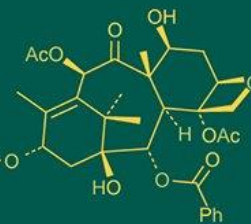
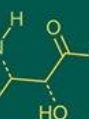
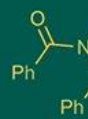


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Prajakta A Phadatre
Department of Entomology,
RCSM Govt. College of
Agriculture, Kolhapur,
Maharashtra, India

Indira A Ghonmode
Department of Entomology,
RCSM Govt. College of
Agriculture, Kolhapur,
Maharashtra, India

Abhaykumar S Bagde
Department of Entomology,
RCSM Govt. College of
Agriculture, Kolhapur,
Maharashtra, India

Ravindra A Karande
Department of Plant
Pathology, RCSM Govt.
College of Agriculture,
Kolhapur, Maharashtra, India

Deepak P Deshmukh
Department of Plant
Pathology, RCSM Govt.
College of Agriculture,
Kolhapur, Maharashtra, India

Mahesh S Pawar
Department of Entomology,
RCSM Govt. College of
Agriculture, Kolhapur,
Maharashtra, India

Mangesh M Ballal
Department of Entomology,
RCSM Govt. College of
Agriculture, Kolhapur,
Maharashtra, India

Corresponding Author:
Prajakta A Phadatre
Department of Entomology,
RCSM Govt. College of
Agriculture, Kolhapur,
Maharashtra, India

Isolation and identification of cellulolytic bacteria from the gut of white grub native to the Kolhapur region

Prajakta A Phadatre, Indira A Ghonmode, Abhaykumar S Bagde, Ravindra A Karande, Deepak P Deshmukh, Mahesh S Pawar and Mangesh M Ballal

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Abstract

The current study was carried out at the Entomology Section Laboratory, Division of Entomology, Rajarshree Chhatrapati Shahu Maharaj Government College of Agriculture, Kolhapur, during the academic year 2025-26. White grubs are important soil dwelling insect pests, and their gut microbiota represents a potential source of microorganisms with diverse enzymatic activities. The present study was undertaken to isolate, screen and molecularly identify cellulolytic bacteria associated with the gut of white grubs native to the Kolhapur region of Maharashtra. Cellulolytic bacteria were isolated from the fermentation chamber of the hindgut using serial dilution and pour plate techniques on cellulolytic agar medium. Three bacterial isolates were obtained from 10^{-5} , 10^{-6} and 10^{-7} dilutions. The isolates were screened on Carboxymethyl Cellulose (CMC) agar using the Congo red staining method. Among the three isolates, HS-1 exhibited the highest cellulolytic index (2.10), followed by HS-2 (1.40), whereas LL-1 did not exhibit a clear hydrolysis zone. The cellulolytic isolates were further characterized and the most promising isolate was selected for molecular identification based on 16S rRNA gene sequencing. The obtained partial 16S rRNA gene sequencing was 445 bp long and showed affiliation with the genus *Hydrogenophaga*. The sequence was deposited in the NCBI GenBank database under accession number PZ783938, with the isolate designated as *Hydrogenophaga* sp. Strain ACK. The findings highlight the insect gut as a promising source of cellulolytic bacteria and provide foundation for further evaluation of their cellulolytic efficiency and potential application in degradation of agricultural residues.

Keywords: 16S rRNA, gut microbiota, Fermentation chamber, cellulolytic bacteria, isolates

Introduction

Cellulose is a natural carbohydrate (a polysaccharide) made of glucose units linked together. It is the main structural polysaccharide of plants and predominant constituent of cell wall of plant cells. Its content ranges approximately 35 to 50 per cent of plant dry weight. This polysaccharide is composed of β -D-glucose units joined by glycosidic linkage. Cellulose is commonly degraded by an enzyme called cellulase (Singh *et al.*, 2023) [13]. Cellulolytic bacteria are capable of producing cellulase enzymes that catalyze the hydrolysis of cellulose into simpler sugars. In recent years, cellulolytic bacteria have emerged as important biological resources for sustainable management of agricultural residues and environmental friendly biomass conversion technologies (Gupta *et al.*, 2011) [6]. The insect gut microbiota performs several important functions including cellulose degradation, nutrient acquisition, detoxification of plant metabolites and maintenance of host fitness (Engel and Moran, 2013) [5]. Due to continuous adaptation to cellulose-rich diets, gut microorganisms frequently possess highly efficient enzymatic systems for lignocellulose degradation. Recent studies have demonstrated that insect digestive systems harbour diverse cellulolytic bacterial groups exhibiting significant cellulase activity (Dar *et al.*, 2021; Schroeder *et al.*, 2022) [4, 11]. Therefore, insect gut microbiota has become an important target for the isolation of novel cellulolytic bacteria with agricultural and industrial significance. Among the various insect groups investigated, white grubs have attracted considerable attention because of their feeding behaviour and digestive capabilities. White grubs are the larval stages of scarab beetles

belonging to the family Scarabaeidae and are widely distributed in agricultural ecosystems. Species such as *Holotrichia serrata* Fabricius and *Leucopholis lepidophora* Blanchard are common in Maharashtra and are considered important pests of sugarcane and groundnut. These grubs spend most of their life cycle beneath the soil surface and feed continuously on roots, decaying organic matter and cellulose-rich plant materials (Bhavani *et al.*, 2024) ^[1]. The remarkable ability of white grubs to survive on cellulose-rich diets suggests the presence of efficient cellulolytic microorganisms within their digestive systems. Previous studies have reported that a substantial proportion of ingested cellulose undergoes degradation within the gut of scarab larvae due to microbial activity (Shengwei *et al.*, 2012) ^[12]. Despite the growing interest in insect-associated cellulolytic microorganisms, information on cellulolytic bacteria associated with the gut of white grubs native to the Kolhapur region of Maharashtra is limited. Isolation and preliminary screening of such bacteria can help identify promising cellulolytic isolates, while molecular characterization provides reliable identification at the bacterial taxonomic level. Therefore the present study provides preliminary information on cellulolytic bacteria associated with white grub gut and establishes a basis for their further characterization and evaluation.

2. Materials and Methods

2.1 Insect Sample Collection

The third instar larvae of white grub species were collected from infested fields in Vasagade (16.67° N latitude and 74.34° E longitude) and Haldi (16.58° N and 74.16° E longitude) village in Kolhapur region. These white grubs larvae were subsequently transported to the Entomology laboratory of R. C. S. M. Govt. College of Agriculture, Kolhapur in sterilized aerated plastic containers. Nearly 60 white grubs larvae of two white grub species were collected and dissected for experiment.

2.2 Insect Dissection

The collected grubs were rinsed by distilled water for 30 seconds followed by 70 per cent ethanol for 60 seconds. Again it was rinsed by distilled water for 30 seconds to remove the disinfectant (Bhavani *et al.*, 2024) ^[1]. The sterilized white grub was mounted on wax dish. The sterilized grubs were dissected under laminar airflow cabinet using sterile microscissors and forceps to extract the gut, specifically isolating and removing the fermentation chamber to extract the gut portion. Different gut sections were incised and separated. The dissected gut section was transferred to sterilized 1.5ml microcentrifuge tubes containing 0.85 per cent NaCl and macerated and used for serial dilution.

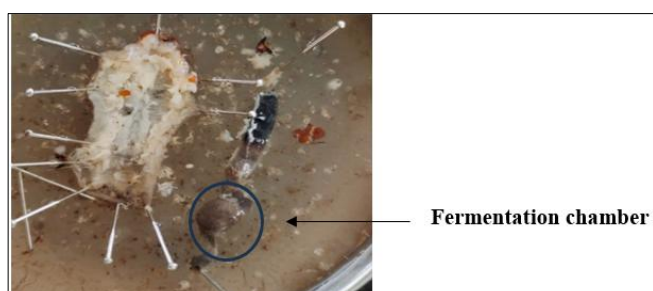


Fig 1: Dissection of white grub

2.3 Isolation of Cellulose Decomposing Bacteria

The cellulolytic media were used for isolation of cellulose decomposing bacteria.

Table 1: Cellulolytic media composition (for 1 Litre)

Chemicals	Quantity (g/L)
KNO ₃	2.00 gm
K ₂ HPO ₄	0.05 gm
MgSO ₄ .7H ₂ O	0.5 gm
MnSO ₄ .H ₂ O	0.02 gm
CaCl ₂ .H ₂ O	0.02 gm
CMC	10 gm
Agar	20 gm
Distilled water	1L.

Isolation of pure culture of cellulose decomposing bacteria was carried out from dissected gut section on cellulolytic medium. Serial dilution were made from 10⁻¹ to 10⁻⁷ dilution. Subsequently, the autoclaved and cooled cellulolytic medium was poured in sterile glass petri plates (approx.. 20ml/plate) and were allowed to solidify. The serially diluted content from 10⁻¹ to 10⁻⁷ was plated with the help of sterilized glass loop in petri plates containing cellulolytic media. The bacterial plates were incubated for 24-48 hrs. at 37 °C and 65±5 per cent RH (Bhavani *et al.*, 2024) ^[1]. Bacterial colonies were selected based on the difference in colour, size and morphology. A single representative isolate of each morphotype was selected and purified by streaking on cellulolytic medium plates repeatedly until the pure bacterial culture was obtained. The purified bacterial colonies were subcultured on agar slants.

2.4 Screening of Cellulolytic Activities of Bacterial Isolates

The ability of the bacterium to degrade cellulose were tested on a CMC (Carboxymethyl cellulose) agar medium with the following composition:

Table 2: CMC agar media composition

Chemicals	Quantity (g/L)
Yeast extract	0.2 gm
CMC	5.00 gm
Agar	15.00 gm
Distilled water	1 L.

The bacterial colonies were transferred on CMC agar medium to check its activity. The bacterial plates were incubated at 28 °C for 4 days and then treated with Congo red i.e. bacterial plates prepared with CMC agar medium were flooded with an aqueous solution of 0.2 per cent Congo red (w / v) for 30 min to confirm the presence or absence of endoglucanase activity. After 30 min, the Congo red solution was poured off and plates were treated by flooding with 1 M NaCl for 15 min. The NaCl solution then poured off and the degradation of CMC was observed as a clear halo around the bacterial colony (Danu *et al.*, 2022) ^[3]. The cellulolytic index was calculated by the following formula (Bhavani *et al.*, 2024) ^[1].

$$\text{Cellulolytic index} = \frac{\text{Diameter of zone of clearance} - \text{Diameter of bacterial colony}}{\text{Diameter of bacterial colony}}$$

Among the purified bacterial isolates, the isolate exhibiting the highest cellulolytic index on CMC agar medium was selected for molecular identification.

2.5 Genomic DNA Extraction

A pure culture of isolate ACK was grown in appropriate bacterial growth medium. Bacterial cells were harvested and genomic DNA was extracted using the DNA extraction procedure followed in the laboratory of Agharkar Research Institute. The extracted genomic DNA was assessed for quality and suitability for PCR amplification.

2.6 Amplification of 16S rRNA gene

The bacterial 16S rRNA gene was amplified using universal bacterial primers. Genomic DNA obtained from isolate ACK was used as the template for PCR amplification. The PCR product was examined by agarose gel electrophoresis. The amplified product was subsequently purified and submitted for sequencing.

2.7 16S rRNA gene sequencing

The purified PCR product of isolate ACK was subjected to nucleotide sequencing. The resulting sequence was processed and used for molecular identification. A partial 16S rRNA gene sequence of 445 bp was obtained from isolate ACK.

2.8 Molecular Identification

The obtained nucleotide sequence was compared with sequences available in the NCBI GenBank database using BLASTn. Based on sequence similarity and taxonomic comparison, isolate ACK was assigned to the genus *Hydrogenophaga* and designated: *Hydrogenophaga* sp. strain ACK

2.9 Sequence Submission to GenBank

The partial 16S rRNA gene sequence of isolate ACK was submitted to the NCBI GenBank database. The sequence was assigned accession number: PZ783938

The GenBank record describes it as *Hydrogenophaga* sp. strain ACK 16S ribosomal RNA gene, partial sequence, with a sequence length of 445 bp.

3. Result and discussion

3.1 Isolation of Cellulolytic Bacteria from the Gut of White Grubs

The gut of white grubs harbours a diverse community of microorganisms that plays an important role in the degradation of cellulosic plant materials. Among these microorganisms, cellulolytic bacteria produce cellulase enzymes that hydrolyzed cellulose into simple sugars, thereby contributing to digestion of plant biomass. The cellulolytic bacteria were isolated from the fermentation chamber of the hindgut of white grub (*Holotrichia serrata*) collected from the Kolhapur region of Maharashtra.

The isolation of cellulolytic bacteria was done on cellulolytic medium by serial dilution and pour plate technique. Carboxymethyl Cellulose (CMC) served as the sole carbon source in the medium, thereby selectively supporting the growth of cellulolytic bacteria capable of producing cellulase enzymes while suppressing non-cellulolytic microorganisms. Therefore, the bacterial isolates recovered on CMC medium were considered potential cellulolytic bacteria. Three distinct cellulolytic bacterial isolates were successfully obtained from the 10^{-5} , 10^{-6} and 10^{-7} serial dilution plates. The isolates were named as HS-1, HS-2 and LL-1.

These findings are in close agreement with Bhavani *et al.* (2024) ^[1], who isolated 17 cellulolytic bacterial isolates from the fermentation chamber of white grub. Among these 7 isolates were derived from *Leucopholis coneophora*, while

10 were obtained from *Holotrichia serrata*. Similarly, Shengwei *et al.* (2012) ^[12] isolated 207 strains of aerobic and facultatively anaerobic cellulolytic bacteria from the gut of *Holotrichia parallela* larvae. Handique *et al.* (2017) ^[7] found 30 cellulolytic bacterial isolates from the gut of *Lepidiota mansueta* larvae.

3.2 Screening of Cellulolytic Bacteria

The bacterial isolates obtained from the gut of white grubs were screened for cellulolytic activity on Carboxymethyl Cellulose (CMC) agar medium using the Congo red staining method. Cellulase producing bacteria hydrolyzed the cellulose present in the medium, resulting in the formation of clear halos around the bacterial colonies after staining. The appearance of these hydrolysis zones confirmed the production of extracellular cellulase enzymes by the isolates.

The cellulolytic index was calculated by observing diameter of zone of clearance and diameter of bacterial colony. Among the 3 bacterial isolates examined, 2 demonstrated cellulolytic activity. The maximum cellulolytic index of 2.1 was exhibited by isolate HS-1. The isolate HS-2 shows 1.4 cellulolytic index. The isolate LL-1 did not show any clear halo around bacterial colony. Based on these results, isolate HS-1 was chosen for further molecular identification because of their promising cellulolytic activities. The cellulolytic indices of the gut bacteria isolated are represented in the Table 3

Table 3: Cellulolytic index of gut bacteria isolated from species of two white grubs

Sr.no.	Bacterial Isolates	Cellulolytic Index
1.	HS-1	2.10
2.	HS-2	1.40
3.	LL-1	0.00

The present findings are in accordance with Gupta *et al.* (2011) ^[6] who found 8 bacterial isolates positive on screening media. Similarly, Handique *et al.* (2017) ^[7] tested 30 bacterial strains for cellulolytic activity using CMC agar medium. Of these strains, 5 degrade cellulose. Biswas *et al.* (2019) ^[2] screened 19 cellulolytic bacterial isolates from the gut of two insect species on CMC agar medium. Likewise, Msango Soko *et al.* (2020), Miyayo *et al.* (2022) and Bhavani *et al.* (2024) ^[1, 8, 9] screened the bacterial isolates for cellulolytic activity using Congo red staining on CMC agar medium.



Fig 2: Screening of bacterial isolate HS-1 on CMC agar by Congo red staining

3.3 Molecular Characterization of Isolate HS-1

The isolate showing highest cellulolytic index i.e. HS-1 was named as isolate ACK for molecular identification. Genomic DNA extracted from isolate ACK was successfully used for amplification of the bacterial 16S rRNA gene. The amplified product was purified and subjected to nucleotide sequencing. The sequencing analysis generated a 445-bp partial 16S rRNA gene sequence.

3.3 Identification of Isolate HS-1

The obtained 16S rRNA sequence was compared with sequences deposited in the NCBI GenBank database. Sequence comparison indicated that isolate HS-1 belonged to the genus *Hydrogenophaga*. The isolate was therefore identified as: *Hydrogenophaga* sp. strain ACK

The corresponding GenBank record identifies the organism as *Hydrogenophaga* sp. strain ACK and records the sequence as a partial 16S rRNA gene sequence.

3.4 GenBank accession number

The 445-bp partial 16S rRNA sequence of isolate ACK was deposited in GenBank under accession number: PZ783938

Table 4: Molecular Identification of Isolate ACK

Parameter	Observation
Host insect	White grub (<i>Holotrichia serrata</i>)
Developmental stage	Third instar
Collection region	Kolhapur, Maharashtra, India
Isolation source	Fermentation chamber
Strain	ACK
Molecular marker	16S rRNA gene
Sequencing method	Sanger dideoxy sequencing
Sequence type	Partial
Sequence length	445bp
Molecular identification	<i>Hydrogenophaga</i> sp. Strain ACK
GenBank accession	PZ783938
Family	Comamonadaceae
Order	Burkholderiales
Class	Betaproteobacteria
Phylum	Pseudomonadota

4. Summary

The present investigation was undertaken to isolate and molecularly identify a gut-associated bacterial isolate from the white grub, *Holotrichia serrata*, collected from the Kolhapur region of Maharashtra. The fermentation chamber of the larva was selected as the source for bacterial isolation. The bacterial isolate obtained from the fermentation chamber was purified and designated as HS-1. For molecular identification, genomic DNA of the selected isolate was subjected to amplification of the bacterial 16S rRNA gene, followed by Sanger dideoxy sequencing. A partial 16S rRNA gene sequence of 445 bp was obtained. Sequence analysis and comparison with sequences available in the NCBI GenBank database resulted in the identification of the isolate as *Hydrogenophaga* sp. strain ACK. The sequence was deposited in GenBank under accession number PZ783938. The isolate was taxonomically placed under the family Comamonadaceae, order Burkholderiales, class Betaproteobacteria and phylum Pseudomonadota. The molecular characterization thus provided sequence-based confirmation of the bacterial identity and documented the occurrence of *Hydrogenophaga* sp. associated with the fermentation chamber of *H. serrata* from the Kolhapur region.

5. Conclusion

The present study successfully isolated and molecularly characterized a bacterial isolate, HS-1, from the fermentation chamber of *Holotrichia serrata*. Based on partial 16S rRNA gene sequencing, the isolate was identified as *Hydrogenophaga* sp. strain ACK. The 445-bp sequence was deposited in the NCBI GenBank database under accession number PZ783938. The study contributes to the documentation of cultivable bacterial diversity associated with *H. serrata* from the Kolhapur region and provides a molecularly authenticated isolate for further investigation. Further studies may be undertaken to characterize the physiological, biochemical and functional properties of this isolate.

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