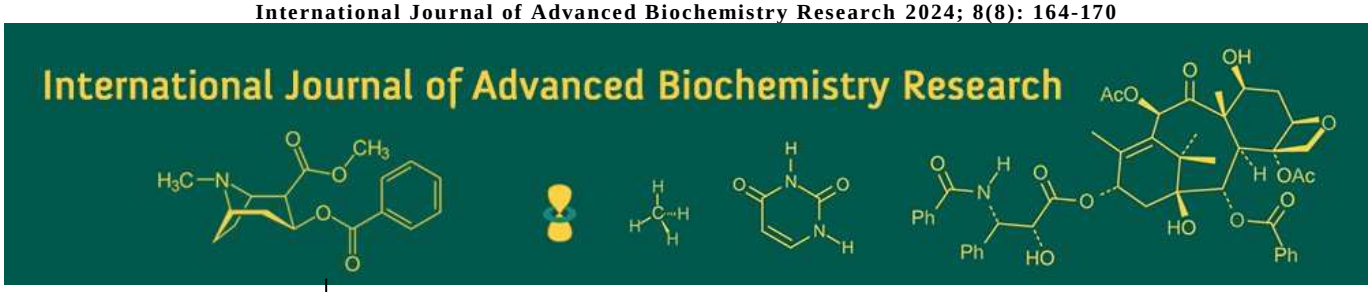

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Qualitative and quantitative estimation of enzymes produced by endophytic bacteria of medicinal plants

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Abstract
Endophytes are microorganisms that reside in plant tissues and cause no evident harm to their host. Medicinal plants have developed importance in the field of research, as well as in the production of biomolecules utilized in pharmaceutical businesses. This research includes isolating and characterizing 17 endophytic bacteria from five medicinal plants: *Moringa oleifera*, *Murraya koenigii*, *Annona muricata*, *Piper betel* and *Justicia adhatoda*. The isolates showed a variety of morphological characteristics and were tested for extracellular enzyme synthesis, which included chitinase, cellulase, and protease. Quantitative enzyme testing indicated considerable differences in enzyme activity, with prominent isolates such as N1 and S4 showing high specific activity. The findings emphasize the potential of these bacterial endophytes for biotechnological use.

Keywords: Medicinal plants, Endophytic bacteria, extracellular enzymes

Introduction
Endophytes, known as the largest unexplored reservoirs forming a "ware house of natural bioactive compounds on the earth" (Nicolaou *et al.*, 1994) [1]. Endophytes are micro-organisms (bacteria and fungi) colonizing themselves in living, interior tissues of host plants without causing any overt negative effects to the host (Bacon and White, 2000) [2]. Endophytes are ubiquitous, found in every plant and shows vast biodiversity (Strobel, 2003) [4]. Endophytes show a great biodiversity of adaptations, developed in special and sequestered environments (Owen and Nicholas, 2004). This endophytic diversity may be similar with the diversity of their host plant. The most common endophytes are fungi and bacteria, but comparatively fungi are the more commonly isolated and studied endophytes (Strobel, 2003) [4]. Medicinal plants are an important resource of isolating endophytic bacteria, which can induce secondary metabolite of very important value. There are reports on numerous new endophytic species may exist in medicinal plants, it follows that endophytes are important components of microbial biodiversity (Zhang *et al*, 2009) [5].
Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties. In recent years, antimicrobial properties of medicinal plants are increasingly reported from different parts of the world. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. However, very little information is available on such activity of medicinal plant. The broadly accepted definition of endophytes that was given by (Bacon and White, 2000) [2] is microbes that colonize intra and intercellular plant tissues without having any negative effects to the host plant. As endophytes reside within plant tissues, they are exposed to a more specific and stable habitat than rhizospheric bacteria (Bafana, 2012) [6].
Studies are conducted to optimize the effectiveness of the decontamination procedures for the isolation of endophytic bacteria from *J. adhatoda*, *P. betel*, *M. koeniqii*, *A. muricata*, *M. oleifera* and to evaluate the microscopic and biochemical properties of the isolate in order to characterize them in different groups.

Materials and Methods
A. Experimental Materials
Description of Sampling Procedure

Sample Collection

The fresh and healthy leaves of five different medicinal plants: *J. adhatoda*, *M. koenigii*, *A. muricata*, *M. oleifera*, *P. betel* were collected from Department of Biotechnology, Junagadh Agricultural University, Junagadh. Each sample was tagged and placed in separate polythene bags and processed within 24 hours of collection. Fresh plant materials were used for isolation of endophytic bacteria to reduce the chance of contamination.

Isolation of Endophytic Bacteria from Medicinal Leaves Samples

Sterilization Procedure of Sample

For the pre-treatment of leaf samples and isolation of endophytic bacteria all the leaf samples were excised and subjected to a surface sterilization procedure described by Arunachalam and Gayathri (2010) [7].

Sample Inoculum

Leaves were crushed in sterile distilled water using mortar and pestle and plant extracts were prepared. About 1 ml of crushed samples were serially diluted and 0.1 ml was spread onto nutrient agar (NA) medium. Plates were incubated at 35 °C for 2-3 days.

B. Characterization of Isolates

Isolated endophytic bacteria were phenotypically characterized for growth characteristics on Nutrient agar, colony morphology and Grams reaction by using standard procedures following Anjun and Chandra (2015) [8].

Cultural Characteristics

Cultural characteristics of the different bacterial isolates were studied by observing and recording different growth parameters *viz*., size, shape, elevation, margin, texture, opacity and pigment about colonial characters.

C. Microscopic Characteristics

Gram's Staining

Each culture was created as a thick smear on a clean glass slide, which was then heat dried for a short while by passing over a burner. After applying 13 drops of crystal violet stain, the smears were allowed to sit at room temperature for one minute. After that, a mild tap water wash was used to get rid of the crystal violet stains. After that, the smears were incubated for 30 to 60 seconds at room temperature with 1-3 drops of gram's iodine applied. Following gram's decolorizer and 1-3 drops of safranine for counterstaining, the smears were incubated for 30 to 60 seconds at room temperature before being gently cleaned with tap water. After that, the slides were left to dry overnight at room temperature and examined under a light microscope.

D. Extracellular Enzyme Production Test

Qualitative Chitinase Enzyme Test

The standard method used by Singh *et al*. (2021) [9], was modified to perform the qualitative chitinase enzyme production test. The preparation of Minimal Media (abbreviated as MM) involved dissolving 0.5% colloidal chitin, 0.05% $MgSO_4.7H_2O$, 0.03% KH_2PO_4, 0.07% K_2HPO_4, 0.0001% $MnCl_2$, 0.001% $FeSO_4.7H_2O$, 0.0001% $ZnSO_4$, and 4.5 g of agar in 1000 ml of distilled water to a final pH of 7.0. The mixture was then autoclaved for 20 minutes at 120 °C and 15 psi, and then poured into autoclaved plates. Colloidal chitin preparation involved dissolving 10 g of chitin in conc. HCl and shaking the mixture for 30 minutes. After adding 200 cc of ethanol, the shaker was once more left overnight. Colloidal chitin in the form of precipitate was periodically rinsed with distilled water till pH rose up to 7. The following day, one liter of distilled water was added and centrifuged at 13000 rpm for 20 minutes at 4 °C temperature.

Screening for chitinase activity involved spot-inoculating isolates onto chitin agar plates, which were then incubated for three to five days at 30 °C. Positivity is seen in the clear zone surrounding the endophytes colony. To conduct the additional quantitative chitinase enzyme assay, these positive endophytes were employed.

Quantitative Chitinase Enzyme Assay

By using a colorimetric technique that Setia and Sohorjono (2015) [10] used with three replications, chitinase activity was ascertained. The reaction mixture contained 200 mM potassium phosphate buffer (pH 6.0), 1 milliliter of crude enzyme, and 2 milliliters of 1.25% (w/v) colloidal chitin substrate. After two hours of incubation at 30˚C, the mixture was heated for ten minutes to halt the reaction, cooled down to room temperature in a cold water bath, and one unit of β-N-Acetylglucosaminidase (NAGase) was added. It was then centrifuged for twenty minutes at 8000 rpm. Following the aforesaid centrifugation, 1 ml of the test supernatant was combined with 1.5 ml of freshly made color reagent solution, which was made by combining 96 mM DNSA (3,5-Dinitrosalicylic Acid) reagent with 5.3 M sodium potassium tartrate solution. The mixture was then diluted to 40 ml using deionized water. After five minutes of boiling, the test supernatant and color reagent solution were allowed to cool to room temperature. Next, at 540 nm, the quantity of GlcNAc (N-acetylglucosamine) released was determined. Plotting the GlcNAc standard curve between GlcNAc absorbance and GlcNAc concentration. The amount of enzyme that can release 1.0 mg of GlcNAc from chitin substrate per hour under reaction conditions is known as one unit of chitinase enzyme activity (U).

The protein content in isolates were determined by Folin-Lowry method using BSA as standard. Data of chitinase enzyme activity, specific activity and protein content was analyzed with single factorial CRD analysis of variance (α = 0.05) using excel sheet.

Calculations

Standard Curve: ΔA540nm Standard = A540nm Std - A540nm Std Blank

Plot the ΔA540nm of the standards versus milligrams of NAG released.

Sample Determination: ΔA540nm Sample = A540nm Test - A540nm Test Blank Determine the milligrams of NAG liberated using the standard curve and calculate the chitinase enzyme activity (U ml^{-1} hr^{-1}) and its specific activity (U mg^{-1} hr^{1}) defined per mg of protein estimated in isolates using the following formula:

$$\text{Chitinase activity (U ml}^{-1}\text{ hr}^{-1}\text{)} = \frac{\text{(mg NAG released) (3 + Volume of NAGase}^{3}\text{)}}{\text{(2 x 1 x 1)}}$$

Where,
3 = Initial reaction volume of assay
2 = Conversion factor for converting 2 hours to 1 hour as per the unit definition
1 = Volume (ml) of supernatant used in colorimetric determination
1 = Volume (ml) of crude enzyme used
Volume of NAGase = 0.5 ml
Specific activity (U mg^{-1} hr^{-1}) = Enzyme activity (U ml^{-1} hr^{-1}) / Protein content (mg ml^{-1})

Qualitative Cellulase Enzyme Test

To identify the isolate strains exhibiting positive cellulase activity for additional quantification of the enzyme produced by positive strains, qualitative cellulase analysis was carried out as recommended by Lodi *et al*. (2023) [11]. Using a medium consisting of 3 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, 1% cellulose powder, and 1.5% agar, pH 7.0, the test was conducted by inoculating bacteria into it and then incubating it at 30 °C or 25 °C for 24 hours or 7 days. The plates were then saturated with 0.1% Congo red, and after 20 minutes, the presence of a translucent zone was examined.

Quantitative Cellulase Enzyme Assay

The activity of cellulase was assayed using DNSA method followed by Lay Mg Mg *et al.* (2015) [12]. To make a 0.05 M citrate buffer (pH 4.8) solution, 1 ml of culture supernatant was combined with 1 ml of 1% cellulose substrate in test tubes. An 80–85 rpm water bath shaker was used to incubate the resultant reaction mixture for 60 min. at 50 °C. The reaction was stopped in a vigorously boiling water bath by adding 3 millilitres of DNSA reagent to the reaction mixture and heating it for precisely 5 minutes after the reaction period. After that cool in a cold-water bath, then record the absorbance which was measured by spectrophotometer at 540 nm against the blank without enzyme filtrate. Anhydrous glucose was used as the standard (Adney and Baker, 1996) [13]. The cellulase enzyme activity and specific activity were then determined by calculating with the following formula:

$$\text{Cellulase activity (U ml}^{-1}\text{ min}^{-1}\text{)} = \frac{\text{mg of glucose released x 5 x 0.5)}}{\text{(1 x 2 x 60)}}$$

Where,
5 = Total volume of assay (ml)
0.5 = Dilution factor (DF)
DF = (Vol^m of enzyme extract / Vol^m of enzyme + buffer)
1 = Volume of enzyme extract used (ml)
2 = Volume of reaction mixture taken in cuvette (ml)
60 = Incubation time (min)
Specific activity (U mg^{-1} min^{-1}) = Enzyme activity (U ml^{-1} min^{-1}) / Protein content (mg ml^{-1})

Qualitative Protease Enzyme Test

The culture media used for the qualitative protease enzyme production test contained 3% nutritional gelatin, 0.8% nutritional broth, 0.5% casein, 0.01% MnCl2, 4 g of agar, and 1.2 ml of 20% glycerol. The mixture was then incubated at 37 °C for 24 to 72 hours in a shaking incubator (150 rpm). The isolates that demonstrated positive growth in the broth used to produce protease after incubation were classified as protease-producing strains. The cells were harvested after growing for 72 hrs at 10,000 rpm for 15 min., and the resulting supernatant was utilized as a crude enzyme for a quantitative analysis.

Quantitative Protease Enzyme Assay

The protease enzyme assay was carried out using Cupp-Enyard's (2008) [14] non-specific protease assay technique from Sigma. Each isolate was tested three times, with one test tube used as a blank. Each of the four test tubes received five milliliters of 0.65% casein solution and were equilibrated for five minutes at 37 degrees Celsius. One milliliter of enzyme extract was then added to three test tubes, leaving the blank empty. The mixtures were incubated at 37 °C for ten minutes to assess protease activity and tyrosine liberation. To stop the reaction, 5 ml of 110 mM TCA reagent was added after incubation, followed by another 30-minute incubation.
Tyrosine standard dilutions were prepared by adding various amounts of 1.1 mM tyrosine stock solutions to six test tubes and incubating them for 30 minutes. After incubation, solutions were filtered using a 0.45 µm polyethersulfone syringe filter. Following that, 2 ml of filtrates from test isolates and the blank were transferred to new test tubes, and 5 ml of sodium carbonate and 1 ml of Folin's reagent were added. After mixing and incubating at 37 °C for 30 minutes, a color gradient corresponding to tyrosine concentration was observed.
A spectrophotometer was used to measure the absorbance of standards, standard blanks, test isolates, and test blanks, using a light path of 1 cm. The standard curve was created by charting the micromoles of tyrosine in each standard on the X-axis and the absorbance changes on the Y-axis. These data points were used to construct a slope equation and a best-fit line.
The absorbance difference between the test isolate and the test blank was used to compute the tyrosine produced during the proteolytic reaction using the slope equation. In addition, the protein content was evaluated using Lowry's technique, with bovine serum albumin (BSA) as the standard. The protease enzyme and specific activity were then determined by calculating with the following formula:

$$\text{Protease activity (U ml}^{-1}\text{ min}^{-1}\text{)} = \frac{\text{µ mol of tyrosine equivalents released x 11}}{\text{(1 x 10 x 2)}}$$

Where,
11= Total volume of assay (ml)
10 = Time of assay (min) as per the unit definition
1 = Volume of enzyme used (ml)
2 = Volume taken in cuvette for colorimetric determination
The specific activity was determined by the same formula used in chitinase and cellulase enzyme.

Qualitative Lipase Enzyme Test

The lipase activity of bacterial isolates was determined by the diffusion agar methods, where nutrient agar medium was supplemented with 0.01% CaCl2.H2O. Tween 80 sterilized for 20 min at 120 ºC was added to the molten agar medium at 45 ºC to give a final concentration of 1%. The medium was shaken until the Tween 80 had dissolved completely

and then was poured onto Petri plates. An opaque halo formed around the colonies was considered as positive test.

Qualitative Gelatine Enzyme Test

Inoculate the gelatine media agar plates with the endophytic bacterial isolates using sterile techniques. Streak for isolation if necessary. Using a sterile inoculating loop, transfer a small amount of bacterial growth from each nutrient agar plate onto separate gelatine agar plates. Incubate the gelatine agar plates at the appropriate temperature for 48-72 hours. After the incubation period, examine the gelatine agar plates for zones of clearing around the bacterial colonies. Clearing around bacterial colonies indicates gelatinase production, suggesting the ability of the bacterium to degrade gelatine.

Qualitative Starch Enzyme Test

The qualitative amylase enzyme production test for the bacterial isolates were grown on particular media including starch agar medium for amylase activity (soluble starch-20 g/l, peptone-5 g/l, beef extract- 3 g/l, agar- 15 g/l and distilled water. The test was conducted by inoculating bacteria into it and then incubating it at 30 °C or 25 °C for 48 hours or 7 days. The plates were then flooded with Gram's dye solution. The presence of halos around the colonies were indicative amylase production.

Results

Procurement of Leaves Samples

Endophytic microorganism isolated from five different medicinal plants Five different medicinal plants: *J. adhatoda*, *M. koenigii*, *A. muricata*, *M. oleifera* and *P. betel* were collected from the Department of Biotechnology, College of Agriculture, Junagadh Agricultural University, Junagadh.

Isolation of Endophytic Bacteria from Medicinal Plants Samples

Nutrient agar media has been used for the isolation of endophytic bacteria and different colonies have been recovered after culture plate. About 17 bacterial endophytes are isolated in pure form from five medicinal plants and their respective isolation codes are given below: *Moringa oleifera* (Drumstick) produced isolates S1, S2, S3, S4, and S5. *Murraya koenigii* (curry leaves) produced isolates C1, C2, and C3. *Annona muricata* (Soursop/Hanuman phal) produced isolates H1, H2, and H3. *Piper betel* (Nagarvel) produces isolates N1, N2, and N3. Finally, *Justicia adhatoda* (Adulsa/Vasaka) produced isolates A1, A2, and A3.

Characterization of Isolates

Isolated endophytic bacteria were phenotypically characterized for growth characteristics on Nutrient agar, colony morphology and Grams reaction by using standard procedures in accordance with Panigrahi *et al*. (2018) [15].

Cultural Characterization

In vitro multiplications of bacterial isolates were carried out on nutrient agar plates and the colonial characteristics were recorded in terms of size, shape, elevation, margin, texture, opacity and pigment. All 17 isolates showed typical endophytic like colonies on the medium after 24 hr of incubation. The colony morphologies of the isolates ranged from irregular to circular in shape, majority with small to medium in size with flat to raised and convex type of elevations and some of them were pigment producing. The colonial pigmentation of the isolates included pale yellow, creamy, shiny, watery and white. Most of the colonies had entire to irregular margins. Few of them also had undulate margins. These findings are similar to the findings of Anjun and Chandra (2015) [8]. The cultural characteristics of all the isolates is presented in Table 1.

Microscopic Characterization

Microscopic characterization of the isolates was carried out by gram's staining for cellular morphological characterization. Different isolates displayed different cell sizes and morphologies when viewed under the microscope. The cells ranged from coccus to short or thin long rod shape in single and bacilli in organization while few were also found to be filamentous in structure. The microscopic characterization by gram's staining has been shown in the Table 2.

Extracellular Enzyme Production Test

Qualitative Chitinase, Cellulase, Protease, Lipase, Gelatinase and Amylase Test

The results of production of chitinase, cellulase, protease, Lipase, gelatine and Amylase enzyme by the isolates are presented in Table 3. From the given table, it is obvious out of seventeen only seven, five, nine, eleven, seven and six isolates were capable of producing respectively chitinase, cellulase, protease, lipase, gelatine and starch enzyme qualitatively tested in the respective enzyme production agar media as described in section E. These results shows that the isolates were capable of degrading the colloidal chitin, carboxymethyl cellulose and proteose peptone as substrates provided in the respective enzyme production tests.

Quantitative Chitinase Assay

The data about the results of quantitative chitinase enzyme assay as performed by the method described in section E is presented in Table 3. The standard curve obtained by plotting the varying concentration of N-acetylglucosamine against their corresponding absorbance at 540 nm is depicted in Fig 1. The determination of BSA standard curve for estimation of protein content has been shown in Fig. 2.

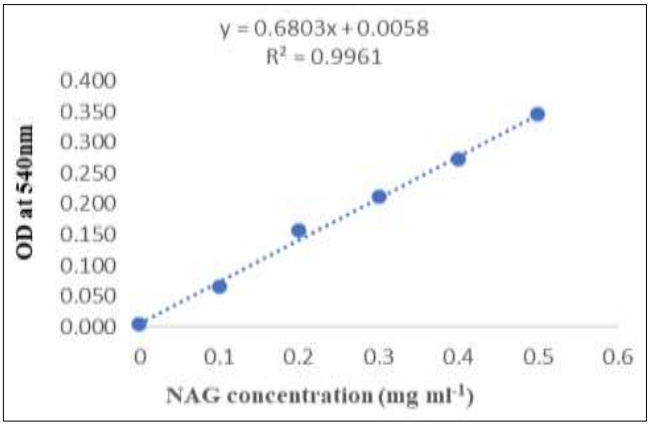

Fig 1: Determination of N-acetylglucosamine standard curve for estimation of chitinase activity

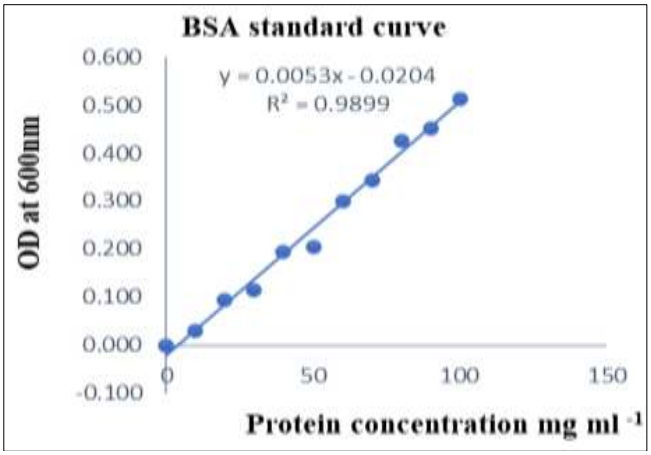

Fig 2: Determination of BSA standard curve for estimation of protein content in isolates

From Table 4., it is clear that chitinase enzyme activity shown by isolates ranged from 0.539 to 0.615 U ml-1 hr-1 while their corresponding specific activity ranged from 0.017 to 0.053 U mg-1 hr-1. The highest chitinase and specific activity was observed in isolate N1 while lowest in isolate S2 and A1 (Fig 3 and 4.). On the other hand, the protein content in isolates ranged from 11.45 to 32.43 mg ml-1. The highest protein content was produced by isolate A1 while lowest by isolate N1.

The statistical analysis of variance (ANOVA) was found with greater value of calculated F than that of table F at both 1 and 5% level of significance revealing the high level of significance.

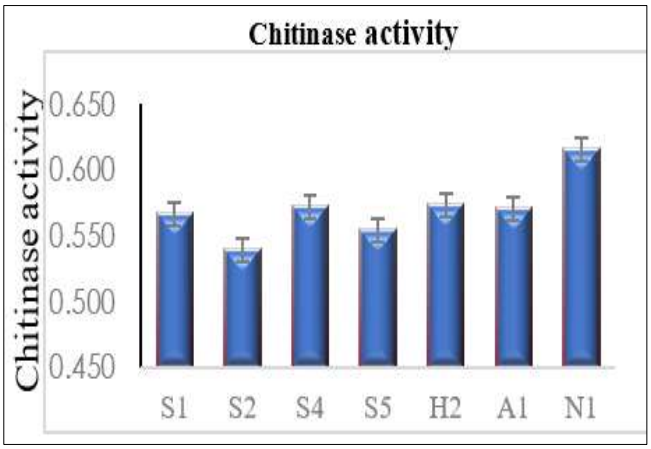

Fig 3: Chitinase activity of endophytes bacterial isolates (U/ml/hr)

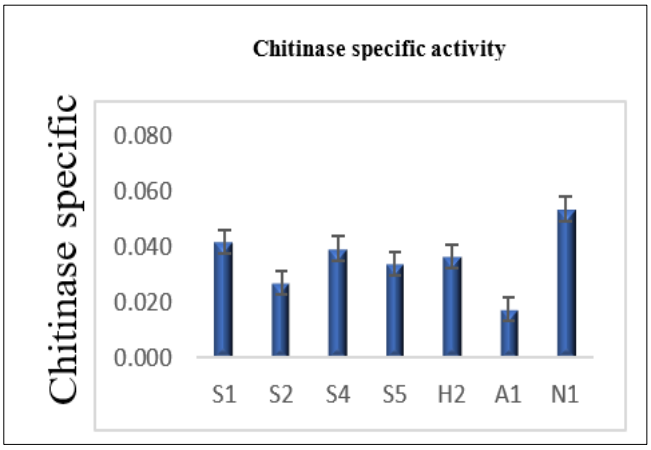

Fig 4: Specific activity of chitinase enzyme of isolates (U/mg/hr)

Quantitative Cellulase Assay

The results of quantitative cellulase enzyme assay as performed by the method described in section E are presented in Table 5. The standard curve obtained by plotting the varying concentration of anhydrous glucose against their corresponding absorbance at 540 nm has been shown in Fig 4. The determination of BSA standard curve for estimation of protein content is plotted in Fig. 2. From Table 5, cellulase enzyme activity of isolates ranged from 1.866 to 5.777 U ml^{-1} min^{-1} while their corresponding specific activity ranged from 0.115 to 0.262 U mg^{-1} min^{-1}.

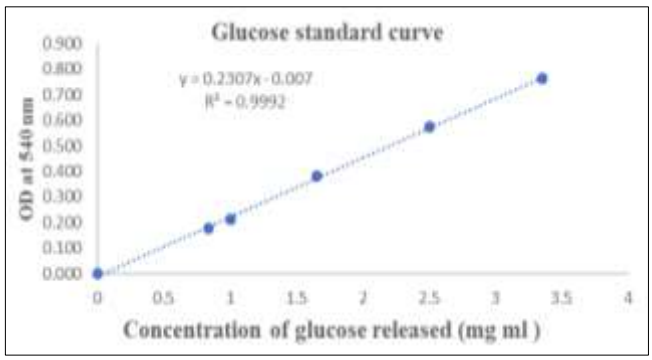

Fig 5: Determination of glucose standard curve for estimation of Cellulose activity

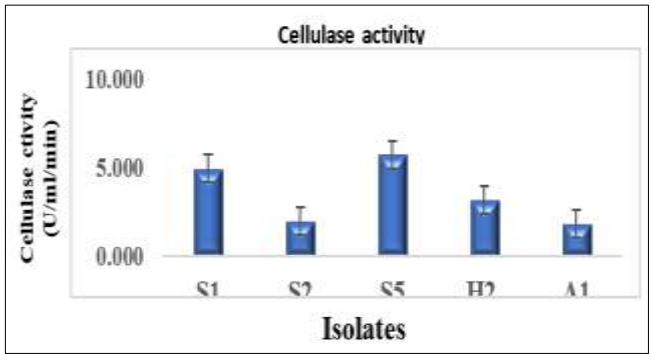

Fig 6: Cellulase activity of endophytes bacterial isolates (U/ml/hr)

The highest cellulose and specific activity was observed in isolate S4 while lowest in isolate H2 (Fig 5 and 6). On the other hand, the protein content in isolates ranged from 14.352 to 22.088 mg ml^{-1}. The highest protein content was produced by isolate S4 while lowest by isolate S2. The statistical analysis of variance (ANOVA) was found with greater value of calculated F than that of table F at both 1 and 5% level of significance revealing the high level of significance. Fig. 4. Determination of glucose standard curve for estimation of cellulase activity.

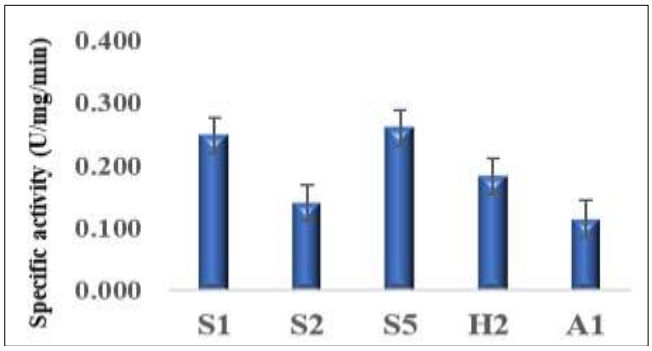

Fig 7: Specific activity of cellulase enzyme of isolates (U/mg/hr)

Quantitative Protease Assay

The data pertaining to results of quantitative protease enzyme assay as performed by the method described in section E are presented in Table 6. The standard curve obtained by plotting the varying concentration of tyrosine against their corresponding absorbance at 540 nm are presented in Fig 7. The determination of BSA standard curve for estimation of protein content has been shown in Fig. 2.

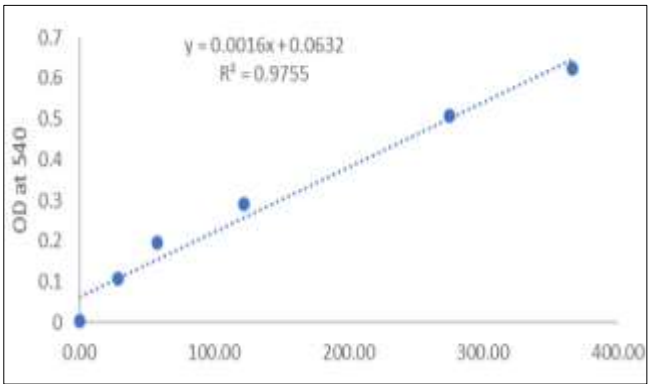

Fig 8: Determination of tyrosine standard curve for estimation of protease enzyme activity

Table 6., depicts protease enzyme activity shown by isolates ranged from 1.171 to 320.61 U ml^{-1} min^{-1} while their corresponding specific activity ranged from 1.171 to 9.112 U mg^{-1} min^{-1}. The highest protease and specific activity N3 and S5 were observed in isolate N1 while lowest in isolate 25A (Fig. 8 and 9). On the other hand, the protein content in isolates ranged from 24.91 to 84.22 mg ml^{-1}. The highest protein content was produced by isolate A1 while lowest by isolate S4 The statistical analysis of variance (ANOVA) was found with greater value of calculated F than that of table F at both 1 and 5% level of significance revealing the high level of significance Endophytic bacteria isolated from medicinal plants have shown significant potential in producing extracellular enzymes such as protease, chitinase, and cellulase.

These enzymes have wide-ranging applications in industries including pharmaceuticals, agriculture, biocontrol, and bioremediation. The qualitative screening.

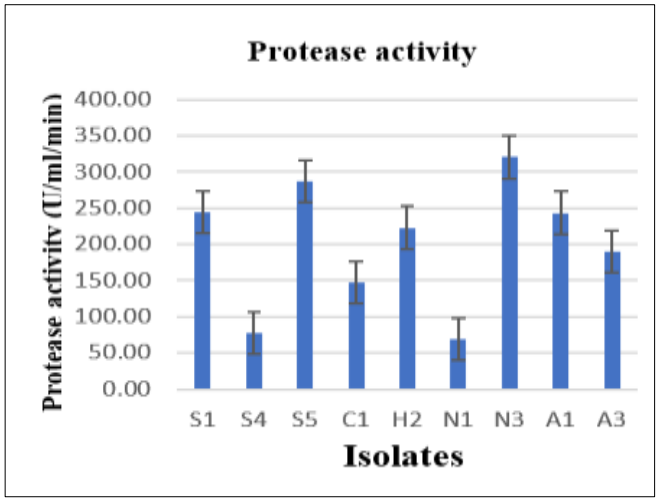

Fig 9: Protease activity oendophytes bacterial isolates (U/ml/hr)

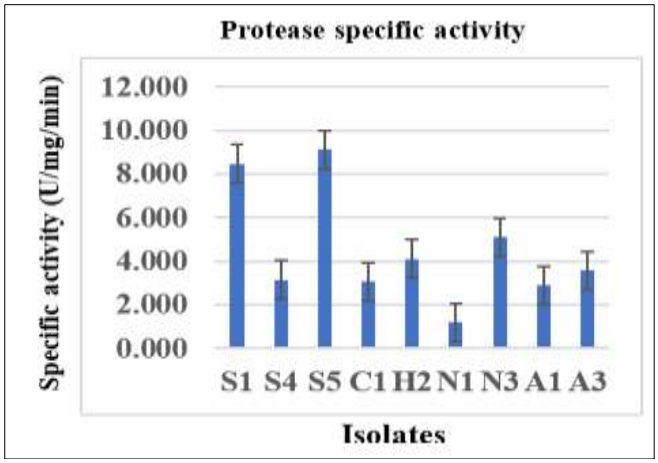

Fig 10: Specific activity of protease enzyme of isolates (U/mg/hr)

methods, such as clear zone formation on specific agar plates, provide initial evidence of enzyme activity. Quantitative assays like the casein digestion assay for protease, DNS assay for chitinase, and filter paper assay for cellulase are essential for determining the specific activity levels of these enzymes.

Conclusion

This study successfully isolated and characterized 17 endophytic bacteria from five medicinal plants: *M. oleifera*, *M. koenigii*, *A. muricata*, *J. adhatoda* and *P. betel*. The isolates exhibited diverse morphological characteristics, including variations in colony size, shape, opacity, pigment, margin, elevation, and texture. Microscopic characterization via Gram's staining revealed a range of cell shapes from cocci to rods and filamentous forms, with both Gram-positive and Gram-negative reactions observed

The isolates demonstrated significant potential in producing extracellular enzymes, with qualitative and quantitative assays confirming the presence of chitinase, cellulase, protease, lipase, gelatinase, and amylase. Notably, chitinase activity ranged from 0.539 to 0.615 U ml-1 hr-1, cellulase activity from 1.866 to 5.777 U ml-1 min-1, and protease activity from 1.171 to 320.61 U ml-1 min-1. The highest specific activities were observed in isolates N1, S4, and N3 for chitinase, cellulase, and protease, respectively. These findings highlight the biotechnological potential of endophytic bacteria from medicinal plants in producing industrially significant enzymes, contributing to advancements in pharmaceuticals, agriculture, biocontrol, and bioremediation.

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