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Functional Characterization of Lignocellulolytic Bacillus and Paenibacillus Species Isolated from the Gut Microbiota of the Eri Silkworm (Samia cynthia ricini)

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Abstract

Lignocellulosic biomass represents a major renewable resource for sustainable biofuel production; however, its structural complexity limits efficient enzymatic hydrolysis. Insect gut microbiota serve as natural bioreactors for lignocellulose degradation and are increasingly explored for industrial biotechnology applications. The present study aimed to isolate and functionally characterize lignocellulolytic *Bacillus* and *Paenibacillus* species from the gut microbiota of the eri silkworm (*Samia cynthia ricini*). Gut homogenates were cultured, purified, and subjected to morphological and biochemical characterization. Functional screening for cellulase, xylanase, and ligninase activities was performed using Congo red-based CMC agar, minimal salt medium supplemented with xylan (MSM-L), and minimal salt medium containing lignin, respectively. Semi-quantitative scoring was conducted in triplicate. All isolates exhibited strong cellulolytic and xylanolytic activity, while differential ligninolytic potential was observed among selected strains. The findings indicate that the eri silkworm gut represents a promising microbial reservoir for lignocellulose degradation with relevance to biomass bioconversion and renewable energy research.

Keywords: *Samia cynthia ricini*, lignocellulose degradation, gut microbiota, *Bacillus*, *Paenibacillus*, bioethanol, biomass conversion

1. Introduction

The world is moving toward more environmentally friendly energy systems, which has led to more research into bio-based alternatives to fossil fuels. Lignocellulosic biomass is one of the most promising feedstocks for making second-generation bioethanol because it is cheap, abundant around the world, and doesn't compete with food resources (Sun & Cheng 2002; Zabed et al., 2020). Agricultural residues, forestry wastes, and agro-industrial byproducts form substantial carbon reservoirs; nevertheless, their effective valorisation is hindered by structural recalcitrance and process inefficiencies (Himmel et al., 2007; Kumar et al., 2022).

Lignocellulose is a complex composite matrix made up of cellulose microfibrils that are surrounded by hemicellulose and protected by lignin, a highly cross-linked aromatic polymer that makes it stiff and resistant to enzymes. The crystalline structure of cellulose, the heterogeneous branching of hemicellulose, and the hydrophobic properties of lignin collectively impede enzymatic accessibility, constituting a significant bottleneck in biomass saccharification (Lynd et al., 2002; Santos et al., 2019). To get around this problem, cellulases, hemicellulases (like xylanases), and lignin-modifying oxidative enzymes like laccases and peroxidases must work together (Kumar et al., 2022). As a result, finding microbial enzyme systems that are efficient, strong, and able to adapt to different environments is still a top research goal. Natural ecosystems that break down lignocellulose are good models for biotechnology. Insect gut microbiomes are highly specialized, evolutionarily refined microreactors that can break down plant polymers under normal conditions (Engel & Moran, 2013; Ceja-Navarro et al., 2019). Herbivorous insects depend significantly on symbiotic microorganisms for the digestion of complex polysaccharides that surpass the hydrolytic capabilities of host-derived enzymes (Douglas, 2015).

Recent metagenomic and metatranscriptomic investigations have identified various carbohydrate-active enzymes (CAZymes) encoded in insect gut microbiota, underscoring their industrial significance for biomass conversion and biofuel generation (Berini et al., 2018; Su et al., 2022).

Members of the genera *Bacillus* and *Paenibacillus* have attracted considerable attention among lignocellulolytic bacteria because of their metabolic versatility, stress resistance, and prolific synthesis of extracellular enzymes. These genera are recognized for producing cellulases, endoglucanases, β-glucosidases, xylanases, and other enzymes that modify lignin (Grady et al., 2016; Patel et al., 2021). Recent research indicates that *Bacillus spp*. are applicable in consolidated bioprocessing techniques and bioethanol production systems due to their ability to withstand extreme temperatures, produce spores, and adapt to industrial environments (Zabed et al., 2020; Patel et al., 2021). Similarly, *Paenibacillus species* possess diverse CAZyme methods and are associated with the degradation of lignin and hemicellulose, highlighting their significance in sustainable biomass valorization (Grady et al., 2016; Su et al., 2022).

The eri silkworm, *Samia cynthia ricini*, is a significant lepidopteran cultivated extensively for the production of non-mulberry silk. It mostly consumes the foliage of Ricinus communis, which is rich in structural polysaccharides and secondary metabolites. The ability of this insect to efficiently use lignocellulose-rich foliage suggests the presence of a specialized gut microbiota that is suited for detoxification and polymer breakdown activities. Extensive study has been conducted on the gut systems of insects such as termites and beetles that decompose lignocellulose (Ceja-Navarro et al., 2019), although there is limited investigation into the gut microbial community of *S*. cynthia *ricini*, particularly regarding the production of enzymes beneficial for biofuels.

The lignocellulolytic capabilities of gut-associated bacteria from *S. cynthia ricini* may provide dual (i) advancing ecological insights into host–microbe symbiosis in Lepidoptera and (ii) identifying novel enzyme producers appropriate for biomass pretreatment and saccharification processes. The increasing need for efficient enzyme systems compatible with sustainable biorefineries (Kumar et al., 2022; Su et al., 2022) highlights *Bacillus* and *Paenibacillus* strains derived from insect guts as attractive yet underexplored alternatives.

Thus, the present work aims to identify and functionally characterize the lignocellulolytic species of *Bacillus* and *Paenibacillus* from the gut microbiota of *S. cynthia ricini*. This study evaluates the enzymatic efficacy of cellulase, xylanase, and ligninase activities via systematic screening and investigates their prospective uses in the bioconversion of lignocellulosic biomass and bioethanol production. This work combines ecological microbiology with practical bioenergy research, promoting the advancement of sustainable enzyme resources derived from insect-associated microbial systems.

2. Materials and Methods

2.1 Sample Collection and Dissection

We obtained late-instar larvae of the eri silkworm, *Samia cynthia ricin*i, from a certified sericulture unit, the Central Sericultural Germplasm Resources Center (CSGRC) in Hosur, Tamil Nadu, and maintained them on fresh leaves of Ricinus communis prior to the experiment. We selected only healthy, actively feeding larvae for study. The larvae were initially immersed in 70% ethanol for one minute, subsequently in 0.1% sodium hypochlorite for one minute, and lastly washed three times with sterile distilled water to eliminate external contaminants. Aseptic dissections were performed in a laminar airflow setting. Whole gut tissues were thoroughly dissected using sterile instruments and transferred to sterile phosphate-buffered saline (PBS; pH 7.2).

2.2 Preparation of Gut Homogenate and Bacterial Isolation

We homogenized and removed gut tissues in sterile PBS using a sterile glass homogenizer. We prepared successive dilutions (10^{-1} to 10^{-6}) and inoculated 100 µL of each into Nutrient Agar (NA) plates. The plates were maintained at 37 ± 2°C for a duration of 24 to 48 hours in an aerobic setting.

Distinct colonies were selected based on their features and purified using several streaking procedures. Pure cultures were preserved on NA slants at 4°C for further examination. Standard microbiological methods were followed for isolation and preservation (Cappuccino & Sherman, 2014).

2.3 Biochemical and Physiological Characterization

2.3.1 Gram Staining and Microscopy

Gram staining was performed using known laboratory techniques (Cappuccino & Sherman, 2014). The cellular morphology and Gram reactions were examined under oil immersion at 100× magnification, adhering to the systematic categorization criteria specified in (Whitman, 2015).

2.3.2 Aerobic Growth and Catalase Activity

Aerobic growth was verified by incubating isolates on nutrient agar plates at 37°C for 24 hours. Catalase activity was assessed by applying a drop of 3% hydrogen peroxide (H_2O_2) to newly cultivated colonies. Prompt bubble development signified a favorable reaction (Cappuccino & Sherman, 2014).

2.3.3 Motility Test

Motility was evaluated with semi-solid motility agar (0.4% agar). Isolates were stab-inoculated and incubated at 37°C for 24 to 48 hours. Diffuse development radiating from the stab line was seen as indicative of positive motility (Cappuccino & Sherman, 2014).

2.4 Qualitative Screening of Lignocellulolytic Enzyme Activities

All enzyme tests were conducted in triplicate, and average results were documented.

2.4.1 Cellulase Activity (CMC–Congo Red Assay)

Cellulase activity was assessed utilizing Carboxymethyl Cellulose (CMC) agar with 1% CMC as the substrate. Isolates were spot injected and incubated at 37°C for 48 hours. Plates were immersed in a 0.1% Congo red solution for 15 minutes and subsequently destained with 1 M NaCl for 20 minutes. Distinct hydrolysis zones encircling colonies demonstrated cellulase synthesis (Teather & Wood, 1982; Kasana et al., 2008).

Zone sizes were quantified in millimeters, and enzyme activity was evaluated semi-quantitatively in accordance with known plate assay methodologies (Florencio et al., 2012).

2.4.2 Xylanase Activity (MSM–Xylan Assay)

Xylanase production was evaluated utilizing Minimal Salt Medium (MSM) augmented with 1% birchwood xylan as the exclusive carbon source. Plates were incubated at 37°C for 48 hours and thereafter stained with Congo red as previously outlined. Distinct halos surrounding colonies signified xylan hydrolysis (Teather & Wood, 1982; Bailey et al., 1992).

Zone diameters were measured and scored accordingly.

2.4.3 Ligninase Activity (MSM–Lignin Assay)

Ligninolytic activity was assessed utilizing minimum salt medium enriched with 0.2% alkali lignin. Plates were incubated in aerobic conditions at 37°C for a duration of 3 to 5 days. Lignin degradation was assessed using apparent discoloration, oxidation zones, and alterations in pigmentation surrounding colonies (Pointing, 1999).

The qualitative evaluation of ligninolytic potential was conducted following recognized methodologies for screening lignin-degrading enzymes (Tien & Kirk, 1988).

2.5 Semi-Quantitative Enzyme Scoring

Hydrolysis zones were measured, and enzyme activity was scored using a 0–4 scale:

- 0 = No detectable activity

- 1–2 = Moderate activity
- 3–4 = Strong to very high activity

Results represent the mean ± standard deviation (SD) of three independent experiments. Semi-quantitative plate index evaluation was performed following the method described by Florencio et al. (2012).

2.6 Statistical Analysis

All experiments were performed in duplicate. Data are presented as mean ± standard deviation (SD). Variations in enzyme activity among isolates were examined using one-way analysis of variance (ANOVA) in accordance with established experimental design protocols (Montgomery, 2017). Statistical significance was defined as $p < 0.05$.

3. Results and Discussion

3.1 Biochemical and Physiological Characterization of Gut Isolates

Six bacterial isolates obtained from the intestine of *Samia cynthia ricini* were classified into the genera Bacillus and *Paenibacillus* based on visual and biochemical characteristics (Table 1). Consistent with the established physiological characteristics of the genera, all isolates were Gram-positive rods exhibiting aerobic growth and positive catalase activity. The persistent catalase positive signifies that the insect's gastrointestinal setting, where reactive oxygen species may arise during digestion, has acclimatized to oxidative stress conditions.

Despite the absence of motility under the experimental conditions, this does not exclude the potential for flagellar function, since the medium composition and incubation conditions can influence motility in *Bacillus* and *Paenibacillus* species. The taxonomic classification of *Bacillus*, *paralicheniformis*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus velezensis*, and *Paenibacillus spp*. is corroborated by their biochemical profiles, which typically align with their established characteristics. The intestine of the eri silkworm is predominantly inhabited by spore-forming Gram-positive bacteria, signifying ecological adaptability to fluctuating environmental conditions such as pH, nutritional availability, and host digestive enzymes. These attributes confer industrial advantages, particularly in large-scale enzyme manufacturing, where stability and stress resilience are critical.

Table 1. Biochemical and physiological characterization of gut bacterial isolates from *Samia cynthia ricini*.

This table summarizes the biochemical, physiological, and functional screening assays employed to identify and characterize the *Bacillus* and *Paenibacillus species* isolated from the intestines of the eri silkworm, *Samia cynthia ricini*. Results are classified as variable (V), negative (-), or positive (+) in accordance with established microbiological protocols.

Bacterial isolate	**Gram staining**	**Aerobic growth**	**Catalase test (H_2O_2)**	**Motility**	**Cellulase activity (Congo red)**	**Xylanase activity (MSM-L)**	**Ligninase activity (MSM-lignin)**
Bacillus paralicheniformis	+ (Gram-positive)	+	+	–	+	+	+
Bacillus licheniformis	+ (Gram-positive)	+	+	–	+	+	+
Bacillus subtilis	+ (Gram-positive)	+	+	–	+	+	V
Bacillus velezensis	+ (Gram-positive)	+	+	–	+	+	+
***Paenibacillus* sp.**	+ (Gram-positive)	+	+	–	+	+	+
Paenibacillus lactis	+ (Gram-positive)	+	+	–	+	+	V

Scoring criteria

- +: Positive reaction / visible growth or hydrolysis zone
- **V**: Variable or moderate activity
- –: No detectable activity or negative response

3.2 Qualitative Screening of Lignocellulolytic Enzymes

The obvious hydrolysis zones exhibited by all isolates on CMC-Congo red and xylan-supplemented medium proved their cellulolytic and xylanolytic capabilities (Table 1). This ubiquitous polysaccharide-degrading activity indicates that the gut microbiota of *S. cynthia ricini* is significantly oriented towards the decomposition of plant biomass, consistent with its herbivorous diet of *Ricinus communis* leaves.

Variability across strains was noted in ligninolytic activity. B. *subtilis* and *P. lactis* had differing reactions, but *B. paralicheniformis, B. licheniformis, B. velezensis, and Paenibacillus sp.* showed effective lignin breakdown. This pattern of variation indicates strain-specific differences in oxidative enzyme systems, such as peroxidases or laccases. The ability to partly break down lignin is particularly crucial since its removal enhances cellulose accessibility during biomass saccharification.

The coordinated activities of cellulase, xylanase, and ligninase illustrate the synergistic enzymatic capability of the isolates and support the hypothesis that insect gut ecosystems function as organic bioreactors for lignocellulose breakdown.

3.3 Semi-Quantitative Analysis of Enzyme Activity

The enzymatic performance of the isolates was further differentiated using semi-quantitative grading (Table 2). *Paenibacillus sp.* had the greatest overall activity (cellulase = 4; xylanase = 4; ligninase = 4), demonstrating a significant potential for hydrolysis and oxidation. This isolate is regarded as a viable candidate for biomass bioconversion applications owing to its extensive activity, signifying a substantial repertoire of carbohydrate-active enzymes (CAZymes).

B. subtilis and B. paralicheniformis exhibited considerable ligninase potential (scoring 2) and robust cellulase and xylanase activity (score 3). These findings align with documented industrial use of *Bacillus species* for the production of enzymes, particularly hemicellulases and thermostable cellulases. All enzyme classes exhibited balanced moderate activity in *B. velezensis* (score 2), signifying a less intense yet coordinated lignocellulolytic expression. Conversely, the comparatively lower ligninolytic scores (1–3) of *B. licheniformis* and *P. lactis* indicate that they may specialize in polysaccharide hydrolysis rather than lignin modification.

Table 2. Semi-quantitative assessment of lignocellulolytic enzyme activity (cellulase, xylanase, ligninase) with the CMC–Congo red test.

Activity ratings were designated as 3-4 (strong), 1-2 (moderate), and 0 (none). Values denote the mean ± standard deviation of triple experiments.

Isolate	Cellulase Activity	Xylanase Activity	Ligninase Activity
B. paralicheniformis	3	3	2
B. licheniformis	3	2	1
B. subtilis	3	3	2
B. velezensis	2	2	2
Paenibacillus sp.	4	4	4
P. lactis	3	2	3

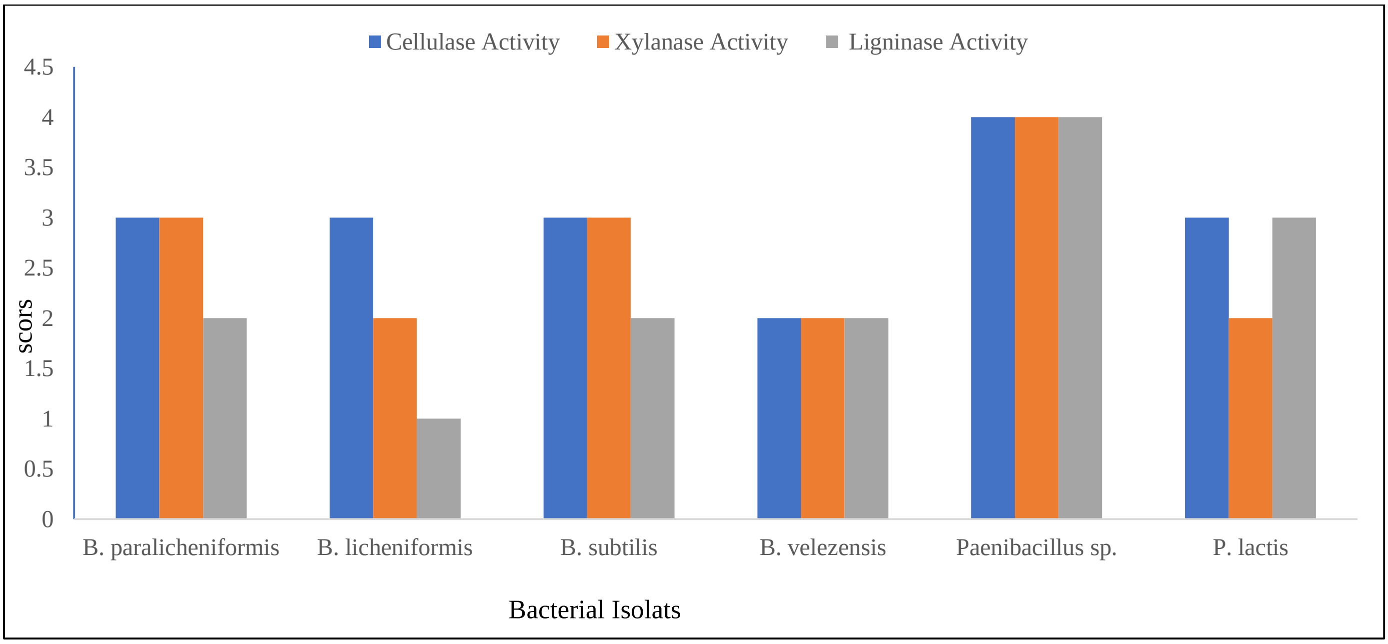

Figure 1. Comparative semi-quantitative assessment of lignocellulolytic enzyme activity in gut bacterial isolates from *Samia cynthia ricini*.

A bar graph depicting the enzymatic activity of cellulase, xylanase, and ligninase in *Bacillus* and *Paenibacillus* isolates obtained from gut microbiota. Enzyme activity was evaluated using the CMC–Congo red test for cellulase, MSM supplemented with xylan for xylanase, and MSM fortified with alkali lignin for ligninase. Activity levels were evaluated using a semi-quantitative scale ranging from 0 to 4, where 0 signifies no activity, 1–2 indicates moderate activity, and 3–4 represents strong to very high activity based on the width of the hydrolysis zone. The figures are the mean of three duplicate tests. Among the examined isolates, *Paenibacillus sp*. exhibited the greatest lignocellulolytic activity across all three enzyme classes, followed by *Bacillus paralicheniformis* and *Bacillus subtilis*, indicating their significant potential for lignocellulosic biomass breakdown.

3.4 Analysis of Heatmaps and Functional Implications.

It graphically depicts differing enzyme strengths among isolates, with deeper color gradients signifying heightened activity levels. The clustering pattern reveals a clear distinction between high-performing strains (*Paenibacillus sp., B. paralicheniformis, B. subtilis*) and intermediate performers (*B. velezensis, P. lactis*). This variance likely signifies differences in regulatory processes, gene expression patterns, and enzyme secretion efficiency. From an applied biotechnology perspective, isolates with high and balanced lignocellulolytic activity are particularly beneficial for integrated biomass conversion approaches, where simultaneous degradation of cellulose, hemicellulose, and partial lignin is desired. The outstanding performance of *Paenibacillus sp*. is noteworthy. Species of this genus are becoming recognized for their diverse CAZyme systems and ability to degrade complex polysaccharides under moderate circumstances. This study's demonstration of their powerful lignin-modifying capability enhances their importance for sustainable pretreatment techniques in second-generation bioethanol production.

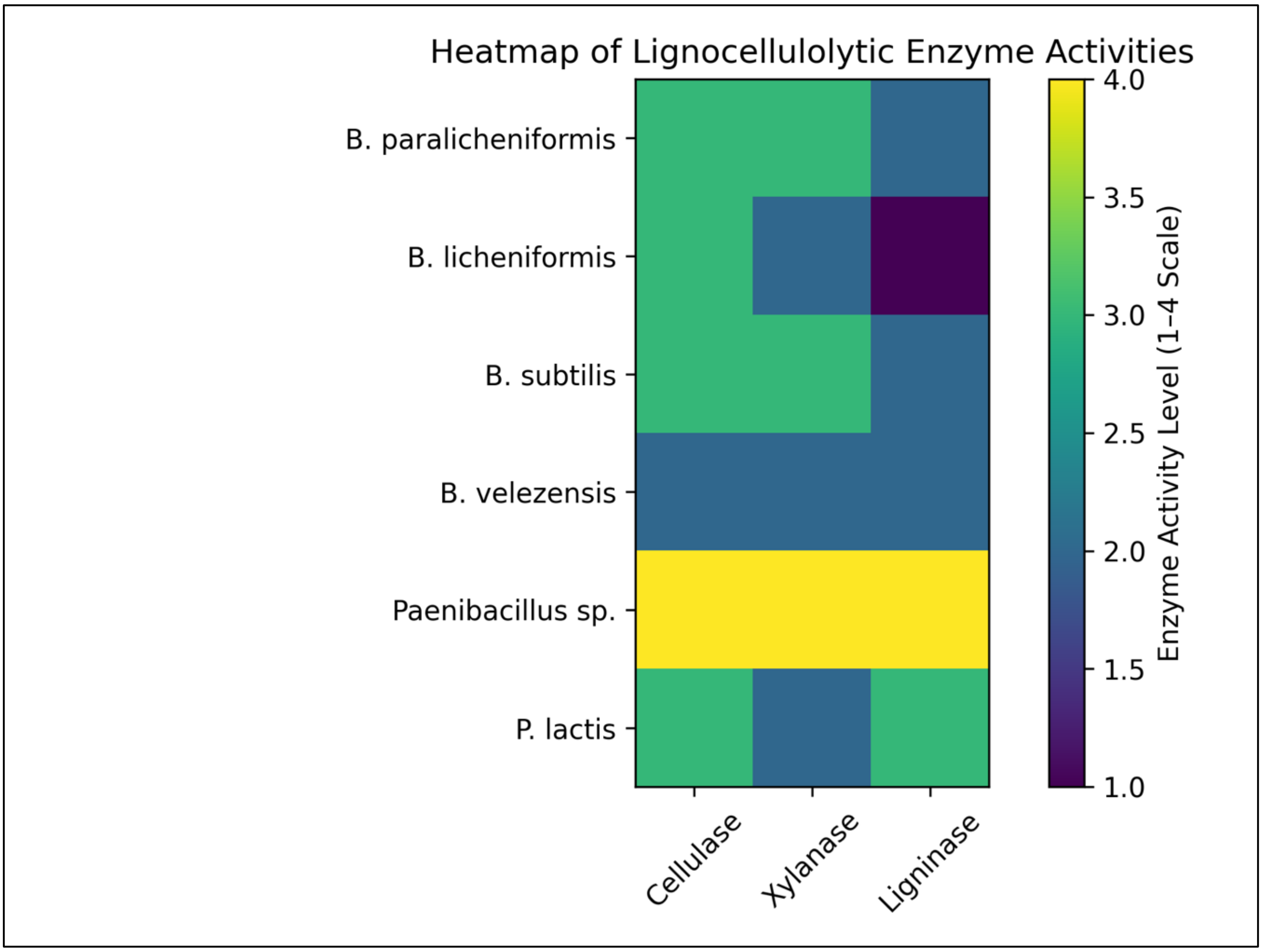

Figure 2: Heatmap depicting the semi-quantitative activities of cellulase, xylanase, and ligninase in bacterial isolates obtained from the intestine of *Samia cynthia ricini.*

Enzyme activity was assessed on a scale from 1 to 4, where 1 indicates low activity, 2 denotes moderate activity, 3 signifies strong activity, and 4 represents extremely high activity. The color intensity escalates with increased enzyme activity, as seen by the color bar.

4. Conclusive Evaluation

This work demonstrates that the gut microbiota of *Samia cynthia ricini* comprises metabolically diversified lignocellulolytic bacteria with significant biomass-degrading ability. All isolates exhibited measurable cellulase and xylanase activity, but ligninase output varied across the strains. Among the assessed bacteria, *Paenibacillus sp*. emerged as the most promising contender due to its continuously excellent enzymatic profile.

These findings support the ecological concept suggesting that the gastrointestinal tracts of herbivorous insect’s function as efficient natural bioreactors for the conversion of plant biomass. The enzymatic capabilities reported in this work suggest possible uses in sustainable bioethanol production and ecofriendly bioprocessing technology. Future study should incorporate molecular gene profiling, quantitative enzyme kinetics, and fermentation-based validation to assess commercial application.

5. Conflict of Interest

Nill

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