Nig. J. Biotech. Vol. 41(1): 97-109 (June 2024)
ISSN: 0189 1731
Available online at
http://www.ajol.info/index.php/njb/index
and https://bsn.org.ng
DOI: https://dx.doi.org/10.4314/njb.v41i1.11

NIGERIAN JOURNAL OF BIOTECHNOLOGY

Extracellular Enzymatic Activities of Endophytic Bacteria Isolates Obtained from *Dioclea reflexa* Hook Seeds

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Abstract

Seeds endophytes play crucial roles in enhancing plants' health and productivity by conferring protection, improving fitness and resilience, and enhancing nutrient uptake. These endophytes are also used for environmental management and production of bioactive compounds for various biomedical applications. In recent years, endophytes are of interest as potential sources of biocatalysts because of their unique properties. In this study, endophytic microbes were isolated from the seeds of an underutilized tree climber, *Dioclea reflexa* Hook, the isolates were biochemically characterized using standard protocols and screened to produce some hydrolytic enzymes of biotechnological importance. Seven distinct Isolates were biochemically characterized. High enzymatic activities were observed from amylolytic enzymes screened in this study. Cellulolytic activities or enzymes were undetected in the isolates at Day 0, which slightly increased in some of the isolates at 24- and 48-hour fermentation period. The highest activities were observed from isolate DRFH102 as 4.64 U/ml at 24-hour for amylase, 0.45 U/ml at 48-hour for cellulase, 1.7 U/ml at 48-hour for Lipase, 2.82 U/ml at 48-hours for β-Amylase, 4.5 U/ml for 24-hours for protease, 3.6-3.8 U/ml between 0-48 hours at glucoamylase and 5.7 U/ml at 24-hour for β-1, 3-glucanase. The isolate (DRFHIS02) with the highest range of enzyme secretion was molecularly identified as *Bacillus subtilis*. This study reveals that endophytic bacteria from *Dioclea reflexa* Hook seeds can be another alternative source of hydrolytic enzyme production for various industrial and biotechnological applications. These enzymes can further be studied to understand their biochemical properties.

Keywords: Endophytes, hydrolytic enzymes, bacteria, amylase, *Bacillus* spp, *Dioclea reflexa*
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Introduction

Endophytes belong to a class of beneficial free-living microorganisms found inside the leaves, fruits, roots, cell walls, stem and seeds of plants generally referred to as plant tissues (Wang et al., 2021). This class of microorganisms can be fungi or bacteria (Malfanova et al., 2013; Samreen et al., 2021), and has been found to play an endo-symbiotic relationship with their host plant by conferring resistance or improving the stress tolerance level of such plant without causing any noticeable harm to the plant (Santoyo et al., 2016). While the plant increases its productivity by protecting the microbes and proving nutrients for them this relationship often improves the health of the plants (Harman et al., 2021).

Seeds are often infected with seed-borne pathogens which can reduce their germination rates and prevent their growth into seedlings

(Gitaitis and Walcott, 2007; Kim et al., 2022). This solemnly leads to a heavy loss in the yield of seedlings and planting materials. Recent studies have revealed that the presence of endophytes in seeds has improved their development into seedlings before their introduction into the soil where their growth is sustained/enhanced by soil microbiota (Kim et al., 2022). Endophytic bacteria have been known to target pathogens and pests through secretions, production of phytohormones (Myo et al., 2019), breaking down complex compounds in either plant roots, seeds, leaves, stems, bark, or flowers to ensure there is no nutrient limitation in such plants. They are also known for ensuring drought stress resilience in several seeds such as millet, and *Zea mays* (Siddique et al., 2022). These actions ensure that the plant has a primed defense mechanism. These endophytes have been reported to be a good source of hydrolytic enzymes (Afzal et al., 2019).

Hydrolytic enzymes are a class of biocatalysts with tremendous applications in various biotechnological industries, this class of enzymes breaks down lipids, starch and carbohydrates, cellulose, fats, nucleic acids, and proteins (Prabha et al., 2013) into their simplest monomers or units for easy applications (Ogundolie, 2021). They are of great importance in various industries ranging from pharmaceutical industries, medical industries where they can be used for analytical, diagnostic and agricultural purposes. They are also essential tools in food (Yadav, 2017), biofuel, medical, baking (Ogundolie, 2015) and beverage industries. These hydrolytic enzymes include lipase, amylase, protease, xylanase, cellulase, pectinase and have been reported to be obtained from different plant parts (Carrim et al., 2006; Dogan and Taskin, 2021).

The marble Vine (*Dioclea reflexa* Hook), a leguminous plant that belongs to the family of Fabaceae (Ogundolie, 2015; Ahmad et al., 2016). This marble plant is an underutilized climber; a leguminous plant native to southern America, south tropical, and Western parts of Africa (Oladosu et al., 2010; Ogundolie et al., 2022). The seed of this plant has been reported for various purposes such as dietary, decorative, and medicinal (Ogundolie, 2015) due to its rich bioactive compounds. Medicinally, bioactive compounds from these seeds have been documented for the management of breast (MCF-7) cancers by Balapangu et al. (2021). Other applications include hepatoprotective activities (Iliemene, & Atawodi, 2014), anti-inflammatory activity (Pinto-Junior et al., 2016), vasorelaxant properties (Pinto-Junior et al., 2017), pharmaceutical excipient (Builders et al., 2012; Mbah et al., 2022). This seed has also been reported for the production of proteins and enzymes such as mannose-specific lectin (Pinto-Junior et al., 2016) and β-amylase (Ogundolie, 2015 and Ogundolie et al., 2022). To the best of our knowledge, there is little information on hydrolytic enzymes activities during fermentation of *Dioclea reflexa* Hook seeds (DRFHS) using its endophytic microbes. With an increasing demand for newer sources of enzymes of biotechnological importance, this explores and reports the first attempt to isolate endophytic bacteria from DRFHS and screen them for the production of some industrially important hydrolytic enzymes.

Materials and Methods

Dioclea reflexa Hook seeds (DRFHS) were obtained from Igbokoda International Market, Ondo State, Nigeria (Caring Heart)(GPS coordinates- 6.3517, 4.80229), Soluble starch, 3,5-dinitro salicylic acid (DNSA), Starch Potato, Soluble (S2630), Sodium Acetate, Trihydrate (S8625), BSA (bovine serum albumin), Monohydrate (M5885), Sodium Potassium Tartrate, Tetrahydrate (S2377), 3,5-Dinitrosalicylic Acid (D0550), Maltose (Sigma–Aldrich Chemical Company, St. Louis, MO, USA), PCR Master mix (OneTaq® 2X Master Mix with Standard Buffer (M0482)) (NEB, USA).

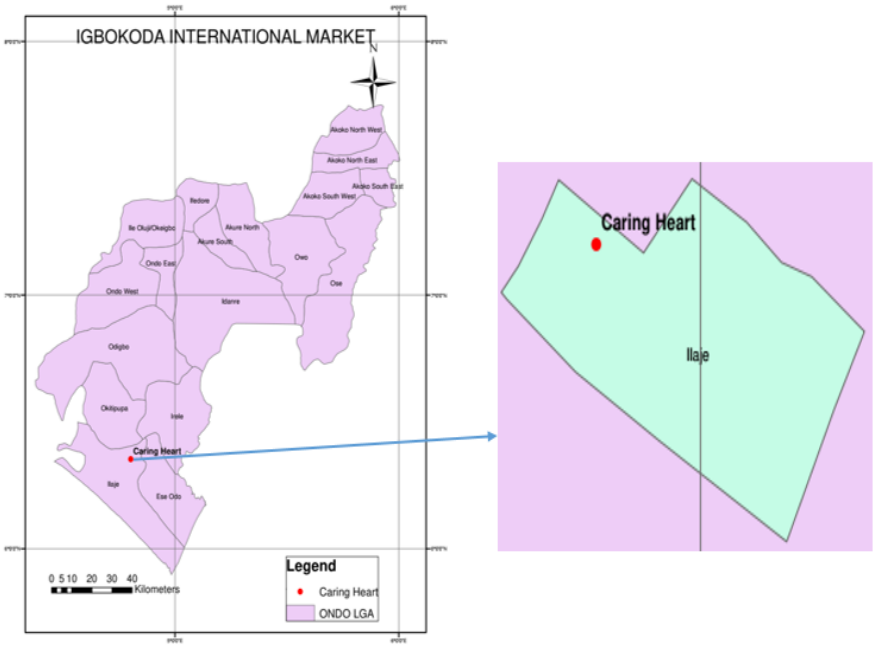

Fig 1: Geographical location of *Dioclea reflexa* Hook obtained from Igbokoda International Market (Caring Heart- GPS coordinates- 6.3517, 4.80229)

Sample Preparation and Surface sterilization

This was carried out according to the method of Acuña et al. (2023) with slight modification. *Dioclea reflexa* Hook seeds were soaked in a solution containing 4% Sodium hypochlorite (NaOCl) for 10 minutes with constant shaking. The seeds were transferred into a sterile 1000 mL beaker and washed with autoclaved distilled water (ADW) before introducing into a solution containing 70% ethanol for one minute. Then the seeds were thoroughly washed thrice using ADW and transferred into 70% ethanol solution for one minute, then rewashed with ADW. To confirm the seeds were sterile, one ml aliquot of the last, autoclaved distilled water rinse was serially diluted and plated on Nutrient agar (NA) plates and no visible growth was observed.

Isolation and Enzyme Screening of Endophytic Bacteria

To isolate the endophytic bacteria, the seeds were aseptically opened and ground into a fine powder using a sterile mortar and pestle. One gram of the seed powder was weighed for serial dilution and the appropriate dilution was plated on NA agar plates and incubated at 27 degrees for 3 to 5 days. Isolates were observed and further sub-cultured to obtain pure cultures. The pure cultures were further characterized biochemically. Each pure isolate was individually inoculated into nutrient broth for 48 hours at 150 rpm, aliquots were taken at 24-hour intervals and enzymes of biotechnological importance were screened from the supernatant. Isolate with the best enzyme production was later molecularly identified. (All procedures and assays unless otherwise stated were carried out in triplicate).

Protein concentration Determination

The total protein concentration of the supernatants obtained from the respective isolates was determined according to the method of Bradford (1976), using BSA as the protein standard.

Determination of Alpha Alpha-Amylase Activity

The alpha-amylase assay was determined as described by Ogundolie (2022). A reaction mixture containing 0.1 mL of the supernatant and 0.1 ml substrate (1 % starch solution in 0.02M sodium phosphate buffer pH 6.5). The mixture was incubated for 30 minutes at 30 °C. DNSA solution (3 mL) was added; all tubes were

incubated in a boiling water bath for 10 minutes and allowed to cool at room temperature.

Determination of Beta Amylase Activity

β- Amylase activity was investigated according to the combined methods of Zhang et al. (2006) and Ogundolie et al. (2022). The assay method was used to determine and monitor enzyme activity by measuring the release of maltose residues from soluble starch (dinitrosalicylic acid {DNSA} method). One β-Amylase activity was defined as the amount of maltose released (µmole) per minute per mL from the conversion of starch during the assay reaction at pH 5.0 and 30 °C.

Determination of Glucoamylase Activity

Glucoamylase activity was determined using the method of Ayodeji et al. (2017) with slight modifications. A reaction mixture containing 1 mL of 1% (w/v) soluble starch solution (pH 5.5, 0.05 M acetate buffer) and 1 mL supernatant obtained from the respective broths (aliquots centrifuged at 12,000 xg) was incubated for 10 minutes at 60°C in a water bath. Miller (1959) was used to quantify the released reducing sugar (glucose).

Determination of Cellulase Activity

The activity of cellulase in the supernatant was quantified by measuring the amount of reducing sugar released during the reaction using dinitrosalicylic (DNS) acid method as described by Miller (1959). A reaction containing 0.1 mL of the supernatant with substrate 0.1 mL (1% CMC in 50 mM citrate buffer, pH 4.8) was incubated for 30 minutes at 50 °C followed by the addition of 3 mL DNSA reagent, boiled for 5 minutes and cooled.

Determination of Protease Activity

Protease activity in the supernatant was determined with colometric assay with using casein as the substrate. A reaction mixture containing 1.0 mL of the supernatant solution and 1 ml of the substrate solution containing (2% casein, pH 7.0) was prepared and incubated for 30 minutes at 40 ºC. To stop the reaction, 1.5 mL of 1% Trichloroacetic Acid (TCA) was added to the reaction mixture. Then it was placed in a refrigerator for an hour, the precipitated casein was filtered off and the filtrate transferred into a test tube. Blanks of the samples were prepared by adding the TCA before the addition of substrate. Tyrosine standard cure was prepared and absorbance were taken using a spectrophotometer at 280 nm

Determination of β-1, 3-glucanase

β-1,3-Glucanase activity was determined in the supernatant by mixing 50 µL of the aliquot with 100 µL of substrate solution containing 0.25% Laminarin (Sigma) in acetate buffer (pH 5.0; 50 mmol l^{-1}). The reaction mixture was incubated for 30 minutes at 40 °C. The reducing sugar produced was quantified according to the method of Miller (1959). One unit (U) of β-1,3-glucanase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar min^{-1} under the assay condition used above.

Determination of Lipase activity

Lipolytic activity was determined in this study using the colorimetric method as described by Odeyemi et al. (2013) with slight modification using p-NPP (p-Nitrophenyl palmitate, pH 8.0) as the substrate. A reaction mixture containing 0.18 mL of solution A, 1.62 mL of Solution B, and 0.2 mL of supernatant. Solution A (contains a mixture of 0.062 g of p-NPP in 10 mL of 2-propanol, sonicated for 2 min before use), solution B (contains a mixture of 0.1 % gum Arabic and 0.4% triton X-100 in 50 mM Tris-HCl, pH 8.0) and 0.2 mL of properly diluted enzyme sample. The product was detected at 410 nm wavelength after incubation for 20 minutes at 37°C. Under this condition, the molar extinction coefficient (410 nm) of p-nitrophenol (p-NP) released from p-NPP was 15000 M−1. One unit of lipase activity was defined as 1 µmol of p-NP (p-nitrophenol) released per minute by 1 mL of the enzyme.

Molecular Identification of Endophytic Bacteria Isolate DRFHIS02

Genomic DNA Isolation DRFHIS02

Isolate DRFHIS02 was molecularly characterized by analyzing the 16S conserved region of the bacteria. The genomic DNA of the overnight grown culture of DRFHIS02 was isolated using Quick Fungal/Bacteria DNA miniprep kit (Zymo

Research, USA) as described by Ogundolie (2022).

PCR Amplification, Sequencing and Data Analysis
To amplify the 16S conserved region of the genomic DNA (gDNA) of DRFHIS02 isolate, a 25 µL reaction volume that contains the PCR Master mix, gDNA as template, nuclease-free water and universal primers (27F: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R: 5'-GGTTACCTTGTTACGACTT-3') was prepared. The amplification was achieved using a Veriti thermal cycler (Thermo Fishers, USA). using under the following reaction conditions; initial denaturation (94°C; 30 seconds), 32 cycles of denaturation (94°C; 30 seconds), annealing (45°C; 55 Seconds), initial extension (68°C; 60 seconds), final extension (68°C; 7 minutes) followed by holding (4-8°C). Amplicons were loaded on 1% Agarose gel electrophoresis and purified before the PCR product was subjected to Sanger sequencing. Nucleotide sequences obtained were analyzed using various bioinformatics tools such as ChromasPro DNA Sequencing Software, BioEdit Sequence Alignment Editor, and Basic Local Alignment Search Tools (BLASTn) respectively. Evolutionary relationship of the isolate was analysed using MEGAX (Molecular Evolutionary Genetics AnalysisX) (Ogundolie, 2022).

Results

Table 1 shows the result of the biochemical tests carried out on the distinct endophytic isolates obtained in this study. All isolates are positive for citrate, catalase, and D-glucose fermentation tests. Three isolates namely DRFHIS01, DRFHIS05, and DRFHIS07 were gram-negative organisms while others are grams positive.

Table 1: Biochemical Characterization of Endophytes Obtained from DRFH seeds

	DRFHIS01	**DRFHIS02**	**DRFHIS03**	**DRFHIS04**	**DRFHIS05**	**DRFHIS06**	**DRFHIS07**
Gram Staining	-	+	+	+	-	+	-
H_2S Production	-	+	+	-	-	-	-
Indole Test	-	-	-	-	-	+	+
Gelatin Hydrolysis	+	+	-	+	+	-	-
Methyl Red	+	-	-	+	-	+	+
Catalase	+	+	+	+	+	+	+
Nitrate Reduction	+	+	-	+	+	-	+
Urease Test	-	-	-	+	-	+	-
Oxidase	-	-	+	-	+	+	-
Citrate	+	+	+	+	+	+	+
Coagulase	+	+	+	+	-	-	-
Voges-Proskauer	+	+	+	+	-	-	-
D-glucose	+	+	+	+	+	+	+
L-rhamnose	+	-	-	-	-	+	+
D-mannitol	+	+	-	+	+	-	+
Lactose	-	-	-	+	+	-	+
Raffinose	-	+	+	-	-	+	-
Maltose	+	+	-	+	-	-	+
Sucrose	-	+	+	+	+	+	-
L-Arabinose	+	+	-	-	-	-	+

Results obtained for the screening of hydrolytic enzymes over 48-hour fermentation period is displayed in Figures 2-4. In Figure 2 which revealed 0-hour period fermentation, the activity of Beta amylase is shown to be the highest for all endophytes tested. Isolates DRFHIS01, DRFHIS04, and DRFHIS07 had the highest cellulolytic activity at day 0 with 0.02, 0.02 and 0.03 U/ml respectively while lipolytic activity was observed to be lowest in DRFHIS07 with 0.06 U/ml. In Figure 3, hydrolytic enzyme screening after 24-hour fermentation, revealed the activities of the hydrolases tested in this study. The highest activities were observed for β-1,3-glucanase with observed activities of 3.21 U/ml, 5.27 U/ml, 2.11 U/ml, 2.32 U/ml, 2.44 U/ml,

1.63 U/ml, and 1.33 U/ml for isolates DRFHIS01, DRFHIS02, DRFHIS03, DRFHIS04, DRFHIS05, DRFHIS06, and DRFHIS07 respectively. While the lowest activities were observed for cellulase and lipase during the fermentation period. The result of the α-Amylase screening at 48-hour of fermentation as shown in Figure 4 are 1.92, 4.55, 2.4, 2.8, 2.1, 2.3, 2.4 U/ml for DRFHIS01, DRFHIS02, DRFHIS03, DRFHIS04, DRFHIS05, DRFHIS06, DRFHIS07 respectively. The protease activity screening at 48-hour in U/ml are 3.22, 4.32, 1.66, 1.50, 2.24, 2.42, and 1.33 for isolates DRFHIS01, DRFHIS02, DRFHIS03, DRFHIS04, DRFHIS05, DRFHIS06, and DRFHIS07. Molecular identification of isolate DRFHIS02 in comparison with isolates obtained from the Genebank sequence database via the blast engine showed close similarities with various microorganisms of the Bacillus genus. Phylogenetic analysis using MEGAX bioinformatics tool as shown in figure 5 revealed that the DRFHIS02 is closely related to MH475940.1 with a 97% similarity in identity.

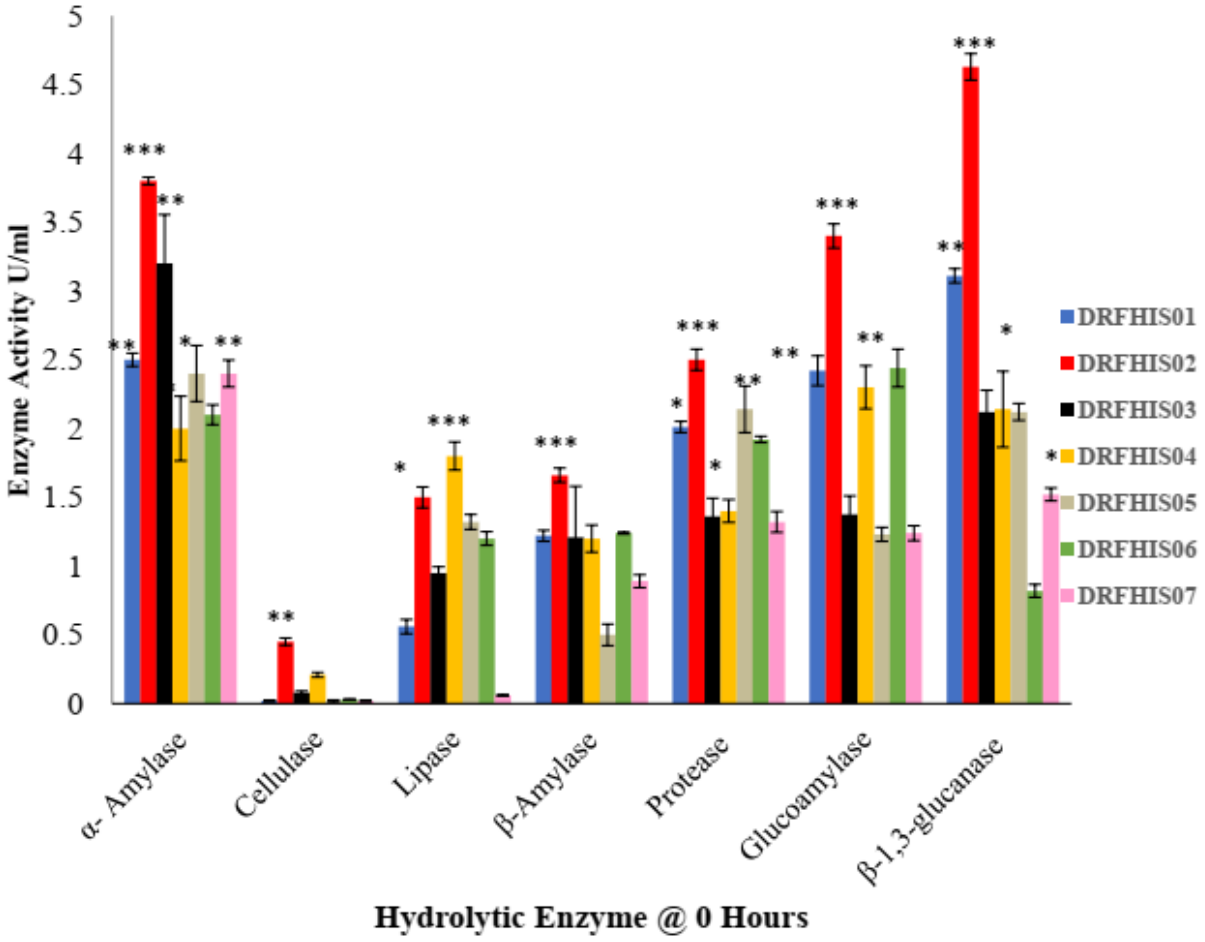

Fig 2: Screening for hydrolytic enzymes in endophytes obtained from DRFH at 0-hour fermentation.

*** indicates the media which shows the most significant expression of enzymes
** indicates the medium with a significant expression of enzymes
* Indicates the medium with a mild expression of enzymes

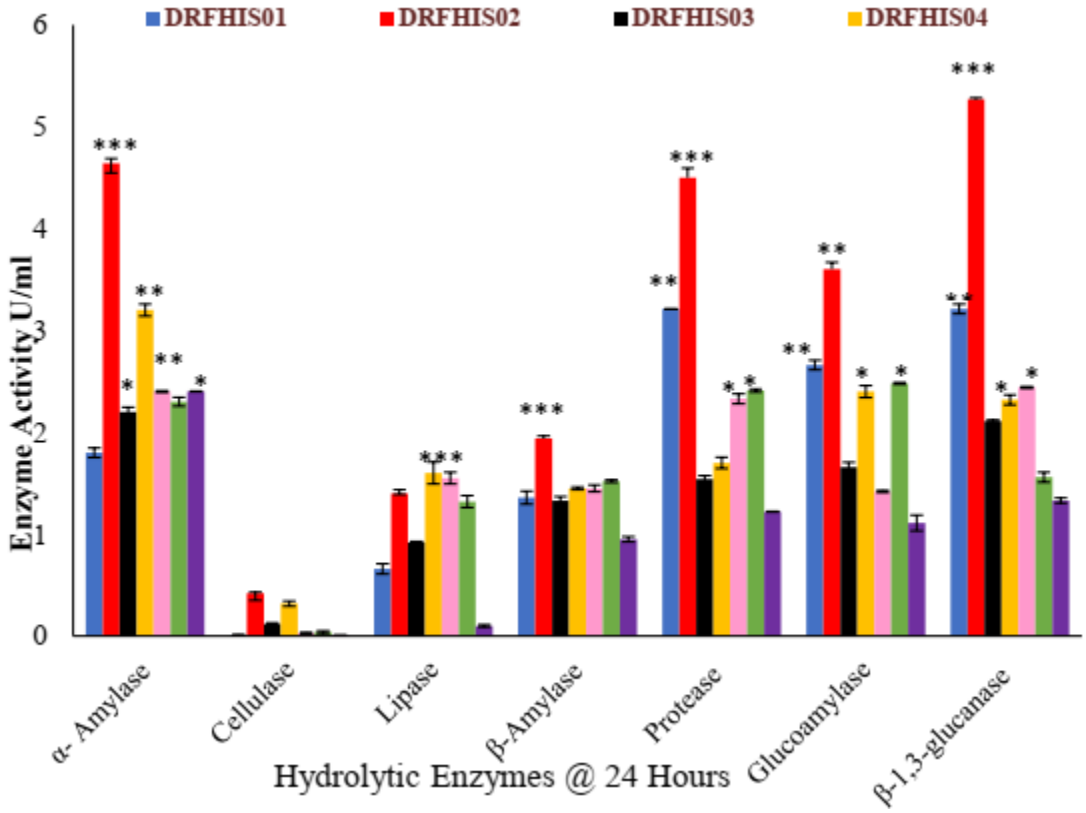

Fig 3: Screening for hydrolytic enzymes in endophytes obtained from DRFH at 24-hour fermentation.

*** indicates the media which shows the most significant expression of enzymes
** indicates the medium with a significant expression of enzymes
* Indicates the medium with a mild expression of enzymes

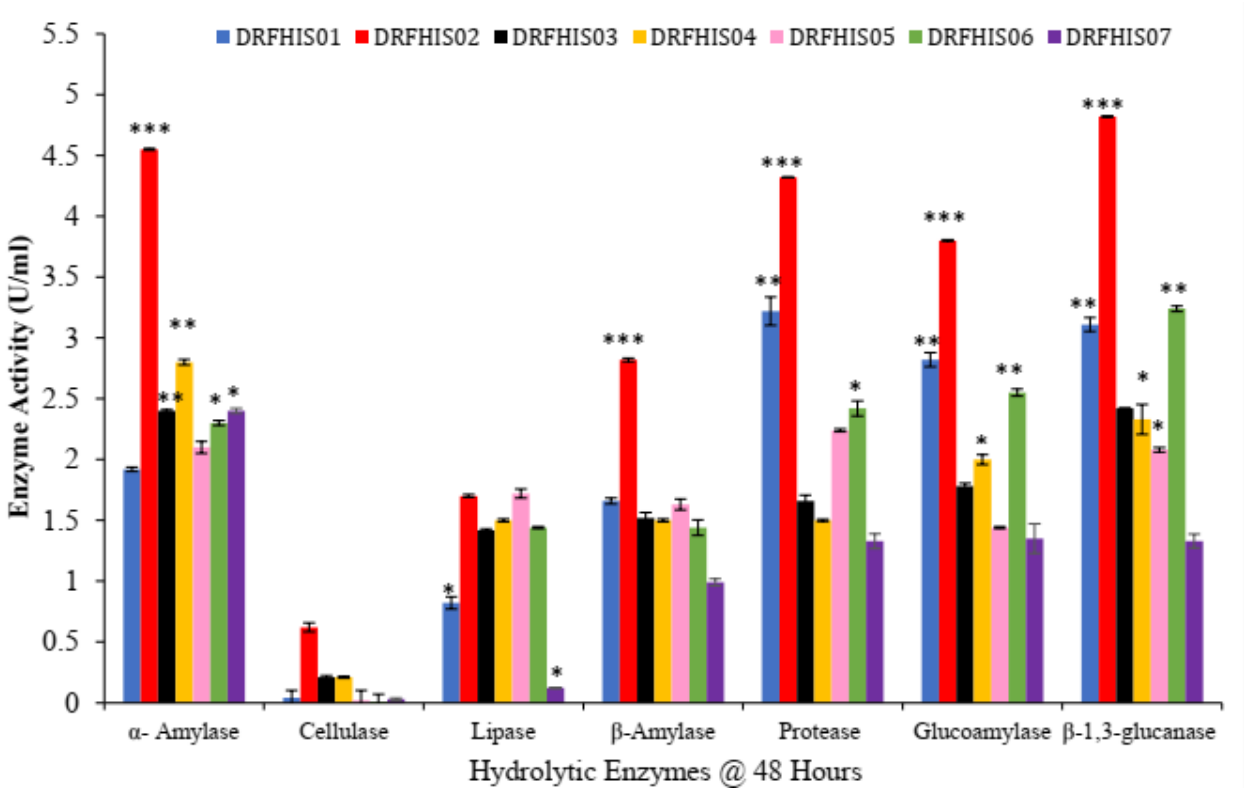

Figure 4: Screening for hydrolytic enzymes in endophytes obtained from DRFH at 48-hour fermentation.

*** indicates the media which shows the most significant expression of enzymes
** indicates the medium with a significant expression of enzymes
* Indicates the medium with a mild expression of enzymes

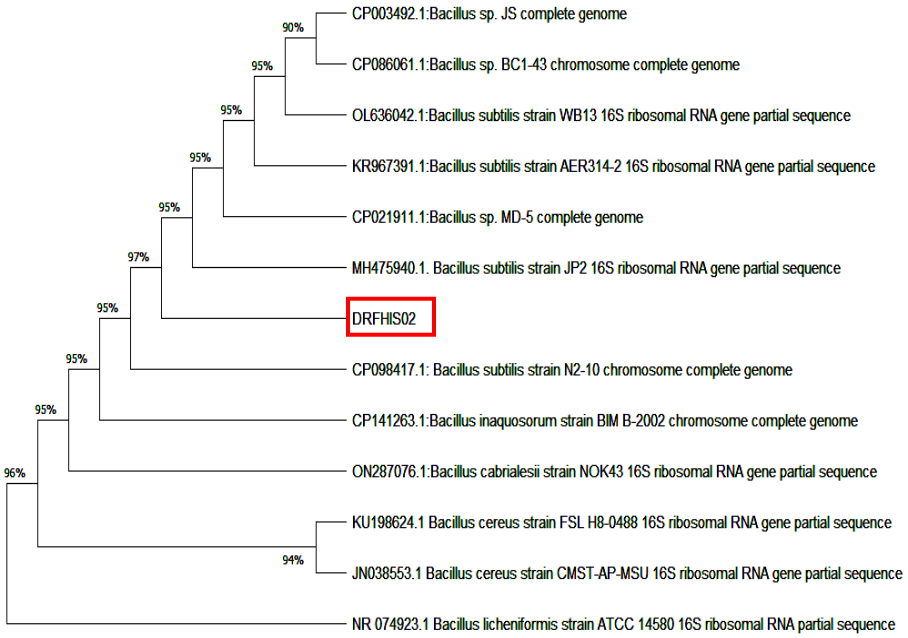

Fig 5: Phylogenetic tree of DRFHIS02 showing the evolutionary relationship of the isolate

Discussion

In this study, the absence of growth after 30 hours of incubation of the aliquot obtained after the final wash during the surface sterilization showed an effective sterilization process, the distinct isolates obtained in this study are similar to the endophytic microbes obtained from pearl millet as reported by Kumar et al. (2021).

Several studies have shown that the seeds-associated microbiota hosts different varieties of microorganisms (Sinclair, 1979) and are dominated by different phyla (Johnston-Monje et al., 2021) which include the Ascomycetes, Bacillota, Bacteroidota, and Proteobacteria (Chen et al., 2018; Acuña et al., 2023). With common genera and species of microbes such as *Staphylococcus spp, E. coli, Acinetobacter spp, Lactobacillus spp, Pseudomonas spp, Streptomyces spp*, *Bacillus spp, Micrococcus spp* been isolated (Hardoim et al., 2015; Nelson, 2018; Tkalec et al., 2022)

In this study, the endophytic isolate with the highest hydrolytic activity across the screened isolates was molecularly identified as *Bacillus subtilis*. *Bacillus subtilis* has been reported from several seeds. This microbe has been reported to be involved in seed priming (Lubyanova et al., 2023), improved salt tolerance (Abd_Allah et al., 2018) plant defense system and protection, plant growth, nutrient, biocontrol (Bolivar-Anillo et al., 2021; Deng et al., 2019) and bioaccumulation. *Bacillus subtilis* is of great biotechnological importance, and it is being explored for the production of bioactive compounds such as antibiotics, peptides, enzymes and through direct application (Gond et al., 2015; Lastochkina et al., 2021).

Endophytes are a group of microbes with tremendous biological resources which are largely underutilized. They have been reported to be valuable in various industries. In plants, they regulate seed dormancy and germination, and are involved in plants' responses to various forms of stress. The process of colonization of plants or seeds biosphere by these microbes (endophytes) requires some hydrolytic enzymes to break down the cell walls (Rajesh, and Rai,

2013). These enzymes are largely substrate-induced; therefore, their presence during screening may be an indicator of the composition-type of the seed/plant. This class of bacteria forms a symbiotic relationship with their hosts and is responsible for the protection of plants through the production of several bioactive compounds such as proteins, phytohormones, short peptides, phytochemicals, and enzymes during their metabolism.

To ensure good seedling development, starch-based seeds normally store their food in reserves which contain lipids, carbohydrates, and proteins, the nature of the carbohydrates will determine the activity of carbohydrase during hydrolysis. Oil-based seeds have more of oil bodies and protein bodies which they use for energy generation. They also provide the seeds, carbon and nitrogen sources for activity (Zienkiewicz et al., 2014). The presence of hydrolytic enzymes such as proteases, cellulase, lipases, and amylases are similar to those observed in the studies of other scientists such as Carrim et al. (2006), Rajesh and Rai (2013), Mamangkey et al. (2019), Dogan and Taskin (2021), Mamarasulov et al., (2022) and Siddique et al., (2022) who reported the presence of these hydrolytic enzymes from various endophytic microbes such as fungi and bacteria.

However, in this study, the types of hydrolytic enzymes under the subclass of hydrolases called amylases such as alpha-amylase, β -amylase, and glucoamylases were investigated to consider the substrate availability in DRFHS. Moreover, the decomposition within the plant tissue does not occur by endophytes unless a suitable or respective substrate/ carbon source is present (Rajesh, and Rai, 2013).

Various levels of enzyme activities were observed during the 48 hours of incubation of the isolates. The low presence of β-amylase observed in this study can be due to the developmental stages involved. In seeds, seed germination process has been associated with an elevated level of β-amylase and β-1,3-glucanases. The findings of Leubner-Metzger et al. (1995) and Gupta et al. (2013) which revealed a high presence of β-1, 3- glucanases is similar to the findings of this study.

β-1,3-glucanase is of biological significance and have the ability breakdown the membrane cell wall of pathogenic fungi thereby conferring resistance to the seed against such pathogenic fungi (Finch-Savage and Leubner-Metzger, 2006; Gupta et al. 2013). This enzyme was observed to be highly produced by six out of the seven isolates obtained. These suggest that this enzyme might be responsible for the defense of the seeds against pathogenic microbes, especially antifungal pathogens.

β -1,3- glucanase has been reported to be responsible for regulating seed germination through its dormancy action on the seeds by hydrolyzing the endosperm and alleviating the inhibitory effects of phytohormone, abscisic acid (ABA) ABA. Its activity in plants tends to keep the seed intact and prevent spoilage due to pathogenic microbes. Also, this enzyme is involved in hydrolyzing the cell wall of seeds and the rupture of endosperm before the radicle projection emerges (Joshi, 2018). Hence the high concentration of this enzyme might suggest the simultaneous germination occurring in the seeds. The high and low activity observed for β-1,3, glucanase and cellulase respectively in this study suggests that the seeds of DRFH is largely consist of β-1,3-glucans than β-1,4-glucans polysaccharides

Conclusion

With increasing demand for new sources of biocatalysts such as enzymes, peptides and other bioactive compounds due their importance in biomedical and various industrial applications. The findings of this study suggests that endophytic microbes obtained from DRFH can be another source of microorganisms for the production of biocatalysts especially lipase, β-1, 3-glucanase, proteases and amylases (glucoamylase, β- amylase, α-amylase) which are enzymes of great biotechnological importance. These enzymes can further be studied towards their utilization in various industries.

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