J. Appl. Biosci., 49(1&2): 17-21, January-December, 2023

ISSN (Print) 0975-685X
ISSN (Online) 0975-864X

PREVALENCE OF AMYLASE PRODUCING BACTERIA FROM THE GUT OF *APIS DORSATA FROM* CENTRAL INDIA

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ABSTRACT

A*pis dorsata* is a wild honey bee as a social insect that represents its unique ability to produce honey by utilizing nectar of flowers with the help of unique gut bacterial community. The present study aimed to indentify the amylase positive gut bacteria in *A. dorsata* using 16S rRNA from the forest around Wardha, Maharashtra, India. More than 30 colonies obtained from gut sample indicated as amylase positive. The better amylase producer identified as *Bacillus aryabhattai* Accession number MW534731 which is reported for the first time in *A. dorsata* from this region.

KEY WORDS: Amylase producer, *Bacillus species*, *Apis dorsata,* 16S rRNA

INTRODUCTION

The commercial use of amylase enzyme finding new avenues and being regularly used in many biotech industries. Presence of amylase in almost all like plants, animals and microbes provides wider diversity to investigate (Engel *et al*., 2016). Amylase is a biocatalyst, able to initiate many biological reactions in organisms. However, few species utilize their amylase for the reaction; instead, they utilize the symbiotic microbial amylase to carry out better properties and yield (Kanmani *et al*., 2011). Wang *et al*. (2014) mentioned amylase producing *Bacillus species* does appear in *Apis cerana* and *Apis mellifera* during rape blooming period which confirms the dominance of *Bacillus species* in the gut. The microbial cell can produce amylase in two forms, termed as exoenzymes and endoenzymes. Exoenzymes release out of the cell and mainly act on any available substrate. These enzymes can hydrolyze high molecular weight substrate into small components that the cells can readily utilize once they are assimilated. Most of the molecules of high molecular weight like starch, pectin, lipids are hydrolyzed by exoenzymes.

Wang *et al.* (2015) investigated honey bees, *Apis mellifera* and reported the presence of *Bacillus species* from the foregut. The study suggests that, α-amylase acts as exoenzyme able to carry out carbohydrate hydrolysis by breaking α-1,4-glycosidic linkage in straw into a low molecular weight product such as glucose, maltose and maltotriose units. Hence α-amylase is in demand from industrial units. The amylase industrial market stands about 30% of the total enzyme market and finds typical applications in pharmaceutical, energy, detergent industry, paper industries and many others.

Taken into consideration, amylase is now extracted from various sources and α-amylase (α-1,4-glucan-4-glucanohydrolase) found in microorganisms, plants, and major animals. α-amylase derived from bacterial community finds its unique place as can be scaled up very quickly (Bankova *et al.*, 2000, Behal *et al.*, 2006). The most-reported genus *Bacillus* mainly the *B. licheniformis, B. amyloliquefaciens,* and *B. stearothermophiles* utilized the most for industrial amylase production.

Among the insect families, Honey bee finds its better place as it gives many useful products to the human. As a social insect, the honey bee represents its unique gut bacterial community. As reported, *Apis dorsata* gut harbours many unique bacterial species and demands further investigation (Niode *et al*., 2021). Using 16S rRNA barcoding studies, many reported the number of phyla in honey bee gut, especially Firmicutes, Proteobacteria and Actinobacteria. The highest count reported with *Bacilli species* and found to be industrially important. The amylase

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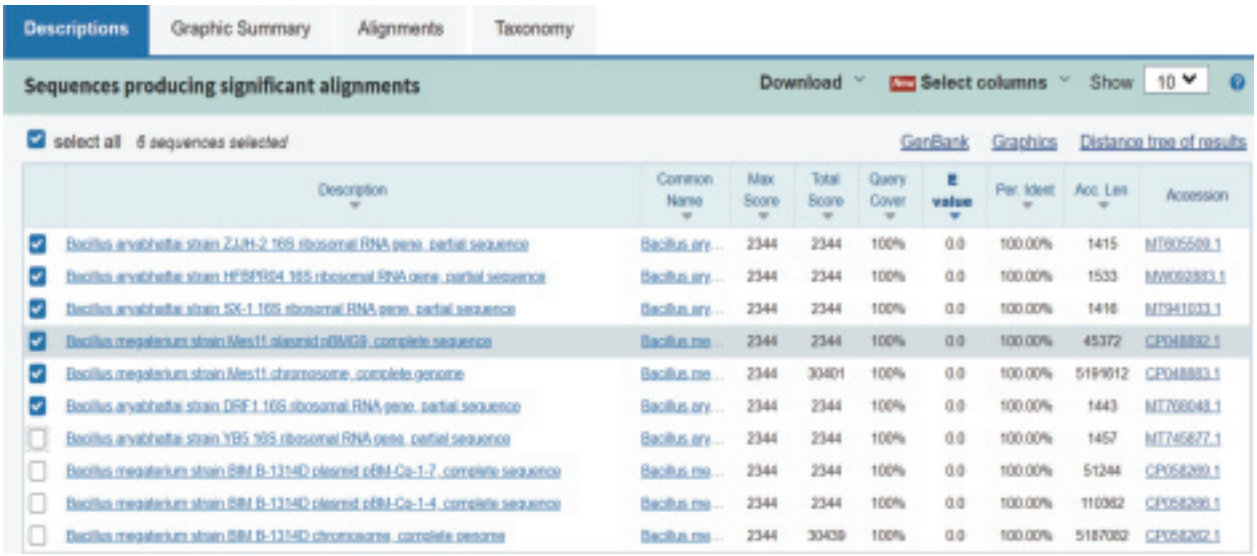

Fig.1: Amylase producing B1 isolate identified as *Bacillus aryabhattai* once isolated from the midgut of the Honey bee *Apis dorsata* as per 16S rRNA gene alignment

of *B. aryabhattai* successfully increased for the yield and activity once supplemented with starch, peptone, yeast extract indicated the mass production is feasible (Ojha *et al*., 2020). Being a social insect honey bee gut possesses a unique microbiota community since honey bee shares a commonplace with many other honey bees; they share common food and close interaction. Therefore, these microbiomes have easy access for easy propagation among each other. Therefore, in the present study attempt been made to isolate and identify the amylase producing bacterial species present in the gut of *Apis dorsata* prevalent in Wardha, Maharashtra, India.

MATERIALS AND METHOD

16S rRNA gene sequencing:

The honey bee midgut spanning amylase positive isolate B1 targeted for 16S rRNA gene by PCR and then sequenced to identify upto species level as per protocol given below.

1) Genomic DNA isolation:

The CTAB protocol used for the genomic DNA separation is as follows: 1.5 ml pre-grown amylase positive isolate B1 in nutrient broth centrifuged to pellet. Pellet suspended in 1.5 ml Tris EDTA and then centrifuged at 10000 RPM for 5 minutes. The step repeated by decanting and further addition of TE buffer and centrifuged. To the pellet 740 µl, TE buffer added with 20 µl Lysozyme (100 mg/ml) and mixed well by incubating for 5 minutes. The sample then added with 40 µl 10% SDS, 8 µl proteinase K (10 mg/ml) and the mixture then incubated at 37°C for 1 hour. The sample then added with 100 µl of 5 M NaCl. The sample supplemented with 100 µl CTAB/NaCl solution and preheated at 65°C. The final mixture then incubated at 65°C in a pre-adjusted water bath for 10 minutes. Upon incubation, the mixture was supplemented with 0.5 ml chloroform: isoamyl alcohol (24:1) and mixed well. Then tubes were spun down at 10000 RPM for 10 minutes at room temperature. The developed upper layer used and added with 0.5 ml phenol: chloroform: isoamyl alcohol (25:24:1). The sample then centrifuged at a maximum speed of 10 minutes at room temperature; the upper aqueous layer then transferred to a fresh tube. The sample then supplemented with 0.6 volume of isopropanol and incubated for 30 minutes at -20°C. The sample then centrifuged at 10000 rpm for 15 minutes. The supernatant then decanted, and the pellet washed with 70% ethanol. The supernatant removed and pellet kept for air drying at room temperature for 5-10 minutes by centrifuging. 20 µl of TE added to dry pellet and after resuspension stored at

Fig. 2: Phylogram showcasing the close alignment of *Bacillus arybhattai* with 16S rRNA query sequence (B1) indicating the best scored homology

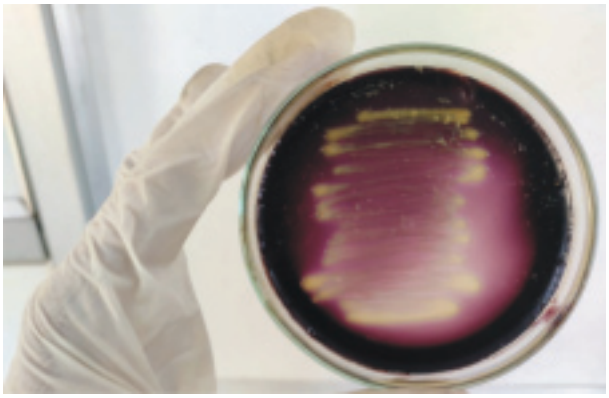

Fig. 3: Amylase positive *A. dorsata* midgut extract spanning isolate B1 showcasing the clear zone aournd the colonies on the starch agar plates flooded with iodine solution

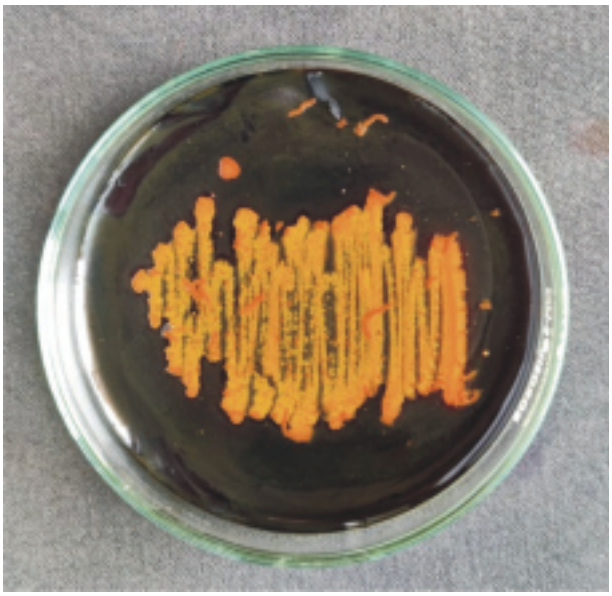

Fig. 4: Amylase negative *A. dorsata* midgut extract spanning isolate B2 showcasing no zone aournd the colonies on the starch agar plates flooded with iodine solution

-20°C. The sample then further used for PCR amplification as described by Rai *et al* (2013).

PCR amplification:

About 200 ng of bacterial DNA targeted for its 16S rRNA gene region using universal primers

16S forward primer 5' AGA GTT TGA TCC TGG CTC AG 3'

16S reverse primer 5' AAG GAG GTG ATC CAG CCG CA 3'

To the total 50 µl reaction mixture, PCR reaction program as 58°C, 57°C, 56°C, 54°C, 52°C, 50°C and 48°C. Scale upcycle sequencing carried out at 54°C using thermal

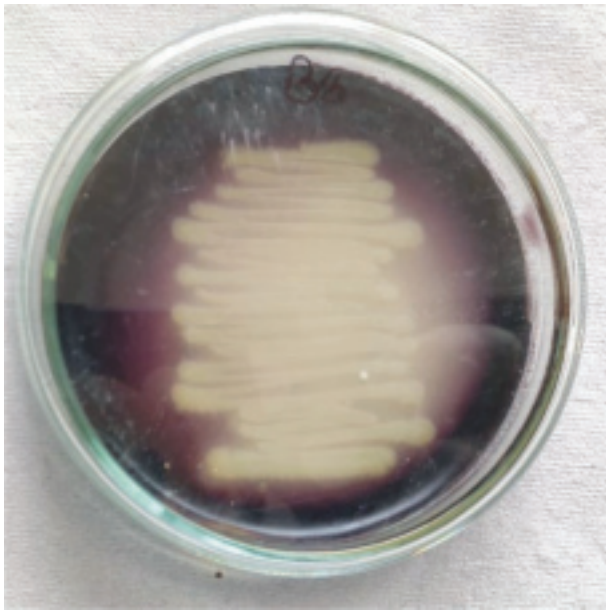

Fig. 5: Amylase positive *A. dorsata* midgut extract spanning isolate B3 showcasing rough colonies on the starch agar plates flooded with iodine solution

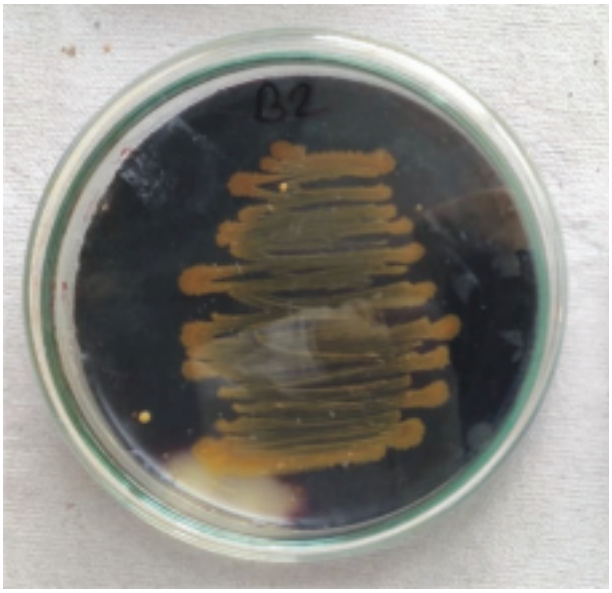

Fig. 6: Amylase negative *A. dorsata* midgut spanning isolate B2 showcasing smooth colonies on the starch agar plates flooded with iodine solution

cycler. The PCR conditions set as initial denaturation of 3 minutes at 94°C, denaturation of 1 minute at 94°C, primer annealing for 1 minute at 54°C, an extension of 2 minutes at 72°C and final extension for 5 minutes at 72°C total 30 cycles carried out. The amplicon stored at 4°C.

The PCR amplicon (50 ng) then processed with 8 µl of ready reaction mix and 5 PMol of forward primer for sequencing. The cycling conditions used as follows 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The sequencing then carried out in ABI Prism 3100 genetic analyzer.

Homology search:

The partial sequencing >1000 base pairs then aligned with public nucleotide database available with the BLAST program at NCBI website. The program nucleotide BLAST N used to obtain the best homology. The best five sequence homologs then used to build the phylogram using MEGA5 software.

RESULTS AND DISCUSSION

The presence of amylase producing bacterial species reported to increase the amylase level in the nectar and useful in processing floral nectar into honey. In the present study, Indian wild honey bee *Apis dorsata* abundant around Wardha, Maharashtra investigated for the presence of amylase producing bacterial species in the gut possibly involved in supplying amylase function for converting nectar to honey. Wang *et al.* (2014) in earlier studies also mentioned amylase producing *Bacillus species* does appear in *Apis cerana* and *Apis mellifera* during rape blooming period which confirms the dominance of *Bacillus species* in the gut. Later Wang *et al*. (2015) reported the presence of *Bacillus species* able to produce amylase once isolated from the foregut of European honey bee, *Apis mellifera* . In the present study as per 16S rRNA gene sequencing >1000 base pairs sequence of bacterium reported BLASTN based homology with *Bacillus aryabhattai* and noted to be another *Bacillus species* prevalent in *Apis* family gut region. This is probably for the first time we are reporting *Bacillus species* capable of amylase production prevalent in *A. dorsata* as shown in Fig. 1 with BLASTN information and Fig. 2 with a phylogram. The *Bacillus arybhattai* identified in the present study assigned with accession number MW534731.

As per initial culturing of midgut on the nutrient agar 90% population, the present study recorded amylase positive on the starch agar plate once flood by iodine solution. The positive amylase nature confirmed by the appearance of no blue colour zone around the confluent colonies, as reported in Fig. 3. In contrast, few amylase negative colonies have also been successfully grown on the starch agar plate, as shown in Fig. 4. During growth on the starch agar plate, it has been observed that those all-amylase positive bacterial species grown with rough, dry colonies while amylase negative isolated give shiny, sticky colonies as can be seen in Fig. 5 and Fig. 6, respectively. This colony characteristics could be the amylase producing bacterial species once mass screened on the starch agar plate.

In recent time, amylase use has increased many folds and specifically *B. aryabhattai* KIIT-BE-1 based amylase finds special attention. The amylase of *B. aryabhattai* successfully increased for the yield and activity once supplemented with 10.0% starch, 5.0% peptone, 5.18% yeast extract indicated the mass production is feasible (Ojha *et al.,* 2020).

In the earlier study, amylase gene of *B. aryabhattai* successfully cloned in recombinant *Brevibacillus choshinensis* and media optimized by involving glucose, pig bone peptone, Mg^{++} and trace elements. They received a yield at 925.4 Uml^{-1} seven-fold higher amylase levels than the initial medium (Duan *at al.,* 2019). Similarly, *A. dorsata* midgut prevalent for amylase producing *B. aryabhattai* strain B1 could be investigated further to produce amylase *in vitro*.

In the present study, amylase producing bacterial species for the first time isolated from the wild honey bee *Apis dorsata* identified as very well-known *Bacillus aryabhattai.* The isolate B1 confirmed as *B. aryabhattai* (Accession No.: MW534731) by 16S rRNA gene sequencing and appeared to be giving rough, dry colonies on the starch agar identifier for amylase producers as noted in 90% of the isolates. Therefore, it would be desirable to either clone amylase gene for overexpression in the selected host or desired media could be set in for increased amylase production so that its industrial applications could be investigated. As per the study, *A. dorsata* once again showcased the amylase producing *Bacillus* species dominance as compared to other bee species.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, CSBR for providing facilities for the research and Dr. M. M. Rai and Dr. M.K. Rathod for their valuable technical support during investigations.

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