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# EXTRACELLULAR ENZYMES PRODUCED BY THE BACTERIAL ASSOCIATES OF *DENDROBIUM OVATUM* (L.) KRAENZL.

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**Abstract**

The associated microbiota of every organism profoundly influences and nurtures the existence of its hosts through various mechanisms. Plant associated microbiota is no exception to this phenomenon. Plants host the phytomicrobiome that include bacteria, fungi, algae, and nematodes. These are reported to be rich source of extracellular enzymes that may be hydrolases, lyases, oxidoreductases, or transferases. The quest for microbial assemblages capable of producing extracellular enzymes of industrial application is currently being thoroughly investigated. The present study reported the capability of the bacterial associates of the wild epiphytic orchid, *Dendrobium ovatum* to produce amylase, cellulase, laccase, lipase, pectinase, protease, and tyrosinase. Screening for extracellular enzymatic activities of the isolates revealed that 76.19 % of the bacterial isolates produced protease, followed by pectinase (52.38 %), amylase (47.62 %), cellulase (19.05 %), and lipase (14.29 %). All the isolates tested negative for laccase and tyrosinase enzyme production. These extracellular enzymes of microbial origin help in symbiosis process, penetrate the plant tissues, and understand their substrate utilization pattern. The stability of microbial enzymes over plant or animal enzymes is one of the striking features that make them a perfect option for industrial purposes. Hence, the bacterial associates of *D. ovatum* emerge as rich source of industrially important extracellular enzymes.

## Introduction

ORCHIDS BELONG to a diverse group of plants that occupy the epitome of evolution and extinction, at the same time. These constitute a magnificent group of flowering plants and are well known for their fascinating, long-lasting flowers (Prakash and Pathak, 2020a, 2022). These plants rank amongst the most significant ornamental plants and are known for their beauty, colour combinations and shape of their flowers (Prakash and Pathak, 2020b, 2023). The family Orchidaceae forms one of the largest and evolutionarily advanced amongst the plant families (Kaur and Sharma, 2021; Prakash and Pathak, 2019). Due to their dependence on a wide variety of life forms throughout its life cycle, orchids can be considered as an *ideal model* organism for co-evolution and *flagship* group for protecting endangered species (Liu *et al*., 2020). The life stages of orchids can be divided into protocorm stage, juvenile stage, vegetative adult stage, and finally reproductive stage. The very dependence on microorganisms starts from the initial germination stage of orchid seeds, as they are minute and lack endosperm. *Dendrobium ovatum*, also known as the *Green-Lipped Dendrobium*, is a relatively understudied species within its genus. It predominantly thrives at altitudes ranging from 50 to 1,520 m above sea level. This orchid, facing threats to its survival, is native to the Western Ghats of India, specifically found in the states of Gujarat, Maharashtra, Goa, Karnataka, Tamil Nadu, Andhra Pradesh, Dadara & Nagar Haveli, and Kerala (Jalal and Jayanthi, 2012; Singh *et al*., 2019).

Extracellular enzymes of microbial origin are the initiator molecules in host symbiosis process, overcoming the host defence mechanisms; these help invade the plant tissues (Khan *et al*., 2017). Thus, the ability to produce extracellular enzymes by plant associated microbiota display the pattern of substrate utilization they prefer and provide evidences regarding their lifestyle (Pointing, 1999). Cellulase, mannanase, or xylanase activity of endophytes point towards their mutualistic or saprophytic nature. Moreover, these studies can also throw light on the evolution and ecology of the associated microbes and their host (Sunitha *et al.,* 2013). The present study deals with the extracellular enzymatic activities of the bacterial associates of the threatened epiphytic orchid, *Dendrobium ovatum* (L.) Kraenzl., an endemic orchid of the Western Ghats of India.

## Material and Methods

### *Qualitative Estimation of Extracellular Enzyme Production*

The bacterial associates of the epiphytic orchid *Dendrobium ovatum* (L.) Kraenzl. described in Raj *et al*. (2024a) were tested for their ability to produce extracullular enzymes were used for the present study. Fresh cultures of bacterial isolates were inoculated onto

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the respective medium with substrates specific for amylase, cellulase, pectinase, protease, lipase, and tyrosinase as proposed by Hankin and Anagnostakis (1975) and Uzma *et al.* (2016). The plates were incubated for 24 to 48 hr and the clear zones formed around the bacterial colonies indicated the potential of the strains to produce enzymes. Diameter of both the clear zone and the colony were noted for all the strains showing a positive result, and the halo zone diameter was recorded. The Enzymatic Index (EI), considered as a semi-quantitative measurement of enzyme activity, was calculated by comparing the halo zone diameter and colony diameter (Abdalla *et al*., 2020; Chamekh *et al*., 2019).

*Quantification of Extracellular Enzyme Produced*

For the quantification of the extracellular enzymes produced, bacterial isolates with highest EI in amylase, cellulase, and protease were cultured in nutrient broth for 24 hr at 28 ± 2°C for 3 days on a rotary shaker. Aliquots of these were centrifuged at 5000-12000 rpm for 10 to 20 min at 4°C and the resultant supernatant were used as the source of enzyme for quantification (Grata, 2020). Reducing sugar method using 3,5-dinitrosalicyclic acid (DNS) was used to estimate the amylase enzyme activity. The absorbance was recorded at 560 nm and one unit of enzymatic activity was defined as the amount of enzyme required to produce 1µmol of glucose per min under the assay conditions. Quantification of the cellulase activity was carried out by the protocol of Grata (2020). One unit of enzymatic activity was defined as the amount of enzyme required to produce 1µmol of glucose per min under the assay conditions. For quantification of protease production, the protocol of Cupp-Enyard (2008) was followed. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1µg of tyrosine per min under the assay conditions.

*Statistical Analysis*

All the tests were performed in triplicates and the statistical analysis was performed using R program version 4.2.1 [R Core Team (2022)]. The comparisons were made by one way ANOVA followed by Tukey's HSD test with significance set at 0.05. The statistical analysis of the association between the extracellular enzyme production of the isolates were performed using Pearson's correlation coefficient using R program version 4.2.1 [R Core Team (2022)].

## Results and Discussion

The quest for the microbial assemblages capable of producing extracellular enzymes of industrial application is currently being thoroughly investigated. These may be attached enzymes that are found adhered to the microbial membrane, or may be free enzymes, that are released to the external environment (Traving *et al*., 2015). In the present study, the screening for the production of extracellular enzymes by the bacterial associates of *D. ovatum* revealed that 76.19% of the isolates produced protease enzyme, followed by pectinase (52.38%), amylase (47.62%), cellulase (19.05%), and lipase (14.29%). All the isolates were tested negative for laccase and tyrosinase enzyme production (Table 1, Fig. 1).

The enzymatic index (EI) for amylase was found to be highest for *Enterobacter* sp., followed by *Enterobacter hormaechei*, *E. mori,* and *Klebsiella* sp. The results are indicative of the capability of these isolates to use carbohydrate as their energy source, by secreting the enzymes to predigest the food outside their cells (Venkatesagowda *et al*., 2012). This property is of particular interest in fostering mutualistic interactions between the microbial associates and hosts plants. For industrial purposes, bacterial amylases are preferred over the fungal amylases, due to their thermostable nature; *Bacillus cereus*, *B. amyloliquefaciens*, and *B.*

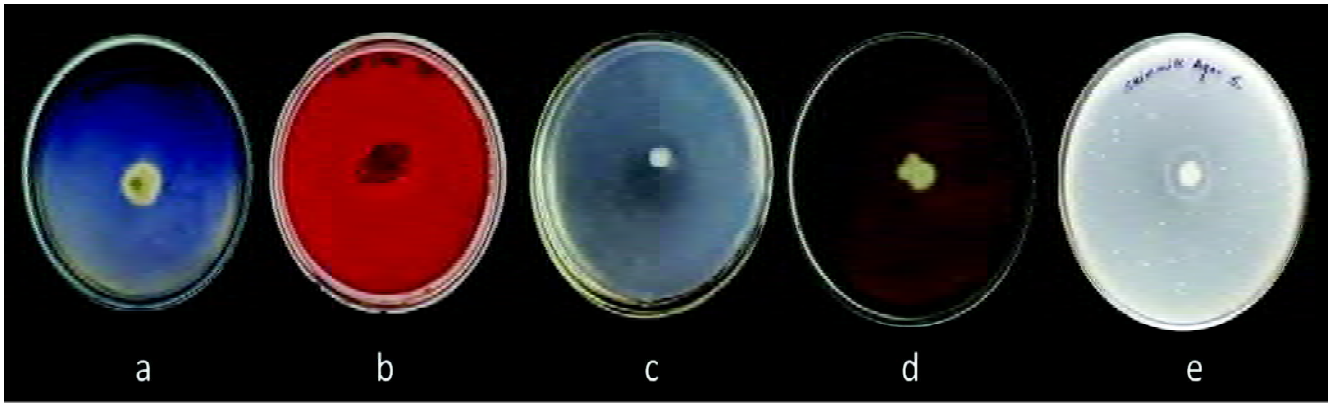


Fig. 1. Extracellular enzyme production by the bacterial associates of *Dendrobium ovatum* on different media: a, Amylase; b, Cellulase; c, Lipase; d, Pectinase; e, Protease.

Table 1. Enzymatic index (EI) of the various extracellular enzymes produced by the bacterial associates of *Dendrobium ovatum.*

| Plant part | Isolate | Amylase | Cellulase | Lipase | Pectinase | Protease |
|---|---|---|---|---|---|---|
| Root | *Bacillus rugosus* | $0.05 \pm 0.03^{de}$ | $0^{e}$ | $0^{d}$ | $0.99 \pm 0.26^{abc}$ | $0.47 \pm 0.16^{cde}$ |
| | *B. cereus* | $0^{e}$ | $0^{e}$ | $0^{d}$ | $1.86 \pm 0.48^{a}$ | $0.36 \pm 0.03^{def}$ |
| | *B. cereus* | $0^{e}$ | $0^{e}$ | $0^{d}$ | $0^{d}$ | $0.23 \pm 0.09^{ef}$ |
| | *Priestia megaterium (Bacillus megaterium)* | $0^{e}$ | $0^{e}$ | $0^{d}$ | $1.58 \pm 0.38^{a}$ | $0^{g}$ |
| | *B. velezensis* | $0.05 \pm 0.02^{de}$ | $1.87 \pm 0.06^{b}$ (0.23 U/ml) | $0^{d}$ | $1.57 \pm 0.54^{a}$ | $0^{g}$ |
| | *B. altitudinis* | $0^{e}$ | $0^{e}$ | $0^{d}$ | $0^{d}$ | $0.18 \pm 0.05^{ef}$ |
| | *B. thuringiensis* | $0^{e}$ | $0^{e}$ | $0^{d}$ | $0^{d}$ | $0.31 \pm 0.08^{def}$ |
| | *B. cereus* | $0.11 \pm 0.02^{cde}$ | $0^{e}$ | $0^{d}$ | $0.46 \pm 0.07^{bc}$ | $0.24 \pm 0.01^{ef}$ |
| | *B. cereus* | $0.22 \pm 0.04^{bc}$ | $0^{e}$ | $0^{d}$ | $0.37 \pm 0.01^{c}$ | $0.63 \pm 0.14^{cd}$ |
| | *B. cereus* | $0.20 \pm 0.02^{bcd}$ | $0^{e}$ | $0^{d}$ | $1.50 \pm 0.11^{a}$ | $0.21 \pm 0.02^{ef}$ |
| | *B. amyloliquefaciens* | $0.10 \pm 0.07^{cde}$ | $2.17 \pm 0.06^{a}$ (0.24 U/ml) | $0^{d}$ | $0.38 \pm 0.04^{c}$ | $0.34 \pm 0.03^{def}$ |
| Leaf | *Enterobacter asburiae* | $0^{e}$ | $0^{e}$ | $0^{d}$ | $1.50 \pm 0.10^{a}$ | $0.39 \pm 0.01^{de}$ |
| | *Stenotrophomonas* sp. | $0^{e}$ | $0^{e}$ | $0.28 \pm 0.07^{c}$ | $0^{d}$ | $0.24 \pm 0.00^{ef}$ |
| | *Enterobacter mori* | $0.28 \pm 0.07^{b}$ (0.76 U/ml) | $0^{e}$ | $1.37 \pm 0.32^{b}$ | $1.42 \pm 1.22^{ab}$ | $0.78 \pm 0.07^{c}$ |
| | *Klebsiella* sp. | $0.23 \pm 0.06^{bc}$ | $0^{e}$ | $1.58 \pm 0.23^{a}$ | $0.87 \pm 0.34^{abc}$ | $0^{g}$ |
| Stem | *Enterobacter hormaechei* | $0.27 \pm 0.03^{b}$ (0.72 U/ml) | $0^{e}$ | $0^{d}$ | $0^{d}$ | $1.21 \pm 0.20^{b}$ (0.64 U/ml) |
| | *Enterobacter* sp. | $0.67 \pm 0.20^{a}$ (0.94 U/ml) | $0^{e}$ | $0^{d}$ | $0^{d}$ | $0^{g}$ |
| | *Bacillus velezensis* | $0^{e}$ | $0^{e}$ | $0^{d}$ | $0^{d}$ | $0.13 \pm 0.02^{ef}$ |
| | *Acinetobacter baumannii* | $0^{e}$ | $0.23 \pm 0.06^{d}$ | $0^{d}$ | $0^{d}$ | $0^{g}$ |
| | *Curtobacterium luteum* | $0^{e}$ | $0^{e}$ | $0^{d}$ | $0^{d}$ | $3.25 \pm 0.47^{a}$ (0.98 U/ml) |
| | *Bacillus cereus* | $0^{e}$ | $0.53 \pm 0.06^{c}$ | $0^{d}$ | $0^{d}$ | $0.78 \pm 0.07^{c}$ |
| No. of isolates producing enzymes | | 10 | 4 | 3 | 11 | 16 |
| Percentage of isolates producing enzymes | | **(47.62%)** | **(19.05%)** | **(14.29%)** | **(52.38%)** | **(76.19%)** |

*megaterium* being prominent producers of the same (Hussain *et al.,* 2013). In the present study, out of the 10 bacterial isolates that produced amylase, 60% were *Bacillus* sp. and the rest were *Enterobacter* sp. and *Klebsiella* sp. These corroborate the previous reports where *Bacillus* sp. was identified as eminent producers of amylase, cellulase, pectinase, and protease (Castro *et al*., 2014; Ferreira *et al.,* 2019; Hussain *et al*., 2013; Singh *et al*., 2019). Cellulase activity was displayed by the endophytic strains isolated from root and stem only, and was recorded highest for the root associate, *Bacillus amyloliquefaciens*. While existing scholarly inquiries into cellulase activity amongst bacterial consortia associated with orchids remain relatively scarce, it is notable that bacterial cohorts linked with terrestrial flora and mangrove ecosystems have been documented as proficient cellulose producers (Anu *et al*., 2014; Kushwaha *et al.,* 2020).

Lipase activity was displayed by the leaf associates only; EI highest for leaf endophytes. The presence of lipolytic activity amongst endophytes may be suggestive of their ability to dissolve cuticular wax, thereby facilitating endophytic colonization (Sopalun and Iamtham, 2020). The ability of leaf associated isolates to produce lipase may be attributed to its capability in coping with the phenolic compounds present in leaves (Carroll and Petrini, 1983). Pectinase activity was displayed by the root and leaf isolates. Amongst bacterial isolates, *Bacillus* sp. and *Enterobacter* sp., especially the endophytic ones showed notable activity. The presence of pectinase activity in an endophytic

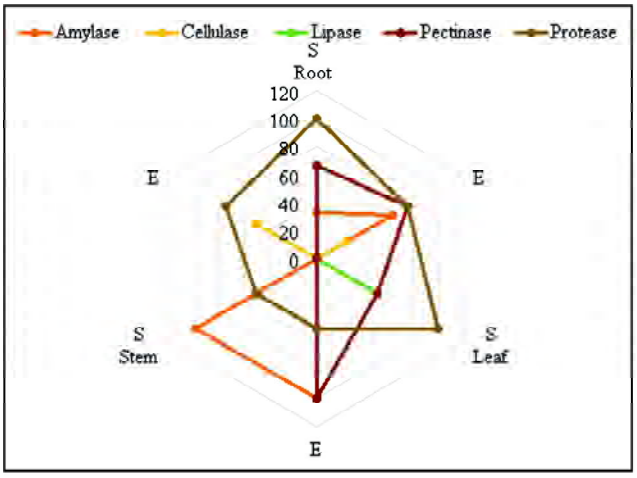


Fig. 2. Percentage of bacterial isolates from different tissues of *Dendrobium ovatum* producing extracellular enzymes on solid nutrient media (S, Surface Associates; E, Endophytes).

microbe may be indication of its latent pathogenic nature (Choi *et al.,* 2005). The presence of cellulase activity along with pectinase activity in an endophyte may be indicative of the ability of the microbial associate to penetrate the tissue and decompose the dead cells (Sieber-Canavesi *et al*., 1991).

The present study showed that 76% of the isolates were producing protease enzyme, where EI was recorded highest for *Curtobacterium luteum*. The root associates were found to be eminent protease producers (77%), followed by stem (75%), and leaf associates (60%). These enzymes garner particular interest for their capacity to degrade proteins, helping in nutrient uptake, and imparting stress tolerance to the plants (Ghosh *et al.,* 2023). The absence of laccase activity in orchid associates may be attributed to their habit, as the enzyme laccase is frequently associated with wood degrading fungi (Singh and Sharma, 2010). The isolates from all the three organs showed amylase and protease activity. A comparative account on the production of enzymes by the microbial associates from different

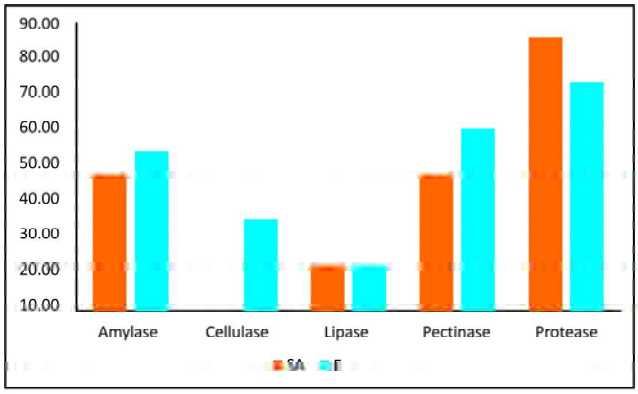


Fig. 3. Percentage of surface associated (SA) and endophytic (E) bacterial associates of *Dendrobium ovatum* producing extracellular enzymes on solid nutrient media.

tissues of *D. ovatum* is depicted in Fig. 2. Fig. 3 represents the percentage of surface associates and endophytes of *D. ovatum* producing extracellular enzymes in solid media. The correlation plot shows positive correlation between amylase and lipase (Pearson correlation coefficient, rs=0.28), cellulase and pectinase (rs=0.11), lipase and pectinase (rs=0.23) (Fig. 4).

In the present study, none of the isolates were found to possess all the enzymatic activities simultaneously. This is in accordance with the findings of Shubha and Srinivas (2017) and Sopalun and Iamtham (2020), who reported similar findings in the endophytes from orchids. The taxonomic affinity of the host, microbe under study, as well as the origin of microbe (surface associate or endophyte) and the tissue from which it was isolated, may influence the substrate preference and consequently, the enzymes produced (Shubha and Srinivas, 2017; Sopalun and Iamtham, 2020; Sunitha *et al*., 2013). Moreover the growth-promoting properties of these microbial associates can be successfully employed in sustainable agricultural practices as discussed in Raj *et al*., 2024b. Carroll and Petrini (1983) suggested that biochemical partitioning of resources

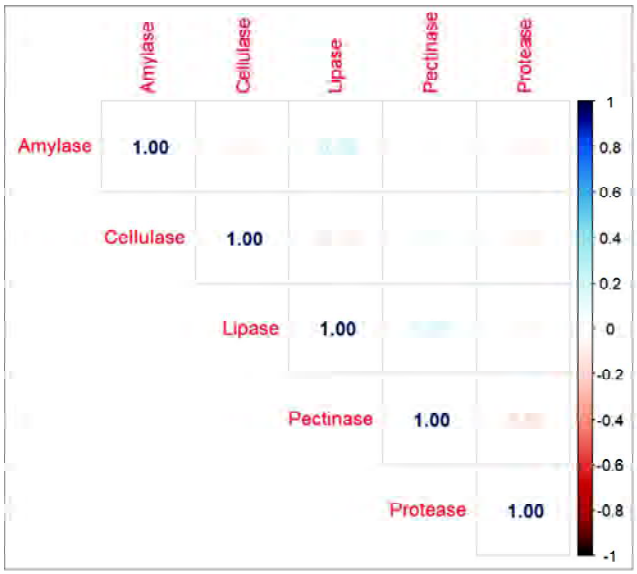


Fig. 4. Correlation plot showing the relation between extracellular enzyme production by the bacterial associates of *Dendrobium ovatum*.

occur where microbes co-occur. Thus, it may be concluded that microbes occupying same niche (organs) may utilize different substrates so as to avoid competition and niche overlapping.

## Conclusion

The present study reported the extracellular enzymatic activities of the bacterial associates of the endemic

orchid, *D. ovatum.* In addition to the industrial applications, these microbial enzymes are pivotal for nutrient cycling, disease suppression, and nitrogen fixation, bolstering plant vitality, development, and yield in both natural and agricultural environments.

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