

Research article

Polysaccharide hydrolyzing enzyme activity of bacteria, native to *Apis florea* gutD. N. Ganeshprasad¹, Yalpi Karthik², H. R. Sachin¹ and A. H. Sneharani¹¹Department of Studies in Biochemistry, ²Department of Studies in Microbiology, Mangalore University, Jnana Kaveri Post Graduate Centre, Chikka Aluvara, 571232, Kodagu, Karnataka, India

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ABSTRACT

Introduction and Aim: *Apis florea* commonly known as “dwarf honey bee” harbors enormous gut bacteria that can digest complex carbohydrates and other food components. In this regard, the present investigation was focused on analyzing the polysaccharide degrading ability of bacteria isolated from the gut of honeybee, for their possible application in nutraceutical and pharmaceutical industries.

Materials and Methods: Nine bacterial isolates were screened for carbohydrate degrading enzymes viz., amylase, pectinase, cellulase, tannase and laccase, using respective substrate by plate assay method. Further activities of amylase and pectinase were measured quantitatively by dinitrosalicylic acid (DNS) method.

Results: All the nine selected isolates exhibited amylase and pectinase activities. However, only two isolates exhibited lignolytic and cellulolytic activity. None of the isolates showed tannin degradation. Maximum amylase activity (4.95 U/mg) was observed in *Bacillus halotolerans* af-M9 followed by *Klebsiella oxytoca* af-G4 (4.62 U/mg). With respect to pectinase activity *Klebsiella pneumoniae* af-E17 displayed higher activity (0.24 U/mg) followed by *Klebsiella oxytoca* af-G4 (0.20 U/mg).

Conclusion: Habitat-specific innovations are being explored for novel compounds for therapeutic applications. This study throws a light on selection of carbohydrate degrading bacteria from a new source i.e., GUT of honeybee.

Keywords: Honeybees; extracellular enzymes; gut bacteria; carbohydrates; fermentation.

INTRODUCTION

The gut environment is a source of unexploited microorganisms and they play an important role in the degradation of macromolecules. Consequently, the insect guts are a rich source of novel compounds employed in pharmaceutical, food, fine chemicals, and enzyme industries. Gut bacteria perform a wide range of actions that have crucial implications for the host metabolism and overall health. Gut microbial populations are vital for many host organisms, from breaking down of indigestible carbohydrates to immunomodulation (1). Understanding the polysaccharide degrading ability of symbiotic gut microbes has the potential in the development of therapeutic and prophylactic treatments for the benefit of human and animal health (2).

Insect diets are rich in plant polysaccharides, but generally lack the enzymes to metabolize these substrates. In general, many rely on gut bacteria that can metabolize these polysaccharides. On hydrolysis, the simple sugars and short-chain fatty acids are easily absorbed and thereby has an indirect influence on, host physiology and health. Honey bees have the similar process for polysaccharide digestion (3).

Bees acquire carbohydrates and amino acids from nectar and pollen, which also includes polysaccharides like pectin, cellulose, and hemicellulose. These potential energetic

compounds are processed by microbial enzymes leading to easy digestion and absorption. However, the role of individual microbiota in polysaccharide digestion has remained unexplored (4).

The abundance of carbohydrate in bee diet explains the ability of its gut microbiota to thrive on such resources. Several of the bee-gut associated bacteria are known to possess genes that can express enzymes which can hydrolyze rarer sugars such as pectin, lignin and cellulose (5). The commercial value of various enzymes has increased over the last two decades, especially in conventional fields such as the food and detergent industries, but it is still improving, especially in areas like pollution control, medical applications, and biosensors. The global enzyme market is estimated to be worth around US \$1.5 billion and growing at a rate of 5- 10% per year (6). Only a few studies have been carried out in detail to elucidate the capacity of bee gut bacteria to produce extracellular enzymes (7).

Such extracellular associations, however, are thought to be susceptible to invasion and replacement by transient microbes. Most of the research was carried out to understand the interactions between the host and the symbiotic microbiota. The discovery of novel genes and enzymes from different natural sources has recently become a subject of both basic and applied biotechnology research. Soil and other niches in the ecosystem were thought to be significant sources of novel biomolecules (8). The multienzymes activities

of bacterial isolates from the bee gut could provide a potential source of enzymes for biotechnological applications (4). The present study was primarily intended to find new strains for potential applications, but it may also partly contribute to the knowledge of the nutritional versatility of the isolates, and to some extent, their ecological importance. In the present study, we report on the screening carried out on nine bacterial isolates (obtained from gut samples of *A. florea* honeybees collected in Kodagu, Western Ghats Forest of India) to assess their ability to produce five extracellular enzyme activities.

METHODOLOGY

Bacterial cultures and growth conditions

The nine bacteria isolated from honey bee gut was grown in nutrient broth at 37 °C for 24 h under constant shaking condition (150 rpm). For determination of extracellular enzymatic activity, the culture grown in nutrient broth was centrifuged at 8000 rpm for 10 min at 4 °C. The cell free supernatant (CFS) thus obtained was stored at -20°C until use.

Amylase activity by plate assay method

Starch media were prepared with 1% soluble starch and 1.5% agar. Plates were spot inoculated (fresh overnight grown isolates) at room temperature. The plates were incubated at 37 °C for 24 h. After incubation, plates were flooded with iodine-potassium iodide solution (1.0g iodine, 5.0g potassium iodide and 330ml H₂O) and observed for the zone of clearance.

Pectinase activity by plate assay method

Pectin media were prepared (0.3% KH₂PO₄, 0.6% Na₂HPO₄, 0.2% NH₄Cl, 0.5% NaCl, 0.01% MgSO₄. 7H₂O, 1.5% agar) and supplemented with 1% pectin. The fresh overnight grown isolates were spot inoculated and incubated at 37 °C for 24 h. After incubation, plates were flooded with 1% aq. hexadecyltrimethyl ammonium bromide and observed for the zone of clearance (9).

Lignolytic activity by plate assay method

The isolates were grown in nutrient broth supplemented with 0.01% guaiacol for 24 h at 37 °C. Azure B media was prepared by supplementation of 6.5 mM Azure B in nutrient media (10). The freshly grown selected bacterial cultures were streak inoculated on the media plates at equidistance plates were incubated at 37 °C for 24 h. After 24-48 h of incubation, a positive reaction indicated by the zone of clearance around the colonies was noted.

The extracellular lignolytic activity was confirmed on Azure B plates by using cell-free supernatant (CFS) of isolates. The wells of 4 mm were made on Azure B plate and the CFS (100 µl) was filled. Plates were carefully incubated at 37 °C for 24 h. The positive

reaction was confirmed by the zone of clearance around the wells.

Cellulolytic activity by plate assay method

Cellulose degrading ability of selected isolates was analyzed by using carboxy methyl cellulose (CMC) as substrate (11). The freshly grown bacterial cultures were streaked on CMC agar plates (1% CMC; 0.1% yeast extract, 0.5% NaCl and 1.5% Agar) at equidistance and incubated at 37° C. After 24-48 h of incubation, plates were flooded with 1% Congo red solution and incubated at room temperature for 5 min. Later, plates were repeatedly washed 5-6 times in 0.1M NaCl solution and observed for the zone of clearance around the positive colonies.

The extracellular cellulolytic activity was confirmed by well diffusion method using CMC plates. The wells of 4 mm were made on CMC plate and the CFS (100 µl) was filled. Plates were carefully incubated at 37 °C for 24 h. The positive reaction was confirmed by the zone of clearance around the wells after Congo red staining.

Tannase activity by plate assay method

Tannase activity was assessed by using nutrient broth supplemented with 1% tannic acid (12). Plates were inoculated with each bacterial isolate on tannin plates and incubated at 37 °C for 24 to 48 h. After incubation, plates were flooded with FeCl₃ solution (0.01 M FeCl₃ in 0.01 N HCl), kept at room temperature for 5 min. However, the positive isolates give a clear zone around the colonies indicating tannin degradation.

The extracellular tannase activity was confirmed on tannin plates by using cell-free supernatant of given isolates. The isolates grown in nutrient broth was centrifuged at 10,000 rpm for 10 min at 4 °C to collect the CFS. The wells of 4 mm were made on tannin plate and the CFS (100 µl) was filled. Plates were carefully incubated at 37 °C for 24 h. The positive reaction was confirmed by the zone of clearance around the wells after FeCl₃ staining.

Protein estimation by Lowry's method

Standard protein graph

The standard graph was constructed by taking the concentration of BSA on X-axis and OD on Y- axis. The scattered points were linearized and trendline was drawn to generate regression co- efficient equation (13).

Protein estimation in test sample

The overnight isolates grown in nutrient broth were centrifuged at 10,000 rpm for 10 min at 4 °C to collect the CSF. CFS (10 µl) was taken in clean test tubes and the volume was made up to 250 µl using distilled water. The same procedure was followed as described above and the absorbance was measured at 650 nm. The absorbance value obtained was then compared

with the standard graph and the values are expressed in mg/ml.

Amylase assay

Amylase activity was measured according to Oluoch et al., with slight modification using DNS reagent (14).

Construction of glucose standard graph

Glucose stock (1 mg/ml) was distributed into clean test tubes at different concentrations (50-500 µg/ml) and the volume was made up to 1 ml with distilled water. The reaction was initiated by boiling in water bath after addition of DNS reagent (1 ml). Later, the reaction was stopped with addition of 5 ml of distilled water and absorbance was measured at 550 nm.

The standard glucose graph was made by taking the concentration of glucose on X-axis and absorbance on Y-axis. The scattered points were linearized and trendline was drawn to generate regression coefficient equation.

Amylase activity of bacterial isolates

The crude enzyme (200 µl of CFS) was allowed to react with 800 µl of 1% starch solution (prepared in 0.05 M sodium phosphate buffer; pH 7.0) at 37 °C for 15 min. After incubation, the amount of glucose released was measured using DNS reagent as described earlier. One unit of amylase is expressed as the quantity of enzyme required to catalyze starch for the formation of reducing sugar under assay conditions.

Pectinase assay

Pectinase activity was measured according to Oluoch et al., with slight modification using DNS reagent (14).

Construction of galactose standard graph

Galactose stock solution of 1 mg/ml was prepared in distilled water and taken in clean test tubes at different concentrations (50-500 µg/ml). The volume was adjusted to 1 ml using distilled water and mixed with 1 ml DNS reagent. The mixture was boiled for 5 min

and mixed with 5 ml of distilled water. The absorbance was then measured at 550 nm.

The galactose standard graph was created by applying the concentration of galactose on X-axis and OD on Y-axis. The scattered points were linearized and trendline was drawn to generate regression coefficient equation.

Pectinolytic action of bacterial isolates

The crude enzyme (200 µl) was mixed with 800 µl of 1% pectin solution (prepared in 0.2 M citrate phosphate buffer; pH 6.0) and allowed to hydrolyze for 15 min at 37 °C. The reaction was stopped by addition of DNS reagent and the released galactose was measured as described earlier. One unit of pectinase activity is defined as the amount of pectinase required to catalyze the formation of reducing sugar, which is equal to one µmol of galactose per min under assay conditions.

Statistical analysis

The experiments were carried out in triplicates, the data were statistically analyzed using one way ANOVA, and mean separation was determined using Duncan's multiple range test.

RESULTS

Screening for extracellular enzyme activities by plate assay method

Bacterial isolates were screened for amylase, pectinase, cellulase, tannase and laccase enzymes by plate assay method (Fig 1-5b). All the nine selected isolates showed zone of clearance in starch and pectin plates (Fig. 2) indicating their ability to produce amylase and pectinase enzymes. Among the isolates, *B. mojavensis* af-L13 and *B. halotolerans* af-M9 were displaying lignin- degrading ability as presented in Fig.3a, b). On the other hand, *B. mojavensis* af-L13 and *B. halotolerans* af-M9 were able to hydrolyze cellulose (Fig. 4a, b). None of the selected bacterial isolates showed tannin degrading activity as tested on tannin plates (Fig.5a, b).

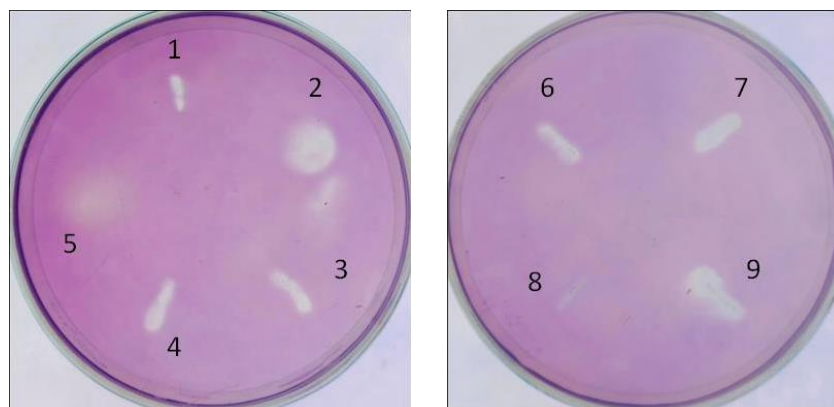


Fig. 1: Amylase activity of bacterial isolates from *Apis florea* gut detected on starch plate (1) *K. oxytoca* af-E5; (2) *K. pneumoniae* af-E17; (3) *K. oxytoca* af-G4; (4) *K. oxytoca* af-L4; (5) *B. mojavensis* af-L13; (6) *K. oxytoca* af-M1; (7) *K. oxytoca* af-M6; (8) *B. halotolerans* af-M9; (9) *E. aerogenes* af-N1.

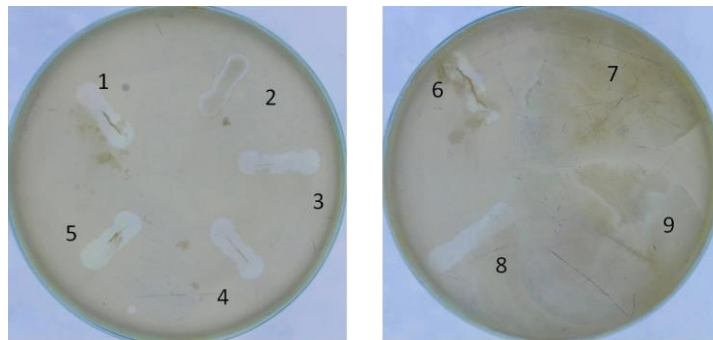


Fig. 2: Pectinolytic activity of bacterial isolates from *Apis florea* gut analyzed on pectin plate. (1) *K. oxytoca* af-M1; (2) *K. pneumoniae* af-E17; (3) *K. oxytoca* af-L4; (4) *K. oxytoca* af-M6; (5) *K. oxytoca* af-G4; (6) *E. aerogenes* af-N1; (7) *B. mojavensis* af-L13; (8) *B. halotolerans* af-M9; (9) *K. oxytoca* af-E5.

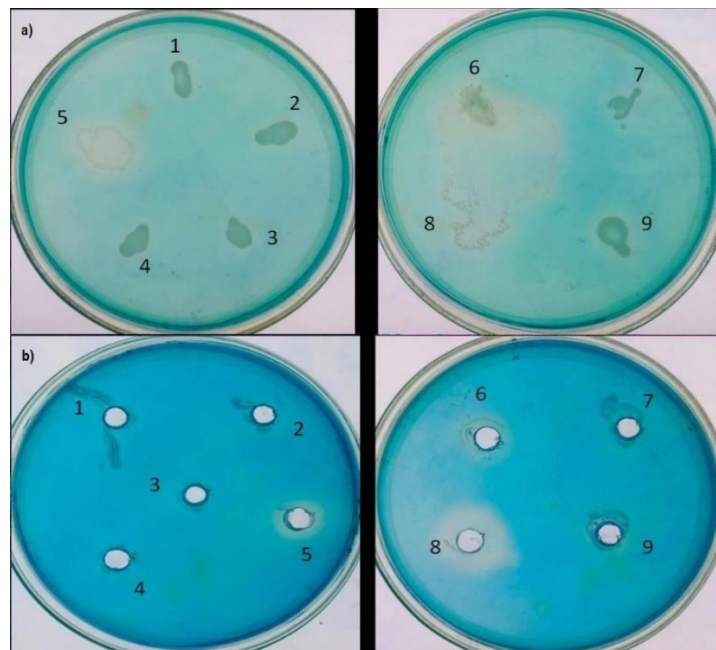


Fig. 3: (a) Lignolytic activity of *Apis florea* gut bacterial isolates on lignin plate; (b) Lignolytic activity of cell-free supernatant for bacterial isolates: (1) *K. oxytoca* af-E5; (2) *K. pneumoniae* af-E17; (3) *K. oxytoca* af-G4; (4) *K. oxytoca* af-L4; (5) *B. mojavensis* af-L13; (6) *K. oxytoca* af-M1; (7) *K. oxytoca* af-M6; (8) *B. halotolerans* af-M9; (9) *E. aerogenes* af-N1.

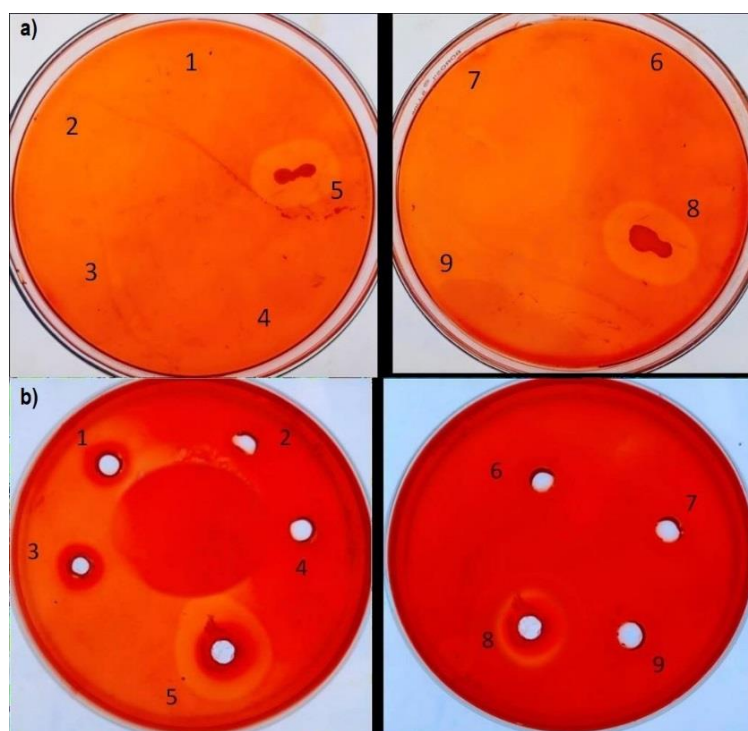


Fig. 4: (a) Cellulolytic activity of *Apis florea* gut bacterial isolates on CMC plate; (b) Cellulolytic activity of cell-free supernatant for bacterial isolates: (1) *K. oxytoca* af-E5; (2) *K. pneumoniae* af-E17; (3) *K. oxytoca* af-G4; (4) *K. oxytoca* af-L4; (5) *B. mojavensis* af-L13; (6) *K. oxytoca* af-M1; (7) *K. oxytoca* af-M6; (8) *B. halotolerans* af-M9; (9) *E. aerogenes* af-N1.

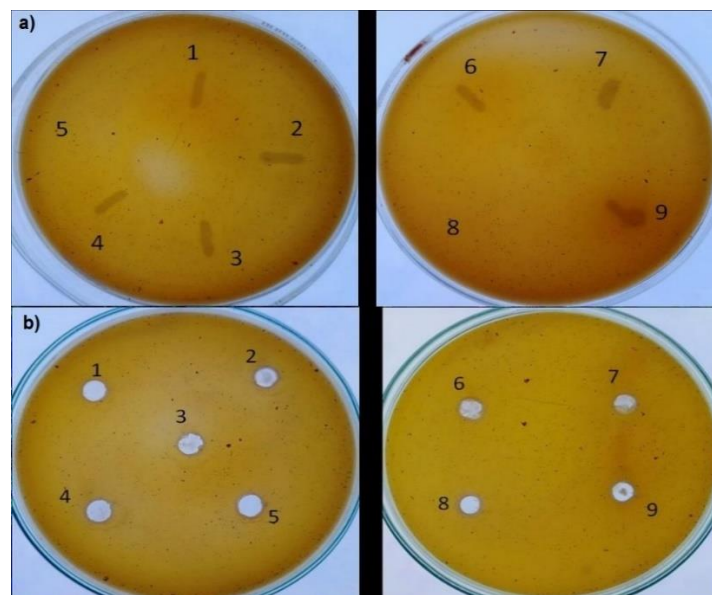


Fig. 5: (a) Tannase activity of *Apis florea* gut bacterial isolates on tannin plate; (b) Tannase activity of cell-free supernatant for bacterial isolates: (1) *K. oxytoca* af-E5; (2) *K. pneumoniae* af-E17; (3) *K. oxytoca* af-G4; (4) *K. oxytoca* af-L4; (5) *B. mojavensis* af-L13; (6) *K. oxytoca* af-M1; (7) *K. oxytoca* af-M6; (8) *B. halotolerans* af-M9; (9) *E. aerogenes* af-N1.

Protein estimation by Lowry's method

Figure 6 displays the standard protein graph constructed using bovine serum albumin (BSA). The results show linear graph with high correlation coefficient ($r^2=0.97$) with $y=0.0164x$. The concentration of proteins in the cell-free supernatant (CFS) of bacterial isolates is shown in table.1. *B. mojavensis* af-L13 demonstrated significantly ($p<0.05$) higher protein content (2.38 mg/ml) compared to other isolates.

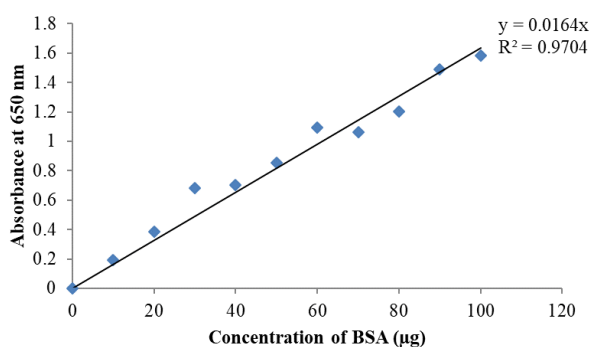


Fig. 6: Standard graph for protein estimation by Lowry's method.

Table 1: Protein content in the CFS of *Apis florea* gut bacterial isolates

Sample	Protein content(mg/ml)
<i>K. oxytoca</i> af-E5	1.95 ± 0.03 ^e
<i>K. pneumoniae</i> af-E17	1.59 ± 0.01 ^b
<i>K. oxytoca</i> af-G4	1.30 ± 0.01 ^a
<i>K. oxytoca</i> af-L4	1.98 ± 0.25 ^e
<i>B. mojavensis</i> af-L13	2.38 ± 0.12 ^f
<i>K. oxytoca</i> af-M1	1.67 ± 0.08 ^c
<i>K. oxytoca</i> af-M6	1.53 ± 0.03 ^b
<i>B. halotolerans</i> af-M9	1.81 ± 0.06 ^d
<i>E. aerogenes</i> af-N1	1.86 ± 0.10 ^d

Note* Values are mean ± SD (n=3); Values in columns with the same superscripts does not differ significantly ($P > 0.05$).

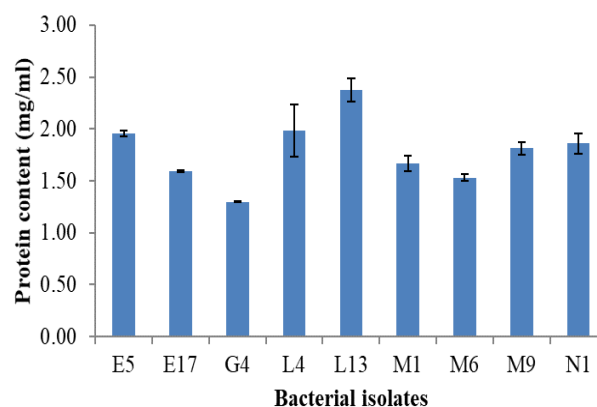


Fig. 7: Protein content in the CFS of *Apis florea* gut bacterial isolates. Note* Values are mean of three independent experiments.

Amylase assay

The standard graph of glucose is been depicted in figure 8. Amylase activity of bacterial isolates are shown in Table 2. According to the results obtained, maximum ($p<0.05$) amylase activity (4.95 U/mg protein) was observed in *B. halotolerans* af-M9 followed by *K. oxytoca* af-G4 (4.62 U/mg protein).

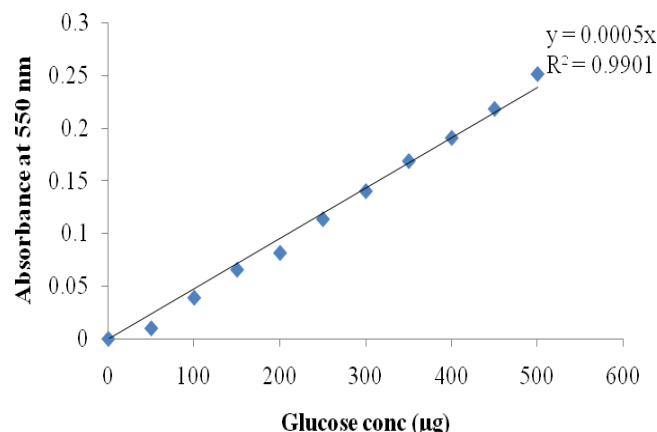


Fig 8: Standard graph of glucose by DNS method.

Table 2: Amylase activity for CFS of *Apis florea* gut bacterial isolates

Sample	Amylase Activity (Unit/ml)	Specific activity (Unit/mg protein)
<i>K. oxytoca</i> af-E5	7.30 ± 0.08	3.74 ± 0.08 ^c
<i>K. pneumoniae</i> af-E17	7.25 ± 0.03	4.55 ± 0.03 ^e
<i>K. oxytoca</i> af-G4	6.00 ± 0.02	4.62 ± 0.02^f
<i>K. oxytoca</i> af-L4	5.91 ± 0.01	2.98 ± 0.01 ^a
<i>B. mojavensis</i> af-L13	7.89 ± 0.03	3.32 ± 0.03 ^b
<i>K. oxytoca</i> af-M1	6.16 ± 0.03	3.68 ± 0.03 ^c
<i>K. oxytoca</i> af-M6	5.86 ± 0.02	3.82 ± 0.02 ^{c,d}
<i>B. halotolerans</i> af-M9	8.97 ± 0.06	4.95 ± 0.06^g
<i>E. aerogenes</i> af-N1	7.40 ± 0.15	3.98 ± 0.15 ^d

Note* Values are mean ± SD (n=3); Values in columns with the same superscripts does not differ significantly (P>0.05).

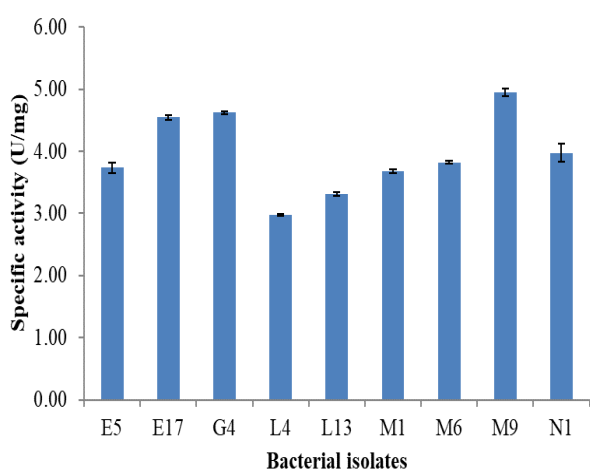


Fig. 9: Amylase activity for CFS of *Apis florea* gut bacterial isolates.

Pectinase assay

Pectinase activity in the bacterial isolates was further quantified by comparing with standard graph of galactose (Fig. 10) Table 3 represents the pectinase activity of bacterial isolates. Accordingly, *K. pneumoniae* af-E17 displayed noticeably higher (p<0.05) pectinolytic activity (0.24 U/mg of protein) followed by *K. oxytoca* af-G4 (0.20 U/mg of protein).

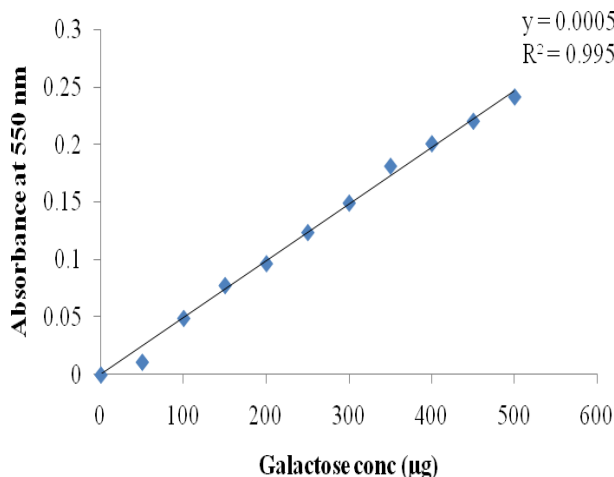


Fig.10: Standard graph of galactose by DNS method.

Table 3: Pectinase activity for CFS of *Apis florea* gut bacterial isolates.

Sample	Pectinase Activity (Unit/ml)	Specific activity (Unit/mg protein)
<i>K. oxytoca</i> af-E5	0.24 ± 0.01	0.12 ± 0.01 ^c
<i>K. pneumoniae</i> af-E17	0.38 ± 0.01	0.24 ± 0.01^g
<i>K. oxytoca</i> af-G4	0.26 ± 0.01	0.20 ± 0.01^f
<i>K. oxytoca</i> af-L4	0.27 ± 0.01	0.14 ± 0.01 ^d
<i>B. mojavensis</i> af-L13	0.17 ± 0.00	0.07 ± 0.00 ^a
<i>K. oxytoca</i> af-M1	0.25 ± 0.01	0.15 ± 0.01 ^e
<i>K. oxytoca</i> af-M6	0.19 ± 0.00	0.12 ± 0.00 ^c
<i>B. halotolerans</i> af-M9	0.16 ± 0.00	0.09 ± 0.00 ^b
<i>E. aerogenes</i> af-N1	0.18 ± 0.00	0.09 ± 0.00 ^b

Note* Values are mean ± SD (n=3); Values in columns with the same superscripts does not differ significantly (P>0.05).

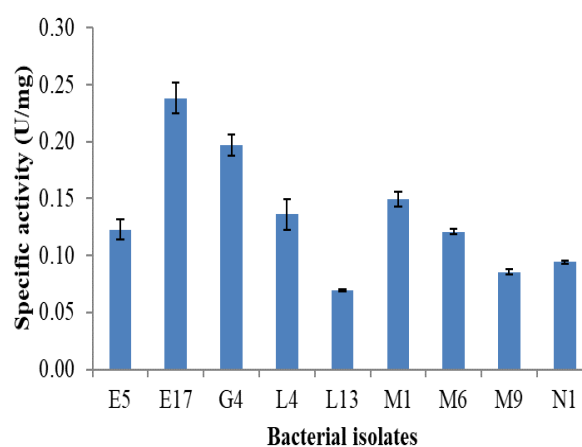


Fig. 11: Pectinase activity for CFS of *Apis florea* gut bacterial isolates.

DISCUSSION

Western Ghats are the global biodiversity hotspot harboring wide distribution of tropical and subtropical forest ecoregions. The diverse flora and fauna of Western Ghats have a great impact on gut flora of insects. *Apis florea*, a wild dwarf bee native to Asia are the critical pollinators of wild ecosystem rather than producing honey (15). Honey bee gut bacteria have a role in neutralizing dietary toxins, nutrient biosynthesis, food component digestion and defense against invading pathogens, which includes the fermentation of complex carbohydrates and other macromolecules that are difficult to digest indigestible (16). The significant modifications that enable this unique microbial community to thrive in the environment of the bee gut have begun to reveal through genomic, transcriptomic, and culture-based investigations. Many gut bacterial species modified their metabolic functions to break down and ferment complex sugars, thereby facilitating host in utilizing carbohydrate-rich diet (17). Many insects lack enzymes capable of breaking down complex and recalcitrant plant cellular components. Some insects, like termites, require symbiotic association with the

gut microbiome for degradation and obtaining energy from a plant-based diet (18).

The present study involved the screening of carbohydrate degrading enzymes from bacteria isolated from *A. florea* gut. The selected bacteria were able to degrade starch and complex carbohydrates like lignin and cellulose were degraded by two bacteria viz, *B. mojavensis* af-L13 and *B. halotolerans* af-M9. Alkaline proteases and thermostable α -amylases produced by *B. mojavensis* SA, a newly isolated bacterium is employed in leather processing, laundry detergent additive and other industrial applications (19).

The amylase and pectinase activities expressed by the bacterial isolates were significant. The gut bacterial flora obtained from *A. florea*, wild bees are dominated by opportunistic environmental microorganisms as they are exposed to stress and protruding colonizers from environmental sources and further studies are required to explore these perturbations. Honey bees feed on pollen and nectar as their dietary sources. The gut of bees constitutes microbes, involved in the degradation of these macromolecules. The gut-associated bacteria of the *A. florea* produce polysaccharide degrading enzymes. The presence of carbohydrate degrading microorganisms, which contribute to host nutrition, is an important factor (17). Amylases are one of the most widely used enzymes in food, fermentation, starch, textile and paper industries (21). Pectinases or pectic enzymes can be extracted from bacteria; are an important class of enzymes for their wide application in paper, food and wine industries and for fruits, vegetables, coffee and tea processing units.

Uchima *et al.*, recently reported heterologous expression of β -glucosidase of termite, *Neotermes koshunensis* in *Aspergillus oryzae* and described its use for bio-ethanol production (22). The proteases from insect gut have been extensively studied due to their potential role in digestion, nutrient absorption, tissue decomposition or remodeling during metamorphosis (23). Further, insect-associated microbes have been greatly explored for their ability to eliminate antinutritive components and to reduce dietary incompatibilities such as celiac disease (24).

The work from earlier investigators support the similar carbohydrate hydrolyzing capacity of microbes such as *Sphaerularia bombi* and *Frischella perrara*, members of the Orbales found in bees, (16, 25). Some strains of *Gilliamella apicola*, include genes that degrade pectin, a component of the cell wall of pollen grains. Pollen is an essential component of the bee diet, and its degradative activity demonstrated in the present isolates may aid in pollen digestion, (16). This is the first report presenting the carbohydrate digesting enzymes from the gut bacteria isolated from wild dwarf honeybee, *A. florea* from Western Ghats. Hence, this study proves that exploration of

unexploited gut inhabitants and their vital role in therapeutics are inevitable.

CONCLUSION

In conclusion, the present study reveals honeybee gut bacterial flora has evolved symbiotically in a foraging environment, rich in dietary plant polysaccharides as a major source of energy. Access to this resource depends greatly on the ability of gut bacteria to ferment and degrade these plant polysaccharides. All of the nine bacterial isolates obtained from *A. florea* possessed amylase and pectinase to some extent as they feed on pollen and nectar sources. The maximum amylase activity (4.95 U/mg) was observed in *Bacillus halotolerans* af-M9, followed by *Klebsiella oxytoca* af-G4 (4.62 U/mg). With regard to pectinase activity, *Klebsiella pneumoniae* af-E17 showed a higher activity (0.24 U/mg), followed by *Klebsiella oxytoca* af-G4 (0.20 U/mg). Cellulase and lignin-degrading activities were expressed by only two bacterial isolates.

This study includes a new source for the isolation of economically significant enzymes such as amylase, pectinase, cellulase and laccase. Further characterization studies are required to investigate the potential sources for industrial applications.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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