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Enzymatic activity of endophytic bacterial isolates of *Morus* Spp.

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Abstract
Endophytic bacteria have been known to inhabit the internal structures of plants throughout the different growth and developmental stages. In this study, the endophytic bacteria were isolated from mulberry cultivars, collected from Jaipur, and their ability to produce various extracellular enzymes was assessed. A total of 52 bacteria were isolated out of which twenty-one (40.38%) isolates were amylase positive, twenty (38.46%) cellullase and twenty-three and (44.23), twenty-eight (53.85%) isolates were protease and lipase positive respectively. Protease and lipase production was found to be highest among the isolates.

Keywords: Endophytic bacteria, *Morus* Spp., extracellular enzymes, actinomycetes

Introduction
Endophytes are microorganisms (bacteria, fungi, actinomycetes, and algae) that live inside of plant tissues without causing any harm to the host plant. Since their discovery, they have received significant attention due to their ability to protect their hosts against insects, pests, and diseases. Nearly every plant species hosts endophytic organisms (Tan and Zou, 2001) [12] and can be found in stems, leaves, roots, seeds, fruits, or flowers at almost any stage of their life.

The natural products of microbial, plant, and animal origin are becoming increasingly popular. Endophytic bacteria have been shown to be an excellent source of new bioactive natural compounds. The wide array of biologically active substances synthesized by endophytic bacteria may have evolved as a chemical defence mechanism and to facilitate plant growth promotion. Furthermore, endophytic bacteria secrete a variety of enzymes that aid in the colonization of the host tissues. Extracellular enzymes from endophytes are gaining interest due to their stability, low cost, and rapid manufacture in large quantities using various fermentation techniques (Shubha and Srinivas, 2017) [11].

Endophytes associated with medicinal plant synergistically produce important metabolites in their host plant. Exploring a wide variety of plants in different settings to discover novel and beneficial endophytic microbes is a very promising activity. These microorganisms or their enzymes have diverse applications in biology, industry, and the environment. In fact, some key enzymes produced by endophytic bacteria include xylanases, phytases, proteases, asparaginase, cellulases, pectinases, gelatinase, chitinase, amylases, and many more. (Khan *et al*., 2017) [5].

The plant targeted for the isolation of endophytes in this study was Mulberry. The mulberry tree, scientifically known as *Morus*, is a well-known medicinal plant that belongs to the *Moraceae* family. It is highly regarded for its economic as well as medicinal value. It is primarily valued for its foliage, which serves as the primary food source for the mulberry silkworm (*Bombyx mori*). Many different organic compounds (anti-microbial, anti-hyperglycemias, anti-hyperlipidemias, antidiabetic, antioxidative) with high medicinal value have been found in mulberry leaf, fruit, stem, and root. There are limited reports on its endophytic community. Hence it is important to look into the endophytic diversity of mulberry.

The aim of this study was to evaluate the potential of the isolates to produce four selected hydrolytic industrially important enzymes.

Materials and Methods

To explore the possibility of isolating bacterial endophytes producing enzymes if any, leaves and stems of mulberry were collected from Jaipur (Latitude 26.50'26''N and Longitude 76.02'57''E) for two consecutive years, 2017 and 2018.

Amylase production

The endophytes were screened for amylase activity on a starch agar medium (10 g starch, 5 g gelatin, 3 g beef extract and 15 g agar/L distilled water). 24 hr old bacteria were spot inoculated on the starch agar medium and were incubated at 32 ± 2 °C for 3-5 days. After incubation, the plates were flooded with iodine solution for 5 min. to identify the starch hydrolyzing zone (Pascon *et al*., 2011) [9].

Cellulase production

Screening of isolated bacteria for cellulase enzyme production was done on CMC (Carboxy-Methyl Cellulose) agar medium. Isolated bacteria were inoculated in a CMC agar medium containing Congo Red as an indicator of cellulose degradation. The petri plates were then placed in an incubator at 32± 2 °C for 3-5 days.

Protease production

Bacterial isolates were subjected to screening of extracellular protease production by Skim Milk agar (28 g skim milk powder, 2.5 g yeast extract, 1 g dextrose, 5 g casein, and 15 g agar/L distilled water) assay. The cultures were inoculated on skim milk agar plate and incubated at 32±2 ℃ for two days (Sharma *et al*., 2015) [10].

Lipase production

The strains were inoculated onto the medium containing (g/l): Nutrient Broth 8 g, $CaCl_2$ H_2O 0.1 g, agar 15 g, and 20 ml Tween 20. Tween 20 was separately added into the medium after sterilization. Cultures were incubated at 32±2 °C for two to three days and the plates were kept at +4 °C for 30 min. Variants showing opaque zone around colonies were evaluated as lipase positive (Hankin and Anagnostakis,1975) [3].

The Enzymatic Index is a useful tool that makes it easier and faster to choose and compare the enzymatic production of different microbial isolates. The EI for each enzyme was calculated at the end of a specific incubation time. EIs were calculated as a mean ratio of halo zone diameter to colony diameter.

Statistical Analysis

All experiments were performed in triplicates. The enzyme production values are given with standard deviations.

Results

In this study, a total of 52 endophyte bacterial isolates were isolated and checked for extracellular enzyme production in solid media and specific substrates based on the presence of hydrolytic halos (Fig. 1). The isolates were named as MW (Mulberry white), and MR (Mulberry red) and S and L denote stem and leaf, respectively, tissues they were isolated from.

The Enzymatic Index (EI) for all isolates was obtained following observation of their ability to produce at least 1 of 4 evaluated enzymes. For all the isolates, the EI of each enzyme activity is given in Table 1.

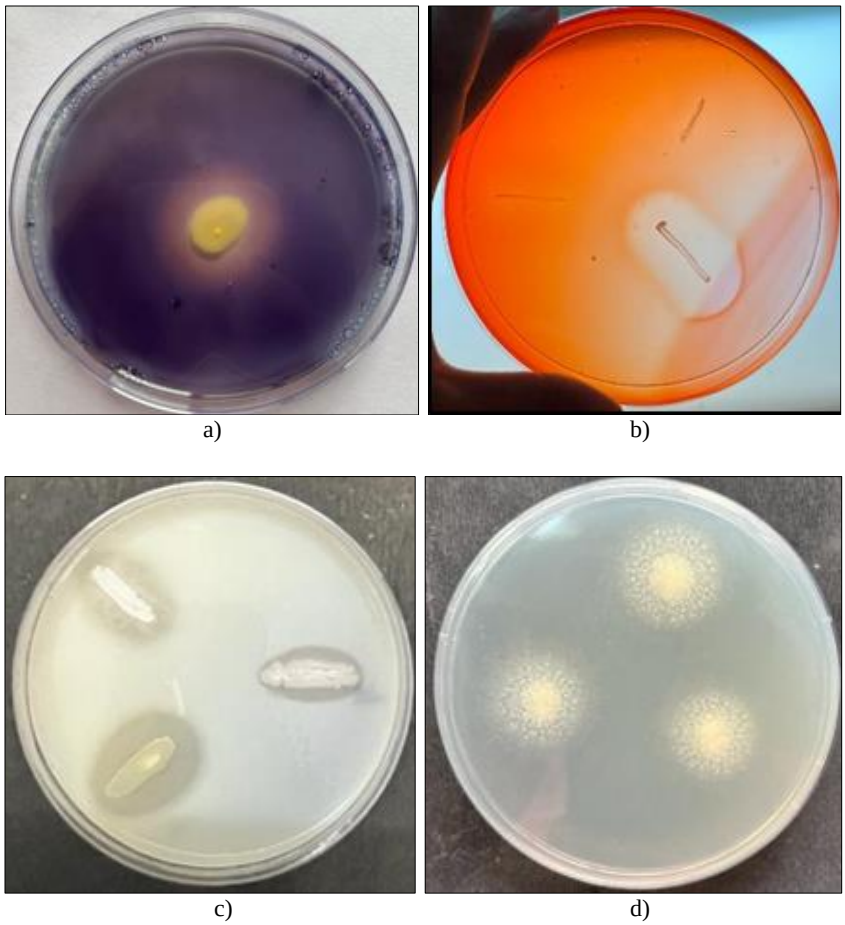

Fig 1: Extracellular enzymatic activity of isolates; (a) amylase, b) cellulase, (c) protease, (d) lipase.

Observation of starch degradation in the petri-plate was observed in 21 (40.38%) isolates. Isolate MRL12, MRS1 and MRS5 presented the highest amylolytic activity, with EI of 3.8, 3.28 and 3.27 respectively. Of the 52 isolates tested, 20 (38.46%) isolates were able to degrade the CMC present in the culture medium. Isolate MWS12, MWL3 and MRL3 presented the highest cellulolytic activity, with EI of 5.97, 5.32 and 4.27 respectively.

Proteolytic activity on SM agar media was observed to be 44.23% (23) in 52 bacterial isolates. MWS18, MWS16 and MRS4 showed highest proteolytic activity with 4.27, 4.00 and 3.52 EI respectively. Lipolytic activity was observed in 28 (53.85%) isolates out of 52. MWS10, MWS17 and MWS4 isolates showed highest EI of 5.53, 4.97 and 4.41 respectively.

Table 1: Enzymatic activity of the isolated microorganisms (The enzymatic index was measured by determining the ratio of degradation halo diameter/bacterial colony diameter in cm. The results represent the means of three replicates for each isolate ± standard deviation)

S. No.	Strain	Amylase	Cellulase	Protease	Lipase
1	MWS1	-	1.41±0.14	1.96±0.15	2.38±0.05
2	MWS2	2.5±0.20	-	-	1.04±0.04
3	MWS3	1.66±0.04	2.48±0.16	1.89±0.032	-
4	MWS4	-	3.68±0.06	-	4.41±0.08
5	MWS5	-	-	1.47±0.03	1.86±0.07
6	MWS6	2.45±0.04	-	-	2.21±0.14
7	MWS7	2.44±0.04	-	-	2.23±0.12
8	MWS8	1.85±0.03	3.19±0.13	1.39±0.07	1.73±0.10
9	MWS9	2.03±0.04	-	1.34±0.06	1.46±0.03
10	MWS10	-	1.66±0.05	2.15±0.13	5.53±0.15
11	MWS11	-	3.47±0.15	3.46±0.19	-
12	MWS12	-	5.97±0.15	1.68±0.45	-
13	MWS13	1.79±0.17	-	-	1.59±0.08
14	MWS14	3.26±0.05	-	3.16±0.03	-
15	MWS15	-	2.26±0.11	-	-
16	MWS16	-	-	4±0.1	4.3±0.1
17	MWS17	-	-	-	4.97±0.15
18	MWS18	-	3.86±0.05	4.27±0.06	-
19	MWL1	-	-	-	3.7±0.20
20	MWL2	-	-	-	3.47±0.20
21	MWL3	2.28±0.23	5.32±0.33	-	1.67±0.03
22	MWL4	-	-	-	2.75±0.12
23	MWL5	-	-	-	1.69±0.07
24	MWL6	-	-	3.46±0.08	-
25	MWL7	-	2.2±0.1	-	4.22±0.10
26	MWL8	-	-	-	2.24±0.06
27	MWL9	-	1.26±0.05	-	4.14±0.13
28	MWL10	1.39±0.1	2.29±0.04	-	-
29	MWL11	-	-	2.1±0.1	1.10±0.08
30	MWL12	-	-	3.34±0.09	-
31	MRS1	3.28±0.05	-	1.83±0.06	-
32	MRS2	1.86±0.05	1.51±0.08	2.47±0.03	-
33	MRS3	2.52±0.10	3±0.2	1.09±0.08	-
34	MRS4	-	-	3.52±0.09	-
35	MRS5	3.27±0.06	-	-	-
36	MRS6	-	-	-	3.53±0.3
37	MRS7	1.78±0.06	-	1.40±0.1	-
38	MRS8	-	2.45±0.05	-	-
39	MRS9	-	-	2.47±0.02	1.19±0.03
40	MRS10	-	-	-	2.61±0.05
41	MRL1	-	3.07±0.11	-	-
42	MRL2	1.37±0.11	-	-	-
43	MRL3	-	4.27±0.3	-	-
44	MRL4	-	-	2.52±0.11	2.13±0.15
45	MRL5	3.22±0.07	4.17±0.15	-	2.97±0.15
46	MRL6	-	-	-	1.11±0.11
47	MRL7	3±0.1	-	-	-
48	MRL8	-	-	2.09±0.09	-
49	MRL9	-	-	-	3.33±0.05
50	MRL10	1.18±0.06	-	-	-
51	MRL11	2.8±0.1	-	-	-
52	MRL12	3.8±0.2	2.17±0.15	1.34±0.05	-

It was observed that all the isolates possessed at least one enzymatic activity tested. According to Fungaro and Maccheroni (2002) [2], EI values greater than 1.0 serve as a reliable indicator of the existence of enzymes secreted by bacteria, and EI value of 2.0 or higher is regarded a good producer (Lealem and Gashe,1994) [7].

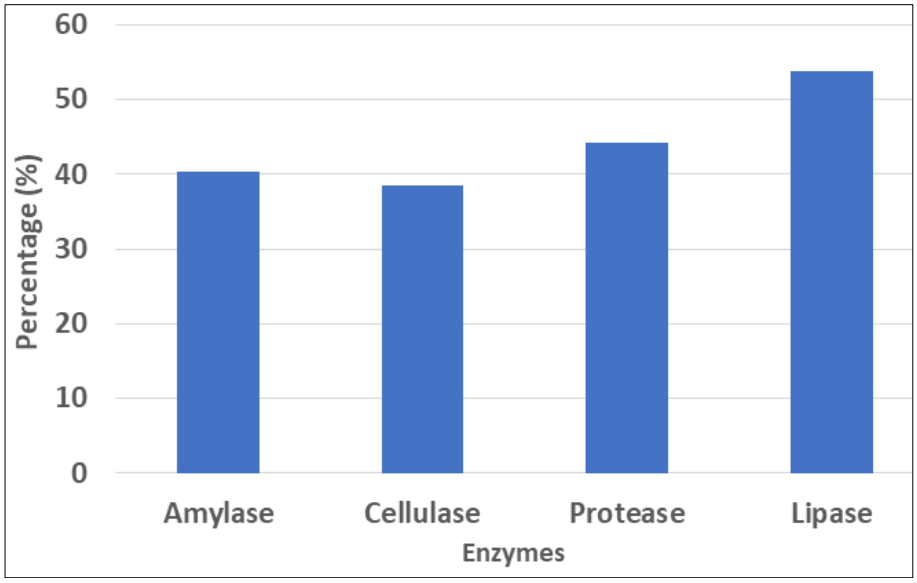

Fig 2: Relative % of strains (from a total of 52) producing individual hydrolytic enzymes

Starch agar medium has been widely used for primary screening of amylase enzyme. To check whether isolated bacteria produced amylase enzyme, the distinct zone of hydrolysis surrounding the isolate's growth on starch agar medium was observed. The breakdown of starch, the primary organic molecule responsible for energy storage, was observed for 40.38% of isolates grown and eight isolates exhibited EI in the range of 1-2, seven in between 2-3 and six showed EI of 3-4.

Cellulose is the predominant carbohydrate found in plant biomass. It has been suggested that the presence of cell wall-degrading enzymes, such as cellulases and pectinases, may play a role in the interactions between plants and microbes, as well as in the colonization of roots at the intercellular level. Additionally, they can serve as agents in the process of biocontrol. (Kandel *et al*., 2017; Lima *et al*., 1998) [4, 8]. Carboxy-Methyl Cellulose (CMC) plates were used for screening of cellulase production by endophytic bacteria. In the current study 38.46% isolates were found to produce cellulase. Yousef and co-workers (2019) [13] isolated a cellulose degrading endophytic bacterium (Chi-04) from medicinal plant *Chiliadenus montanus*. Similarly, Zaghloul and coworkers (2016) [14] isolated 167 endophytic bacteria from many legumes and non-legumes plants. Out of a total of 167 isolates, 55 were shown to possess the capability of producing cellulase and pectinase enzymes. In our results the EI for cellulase production ranged between 1.26 to 5.97. Four isolates exhibited EI in between 1-2, six in the range of 2-3 and ten isolates showed EI of above 3.

The capacity of microorganisms to produce proteases was determined using casein hydrolysis. The medium is opaque due to the casein and formation of a clear zone surrounded the bacterial growth is indication of protease. In our results 44.23% of isolates showed protease activity and 10 isolates showed EI of 1-2, six in the range of 2-3 and seven isolates showed EI in the range of 3-5. In their study, Dogan and Taskin (2021) [1] identified a total of 128 bacteria from *Poaceae* plants. These bacteria were shown to produce several enzymes, including lipases, proteases, amylases, cellulases, pectinases, and xylanases. The relative frequencies of these enzymes were 74.2%, 65.6%, 55.4%, 32%, 21.8%, and 7.8% respectively.

Out of all the enzyme tested lipase was produced by the maximum number of isolates (53.85%) and the EI for lipase ranged between 1-2 for ten isolates, 2-3 for eight isolates and >3 for ten isolates. In their study Khan and co-workers (2022) [6], isolated a total of 11 endophytic bacteria from *Arthrocnemum macrostachyum* and all isolates showed enzymatic potential and lipase was found to be produced by maximum isolates. Out of 52 isolates, only one (MWS8) was able to produce all the four enzymes and nine isolates produced three of the four enzymes tested.

Conclusion

Bacteria residing in the medicinal plant Mulberry showed activity of hydrolytic enzymes. Our work has added to the knowledge on the diversity of endophytic bacterial communities found in mulberry. Further investigation would provide us an insight into the prospective applications of these endophytic bacterial isolates. While there have been reports of numerous bacterial isolates from different sources producing enzymes such as cellulase, protease, amylase, and lipase, there are relatively few studies that investigated endophytic bacteria in relation to their extracellular enzymes. Therefore, endophytic bacteria have the potential to serve as a novel reservoir of enzymes with diverse applications.

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References

1. Dogan G, Taskin B. Hydrolytic enzymes producing bacterial endophytes of some poaceae plants. Polish journal of microbiology. 2021 Sep 1;70(3):297-304.
2. Fungaro MH, Maccheroni Jr. W, Melo IS, Valadares-Inglis MC, Nass LL, Valois AC, *et al*. Melhoramento genético para produção de enzimas aplicadas à Indústria de Alimentos. Recursos Genéticos e Melhoramento-

Microrganismo. Jaguariúna: Embrapa Meio Ambiente. 2002:426-53.
3. Hankin L, Anagnostakis SL. The use of solid media for detection of enzyme production by fungi. Mycologia. 1975 May 1;67(3):597-607.
4. Kandel SL, Joubert PM, Doty SL. Bacterial endophyte colonization and distribution within plants. Microorganisms. 2017 Nov 25;5(4):77.
5. Khan AL, Shahzad R, Al-Harrasi A, Lee IJ. Endophytic microbes: a resource for producing extracellular enzymes. Endophytes: Crop Productivity and Protection. 2017;1:95-110.
6. Khan T, Alzahrani OM, Sohail M, Hasan KA, Gulzar S, Rehman AU, *et al*. Enzyme profiling and identification of endophytic and rhizospheric bacteria isolated from *Arthrocnemum macrostachyum*. Microorganisms. 2022 Oct 26;10(11):2112.
7. Lealem F, Gashe BA. Amylase production by a Gram-positive bacterium isolated from fermenting tef (Eragrostis tef). Journal of Applied Microbiology. 1994 Sep 1;77(3):348-52.
8. Lima LH, Marco JD, Felix JR. Enzimas hidroliticas envolvidas no controle biologico por miciparasitisma. Controle biologico. 1998;11:263-304.
9. Pascon RC, Bergamo RF, Spinelli RX, DE Souza ED, Assis DM, Juliano L, *et al*. Amylolytic microorganism from São Paulo zoo composting: isolation, identification, and amylase production. Enzyme research; c2011.
10. Sharma AK, Sharma V, Saxena J, Yadav B, Alam A, Prakash A, *et al*. Isolation and screening of extracellular protease enzyme from bacterial and fungal isolates of soil. Int. J Sci. Res Environ Sci. 2015;3(9):0334-40.
11. Shubha J, Srinivas C. Diversity and extracellular enzymes of endophytic fungi associated with *Cymbidium aloifolium* L. African Journal of Biotechnology. 2017 Nov 29;16(48):2248-58.
12. Tan RX, Zou WX. Endophytes: A rich source of functional metabolites. Natural product reports. 2001;18(4):448-59.
13. Yousef N, Mawad A, Abeed A. Enhancement the cellulase activity induced by endophytic bacteria using calcium nanoparticles. Current microbiology. 2019 Mar 15;76:346-54.
14. Zaghloul RA, Abou-Aly HE, Tewfike TA, Ashry NM. Isolation and Characterization of Endophytic Bacteria Isolated from Legumes and Non-Legumes Plants in Egypt. Journal of Pure & Applied Microbiology; c2016 Mar 1, 10(1).
15. Chuwa C, Dhiman AK, Thakur NS, Kathuria D, Gautam S, Sharma B. Comparative studies on extraction of starch through physical, enzymatic and alkaline method. International Journal of Food Science and Nutrition. 2020;5(6):116-9.