteria were listed based on the results of the biochemical tests they revealed (Table 3). The entire bacterial strains isolated from the different plant parts are *Bacillus* spp. 57%, *Pseudomonas* spp. 14%, *Achromobacter* 7%, *Enterobacter* 7%, *Coccobacilli* 3%, *Klebsiella* 3%, *Siccibacter* 3%, *Citrobacter* 3%, and *Escherichia* 3% (Fig. 4).

Table 2. Gram staining of the bacterial strains isolated showing both gram positive and gram negative results

Gram positive	Gram negative
GL2	GL1
GL3	GB2
GL4	GST1
GL5	COL3
GB1	COB1
GB3	COR1
ML1	CPL1
ML2	CPF2
MB1	CPS2
MB2	HBL3
MB3	HBST1
COL1	
COL2	
COB2	
COR2	
CPF1	
CPS1	
HBL1	
HBL3	
Total = 19	**Total = 11**

Table 3. Positive and negative results of the biochemical tests, triple sugar iron results of isolates and species identified

Isolates	Ind Test	M-R Test	V-P Test	Ci Test	Ca Test	Oxi Test	Lac Test	Triple Sugar Iron Test				Identified Species
								Slant	Butt	H2S	Gas	
GL1	-	-	-	-	+	+	NLF	Y	Y	-	-	*Achromobacter xylosoxidase*
GL2	-	+	+	-	+	-	GP	R	R	-	-	*Bacillus spp*
GL3	-	+	-	-	+	+	GP	Y	Y	-	-	*Bacillus megaterium*
GL4	-	+	+	-	+	+	GP	Y	R	-	-	*Bacillus cereus*
GL5	-	+	+	-	+	-	GP	R	Y	-	-	*Bacillus pacificus*
GB1	-	+	+	+	-	+	GP	Y	Y	-	-	*Bacillus cereus*
GB2	-	+	+	+	+	+	LF	R	Y	-	-	*Pseudomonas chlororaphis*
GB3	-	-	-	-	-	+	GP	Y	Y	-	+	*Bacillus amyloliquefaciens*
GST1	-	+	-	-	+	+	NLF	Y	Y	-	+	*Citrobacter koseri*
ML1	-	+	-	-	+	-	GP	R	Y	-	-	*Bacillus spp.*
ML2	-	+	+	-	+	+	GP	R	Y	-	-	*Bacillus amyloliquefaciens*
MB1	-	-	-	-	+	+	GP	Y	Y	-	-	*Bacillus mojavensis*
MB2	-	+	+	-	+	+	GP	R	Y	-	-	*Bacillus subtilis*
MB3	-	+	+	+	+	+	GP	R	Y	-	-	*Bacillus pumilus*
COL1	-	+	-	-	+	+	GP	Y	Y	-	-	*Bacillus amyloliquefaciens*
COL2	-	+	+	-	+	+	GP	R	Y	-	-	*Bacillus pumilus*
COL3	-	+	+	+	+	-	LF	R	R	-	-	*Klebsiella terrigina*
COB1	-	+	-	-	+	+	LF	Y	Y	-	-	*Siccibacter colletis*
COB2	-	+	+	-	+	-	GP	R	Y	-	-	*Bacillus anthraciis*
COR1	+	+	+	-	+	+	NLF	R	Y	-	-	*Pseudomonas putida*
COR2	-	+	+	-	+	+	GP	Y	Y	-	-	*Bacillus cereus*
CPL1	-	+	-	+	+	+	NLF	Y	Y	-	-	*Pseudomonas gramnia*
CPF1	-	-	+	+	+	+	GP	R	Y	-	-	*E aerogenes*
CPF2	+	+	+	-	-	+	GP	Y	Y	-	+	*Escherichia coli*

Table 3 continued

CPS1	-	+	+	-	+	+	GP	Y	Y	-	-	*Bacillus oleronius*
CPS2	-	+	-	-	+	+	NLF	Y	Y	-	-	*Pseudomonas chlororaphis*
HBL1	-	-	-	-	+	+	GP	Y	Y	-	-	*Achromobacter Xylosoxidans*
HBL2	-	+	+	+	+	+	GP	Y	Y	-	-	*Bacillus pumilus*
HBL3	-	+	-	+	-	+	NLF	Y	Y	-	-	*Coccobacilli*
HBST1	-	+	+	+	+	+	LF	Y	Y	-	-	*Enterobacter spp*

Codes regarding origin GL: *Psidium guajava* leaf, GB: *Psidium guajava* bark, GBST: *Psidium guajava* stem, ML: *Mangifera indica* leaf, MB: *Mangifera indica* bark, COL: *Cassia occidentalis* leaf, COB: *Cassia occidentalis* bark, COR: *Cassia occidentalis* root, CPL: *Calotropis procera* leave, CPF: *Calotropis procera* flower, CPS: *Calotropis procera* stem, HBL: *Hibiscus rosa-sinensa* leaf, HBST: *Calotropis procera* stem, IND: Indole, M-R: Methyl red, V-P: Voges prokaeur, Ci: Citrate, Ca: Catalase, Oxi: Oxidase, Lac: Lactose, NLF: Non Lactose Fermenters, LF: Lactose Fermenters, GP: Gram Positive, Y: Yellow and R: Red

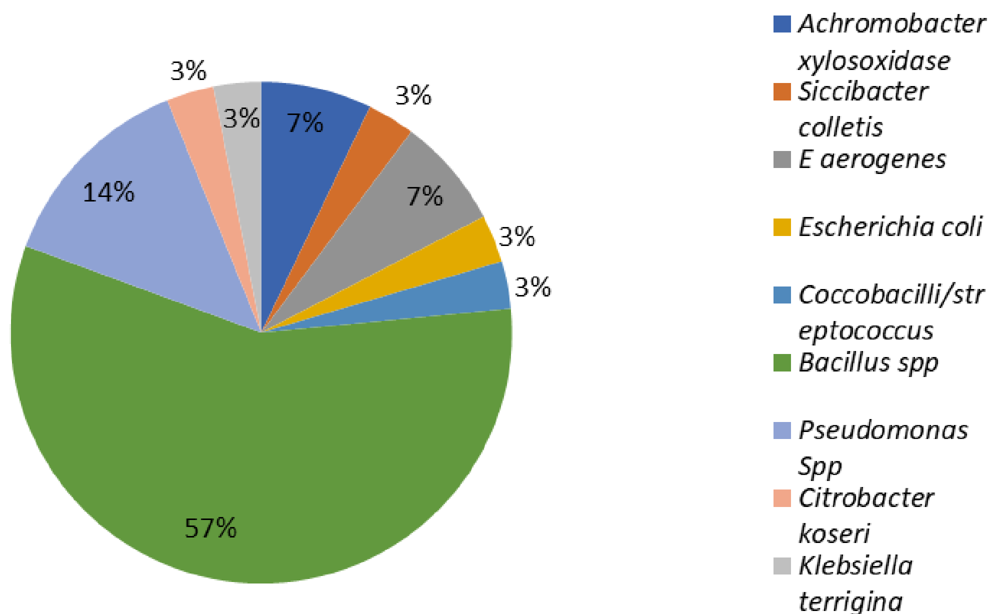


Figure 4. Species percentage of the entire bacterial strains isolated from the different plant parts as identified

3.4. Zone Production and Enzyme Activity

Zone production based on the enzymatic production by the isolates was analysed in percentage (%). Amylase (35%) was seen to be produced by the highest number of the isolates followed by cellulase (19%), protease (18%), pectinase (15%) and lipase (13%) as the lowest (Fig. 5).

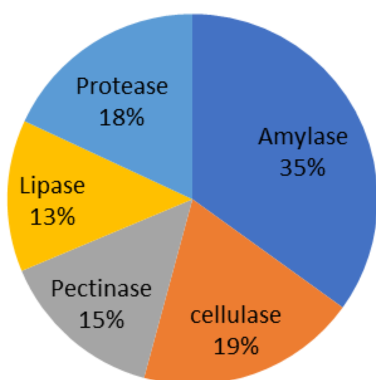


Figure 5. Percentage of the enzymatic activity of the entire bacterial strains isolated from the different parts of plants as based on zone production

3.4.1. Amylase

All the endophytic bacteria isolated showed a significant sign of starch utilization by producing a clear halo zone surrounding their colonies except COB-1 (*Siccibacter colletis*), and zones observed interpret the presence of amylase. 14 amylase producers were selected for assaying their enzymatic activity by the DNSA method. Maltose was used as standard to obtain the amount of reducing sugar (mg/ml) (Fig. 6), which the enzymatic activity was calculated by using the formula below.

$$\text{Enzyme activity} = (\text{mg/ml} \times 1000 \times \text{df}(10)) / (\text{MW of glucose} \times \text{time} \times \text{vol of enzymes})$$

The amylase activity of the selected endophytic bacterial strains was determined independently, and the amylase being released was presented as units/ml. GL-1 (*Achromobacter xylosoxidase*) showed remarkable amylase activity 0.18 U/ml as the highest, followed by GST-1 (*Citrobacter koseri*) and GB-2 (*Pseudomonas chlororaphis*) with similar activity of 0.15 U/ml and 0.14 U/ml, while the remaining isolates showed moderate or low activity ranging from 0.01 U/ml – 0.14 U/ml (Fig. 7).

3.4.2. Protease

Casein agar degrading method resulted in a clear zone on 16 plates surrounding their colonies testifying that they are protease producers. All the 16 endophytic bacteria were selected for further assay (Folin and Ciocalteu method) to know the amount of protease released by each strain as its activity showed variable activities. Tyrosine standard curve was used in obtaining the activity of the protease (Fig. 8). The formula used to calculate the protease produced by the isolated strains is

$$\text{Enzyme activity} = (\mu\text{mol of Tyr} \times \text{reaction volume}) / (\text{Sample vol} \times \text{reaction time} \times \text{vol assayed})$$

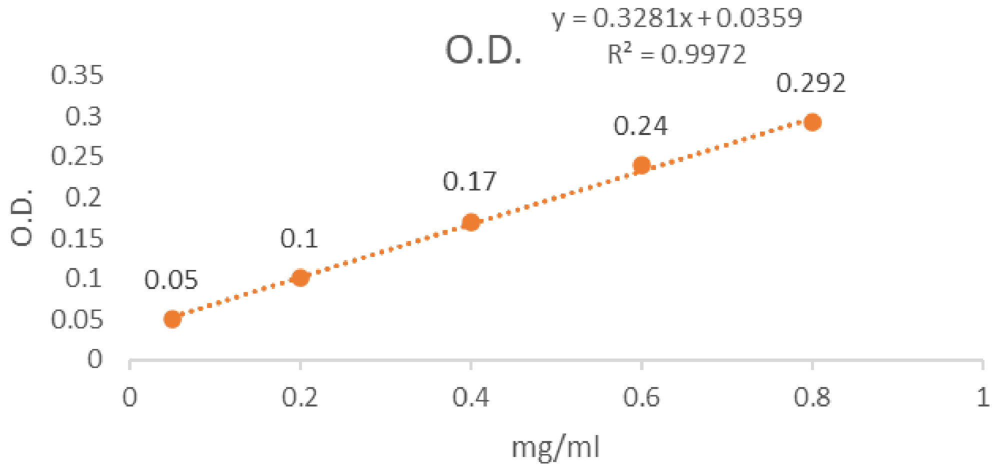


Figure 6. Maltose standard curve calibration based on the amount of maltose sugar reduced

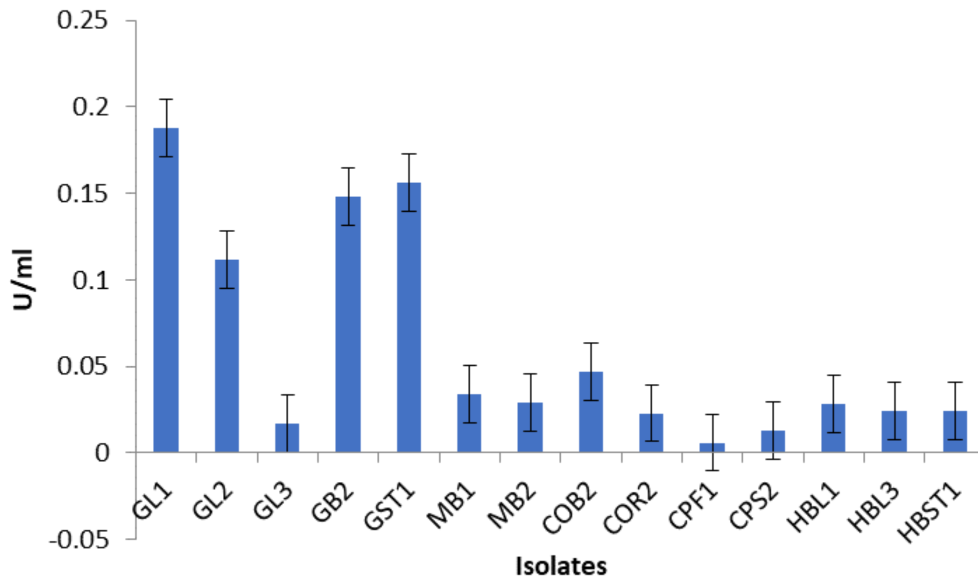


Figure 7. Result showing variable amylase production possessed by 14 bacterial strains isolated from *Psidium guajava, Mangifera indica, Cassia occidentalis, Calotropis procera, Hibiscus rosa-sinensa*

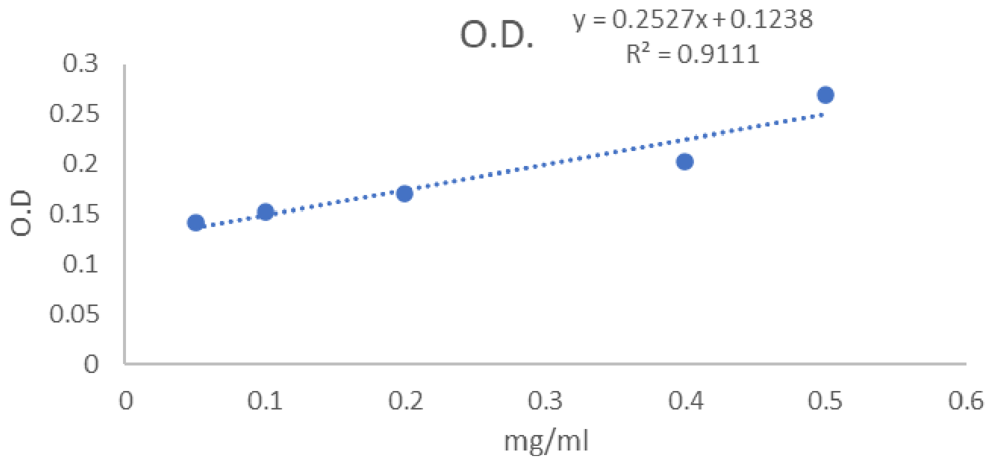


Figure 8. Tyrosine standard curve based on the absorbance of tyrosine solution at 660 nm

ML-1 (*Bacillus spp.*) 0.206 U/ml and MB-2 (*Bacillus subtilus*) 0.152 U/ml showed the highest significant activity while GL-2 (*Bacillus spp*), COB-2 (*Bacillus anthraciis*), HBL-1 (*Achromobacter xylosoxidans*), and CPF-2 (*Escherichia coli*) showed very low activity. CPS-1 (*Bacillus oleronius*), COR-2 (*Bacillus cereus*), MB-3 (*Bacillus pumilus*), COL-2 (*Bacillus pumilus*), CPF-2 (*Escherichia coli*), GL-5 (*Bacillus pacificus*), ML-2 (*Bacillus amyloliquefaciens*), and COL-3 (*Klebsiella terrigina*) showed moderate activities ranging from 0.070 U/ml – 1.400 U/ml (Fig. 9).

3.4.3. Cellulases

16 isolates showed clear zones around their colonies and all were assayed quantitatively in minimal medium with cellulose being the only carbon source using glucose standard (Fig. 10). The formular used in calculating the cellulase activity is as follows:

$$\text{Enzyme activity} = (\text{mg/ml x 1000 x df (10)}) / (\text{MW of glucose x time x vol of enzymes})$$

The results according to DNSA method show GL-4 (*Bacillus cereus*) and GL-2 (*Bacillus sp*) evinced with the highest cellulase activity as 0.112 U/ml and 0.97 U/ml, followed by MB-3 (*Bacillus pumilus*) 0.092 U/ml, GST-1 (*Citrobacter koseri*) 0.073 U/ml, COL-2 (*Bacillus pumilus*) 0.071. HBST-1 (*Enterobacter spp*) 0.040, CPF-1 (*Enterobacter aerogenes*) 0.042 U/ml, COL-3 (*Klebsiella terrigina*) 0.042 U/ml, COL-1 (*Bacillus amyloliquefaciens*) 0.044 U/ml, ML-1 (*Bacillus spp.*) 0.041 U/ml, GB-2 (*Pseudomonas chlororaphis*) 0.040 U/ml possessed very low activities but higher than ML-2 (*Bacillus amyloliquefaciens*) 0.031 U/ml, MB-2 (*Bacillus subtilis*) 0.030, HBL-2 (*Bacillus pumilus*) 0.035 U/ml, COB-2 (*Bacillus anthraciis*) 0.034, COR-1 (*Pseudomonas putida*) 0.037 (Fig. 11).

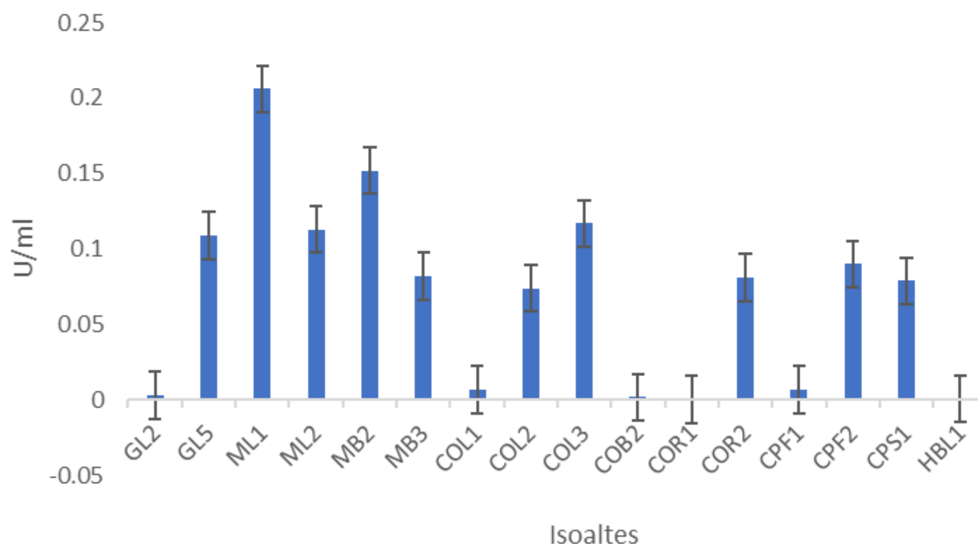


Figure 9. Variable protease production possessed by 15 bacterial strains isolated from *Psidium guajava, Mangifera indica, Cassia occidentalis, Calotropis procera, Hibiscus rosa-sinens*

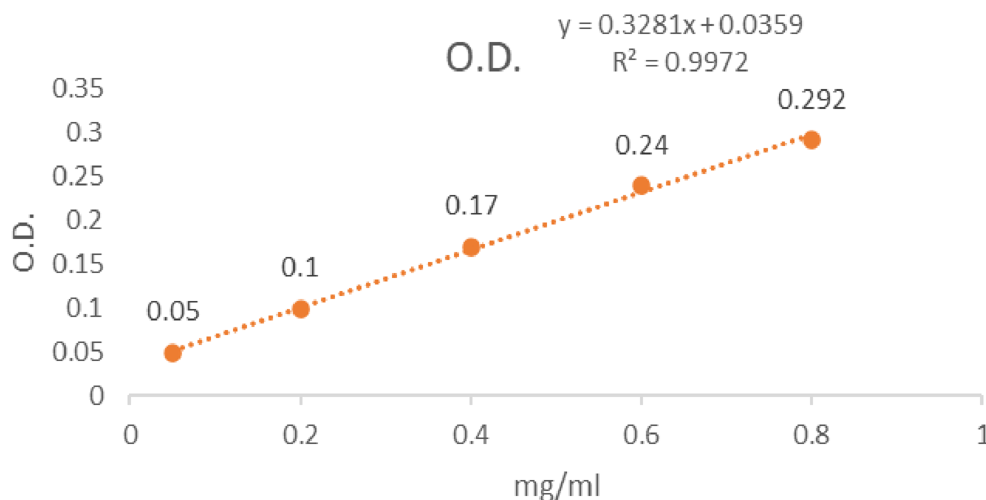


Figure 10. Glucose standard curve calibration based on the amount of glucose sugar reduced

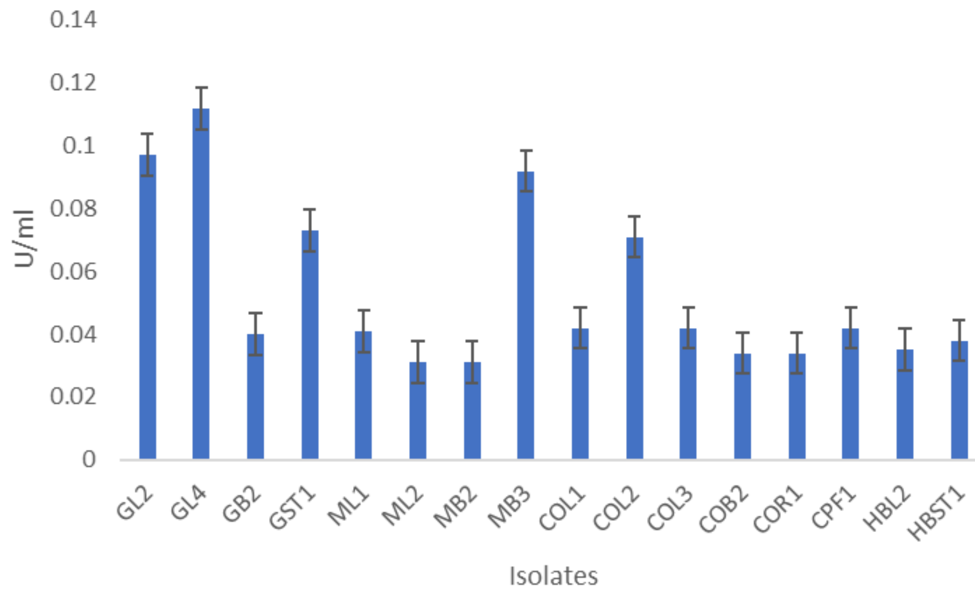


Figure 11. Variable cellulase production possessed by 16 bacterial strains isolated from *Psidium guajava, Mangifera indica, Calotropis procera, Hibiscus rosa-sinens*

3.4.4. Lipases

11 endophytic bacteria from the isolated strains were capable of forming a clear zone surrounding their colonies. Only 6 among the lipase producing endophytic bacteria were selected for the determination of their lipase activity using Cherry and Crandall. The formula used to calculate the activity of the lipase is:

$$\mu\text{mol fatty acid/ml sample} = (\text{ml NaOH for sample} - \text{ml NaOH for blank}) / 5 \text{ x N x } 1000$$

The titrimetric determination of the free fatty acids liberated from triacylglycerols by lipase catalysed hydrolysation showed GL-3 (*Bacillus megaterium*) has the highest activity releasing 150 U/ml enzyme, COB-1 (*Siccibacter colletis*) with slight variation released 110 U/ml enzyme. GB-2 (*Pseudomonas chlororaphis*) 10 U/ml was seen to be the lowest, while GB-3 (*Bacillus amyloliquefaciens*) 60 U/ml was seen with activity higher than GST-1 (*Citrobacter koseri*) 40 U/ml and COR-1 (*Pseudomonas putida*) 30 U/ml (Fig. 12).

3.4.5. Pectinases

GL-2 (Bacillus spp), GL-4 (Bacillus cereus), GL-5 (Bacillus pacificus), GB-2 (Pseudomonas chlororaphis), GB-3 (Bacillus amyloliquefaciens), GST-1 (Citrobacter koseri), ML-1 (Bacillus spp.), HBL-2 (Bacillus pumilus), COL-1 (Bacillus amyloliquefaciens), COL-2 (Bacillus pumilus), COL-3 (Klebsiella terrigina), and CPF-1 (E aerogenes) showed a clear zone surrounding their colonies. Three (3) isolates were chosen among the pectinase producers for further assaying of their activities. The formula used to come up with the activity of each bacterial isolate is:

$$\text{Enzyme activity} = (\text{mg/ml x } 1000 \text{ x df } (10)) / (\text{MW of glucose x time x vol of enzymes })$$

All the tested isolates exhibited very high activity as observed. GST-1 (*Citrobacter koseri*) was seen with the highest activity 1.344 U/ml followed by GB3 (*Bacillus amyloliquefaciens*) 1.141 U/ml and CPF-1(*E aerogenes*) has 1.12 U/ml as the lowest (Fig. 13).

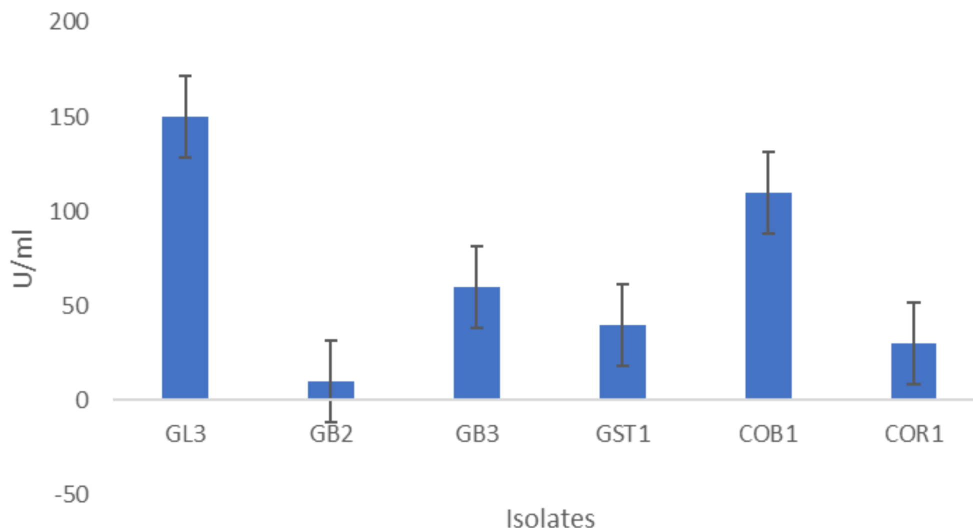


Figure 12. Lipase produced by 6 bacterial strains isolated from different parts of *Psidium guajava* (leaf, bark and stem) and *Cassia occidentalis* (bark and root)

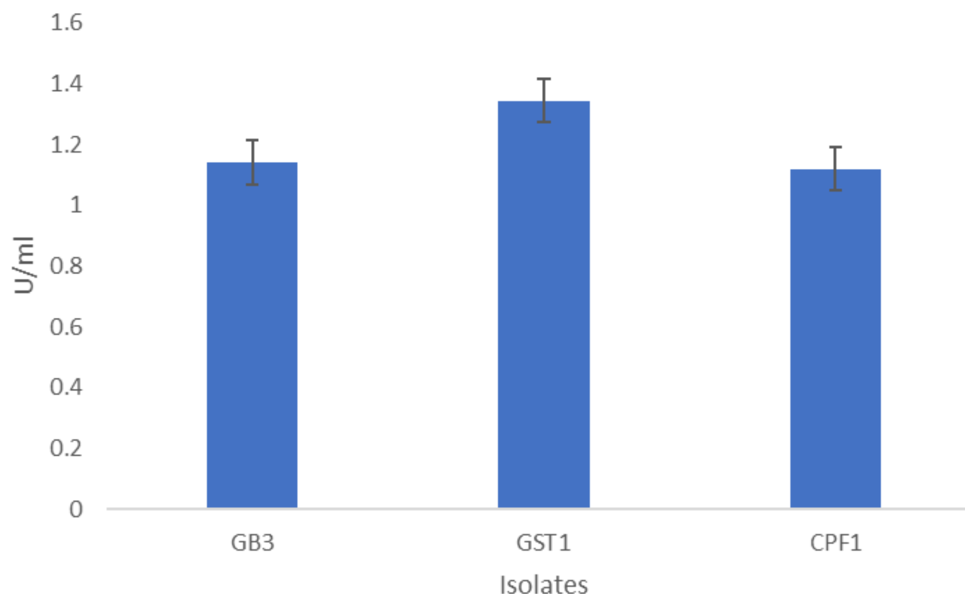


Figure 13. Pectinase produced by 3 bacterial strains isolated from *Psidium guajava* (leaf and stem) and *Calitropis procera* (flower)

4. Discussion

These investigations described the existence of different genera of culturable bacterial endophytes. Thirty endophytes were isolated from different medicinal plants of *Psidium guajava L, Mangifera indica, Cassia occidentalis, calotropis procera and Hibiscus rosa-sinensis.* Based on the morphological physiological and biochemical characteristics results the bacteria isolated fell into categories of numerous genera: Bacillus, Pseudomonas, Enterobacter, Achromobacter, Klebsiella, Escherichia, Siccibacter and Coccobacilli. 35 bacterial strains were realised to have been isolated from f *C. roseus, O. sanctum, M. arvensis,* and *S. rebaudiana* [20]. Another report by Beiranvand et al [33] express 23 bacterial endophytes was isolated from 23 medicinal plants in Iran after which the samples were surface sterilised and crushed. The number of bacteria we isolated is not the same as between [19-33]. This signifies a new consideration of the possible number of bacterial endophytes from 5 different medicinal plants in this work. Moreover, Bacillus displayed the highest number of isolates compared to the remaining genera isolated. The preponderance of Bacillus genus over Micrococcus, Pseudomonas, Stenotrophomonas, Agrobacterium, Microbacterium, and Staphylococcus being isolated from ginseng (*Panax ginseng C.A. Meyer*) has been reported by Vendan et al [34]. Bacillus genus was seen as the only isolated endophyte from *Teucrium polium L.* [35]. The frequent domination of the genus Bacillus has also been reported [36,37,38]. Bacillus is among the commonly found bacterial endophytes [35,39]. Our findings tally with reports

expressing Bacillus as the most dominant endophytic bacteria from plants but differ in the isolated genera of species.

Microbial endophytes are capable of producing secondary metabolites known to be produced by medicinal plants. They can also restrict pathogen incursion into plants through direct or indirect techniques. A competition is seen between endophytes and phytopathogens describing direct mechanisms, where the pathogens have been restricted due to inhibitory metabolites secreted by endophytes. The inhibitory metabolites produced by endophytes are promising feature reliably for controlling pathogens. Endophytic bacteria synthesize different bioactive metabolites that can be used in medicine, controlling pathogens and growth promoting. Half of the new drugs taken to the market are produced by endophytes [40]. They are used as anticancer, antioxidants, antimicrobial agents, insecticides and antidiabetic. Indirect mechanism describes the stimulation of plant's immune system by endophytes thereby increasing the plant's resistance against phytopathogens by upregulating defence genes [41]. Enzymatic activities also describe indirect mechanisms shown by endophytes for defence mechanisms. Our results described that isolated endophytes produced amylase, cellulase, protease, pectinase and lipase enzymes to be processed in the pharmaceutical industry [42,43]. The penetration of endophytes into plant tissue and initiation of symbiotic association between endophytic microbes and host plants is leaned to the hydrolytic actions of their enzymes. Proteolytic and amylolytic enzymes of endophytes are studied to enhance several industrial processes. An endophytic bacteria *Streptomyces* sp. Isolated from grevillea (*Grevillea pteridifolia*) fern-leaved was reported to produce Dzkakadumycin Adz and Dzechinomycindz which describes it as producer of potent antibiotics. Kakadumycin A is basically linked to a quinoxaline antibiotic, echinomycin and expresses better bioactivity towards Gram-positive bacteria than echinomycin as well as amazing activity against *Plasmodium falciparum* (malaria parasite) in human [44].

Carrim et al [27] expressed the enzymatic activity of bacterial endophytes from *Jacaranda decurrens* and determined their production % as: protease (60%), amylase (60%), and lipase (40%). In 2011, Castillo et al [44] presented a result that revealed 50% cellulolytic among proteolytic, amylolytic, esterolytic, lipolytic and asparaginase bacterial endophytes from *Azadirachta indica, Curcuma longa, Musa paradiasiaca*, *Eucalyptus globules, Pongamia glabra, Vinca rosea* and *Withania somnifera.* In 2021, Dogan and Taskin [45] reported a different frequency of 6 potential enzymes produced by bacterial endophytes isolated from Poaceae Plants, and lipase was revealed with highest production percentage (lipase 75%, protease 65%, Amylase 56%, cellulase 31%, pectinase 21% and Xylanase 9%). Our findings expressed amylase with the highest number of endophytic bacterial strains, followed by cellulase, protease, pectinase and lipase (Fig. 5). Regardless of the related studies being limited, the identified isolates we observed with enzymatic production ability varied due to their high species diversity.

Contrarily, our isolates differ from the isolated bacteria by Carrim et al, Dogan and Taskin [27,45] due to the differences of the medicinal plants used. So, our findings bring more endophytic bacteria which are enzyme producers to awareness. Moreover, CPF-1(*E* aerogenes) from *Calotropisprocera* (flower) is expressed to produce all the enzymes assayed expressing high activity in pectinase assay. CPF-2 (*Escherichia coli*) differs from CPF-1 (*Enterobacter aerogenes*) due to its inability to produce pectinase. And CPL-1 (*Pseudomonas gramnia*) was detected only to produce amylase (Fig. 5).

5. Conclusions

This work concludes by revealing the cruciality of the bacterial endophytes residing within medicinal plants (*Psidium guajava, Mangifera indica, Cassia occidentallis, Calotropis procera* and *Hibiscus rosa-sinens*) as good producers of potent biotechnological enzymes (amylase, cellulase, protease, lipase and pectinase). Lipase and pectinase were seen to be the highest enzymes produced by these bacterial endophytes. *Bacillus* spp. revealed to be predominant and the best genus based on the high activity they showed. Potential enzymes produced by our isolates could be used for numerous pharmaceutical industrial purposes due to the wide diversity of endophytic bacterial species and adaptation to various environments. Endophytes could be the best alternative source of multiple medicinal products considering their promising features to withstand pathogenic invasion, drought, salinity, heavy metals and even low pH. Also, their metabolites are toxically less to both humans and animals.

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