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ORIGINAL ARTICLE **OPEN ACCESS**

Isolation and Characterization of a Potential Proteinase Producing *Mammaliicoccus Sciuri* from The Gut Of Eri Silkworm *(Samia Ricini)*

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ABSTRACT
Proteinase, a group of enzymes responsible for the breakdown of proteins into smaller peptides and amino acids, plays pivotal role in various biological processes such as digestion, protein turnover and immune function. Eri silkworms, which are known for their silk production, are of great interest not only in the agricultural industry but also from a microbiological perspective due to the diverse microbial community residing in their gut. This investigation spotlights the isolation, profiling, and enzyme evaluation of putative proteinase-producing bacteria from Eri silkworm. The study pinpoints Mammaliicoccus sciury as a potential proteinase producer, yielding an enzyme activity of 0.115 ± 0.003 U/ml. Characterization unveils its pivotal physiological roles. These findings hold promise for the development of novel probiotic applications aimed at enhancing the health and growth of silkworms and other animal species. Furthermore, exploring protease-producing gut bacteria in Eri silkworms could offer valuable insights for both industrial and ecological applications.
Keywords*: Eri silkworm, proteinase, digestive enzyme, gut.*

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INTRODUCTION

Silkworms (order: Lepidoptera) are the larvae or caterpillar of a moth and they are important as they produce commercial silk. Eri silkworm (*Samia ricini*), a member of the family Saturnidae, is one of the most exploited, domesticated and commercialized non mulberry silkworms. In India, eri silk industry is mainly contributed by North-East India, accounting about 90% of the total eri silk production in India. The gut, as an organ, estimated to contain 10 times more microbes than total cells and 100 folds more microbial genes than animal genes. Such studies and statistics lead to questions, whether animals are super-organisms [1]. The gut is an organ that serves as a main hub for the synthesis of digestive enzymes, which are then used to break down ingested food components and absorb nutrients [2]. The insect gut is inhabited by a wide diversity of microorganisms as a result of its constituting intestinal microbial ecosystem. It has been reported that the intestinal microbiota in insect is involved in the digestion of insect, absorption and may also assist in insect immunity by forming a persistent infection within their hosts [3, 4]. The amount and quality of food intake and their digestibility has great influence on the overall growth and development process of insects. Several crucial digestive enzymes are needed for the breakdown of complex macromolecules found in host plant leaves into simpler, more easily absorbed form. Many gut bacteria have been found in association of digestive enzyme activities in silkworms such as amylase, lipase, cellulose, gelatinase and pectinase [5, 6]. Food plant for eri silkworms are composed of mainly carbohydrate, protein and lipid Proteinase is the key digestive enzyme that cartelizes the conversions of complex protein product in to simpler readily absorbable amino acid forms. However, the role of gut bacteria on proteinase activity of gut has not been reported yet. Therefore, the present study was conducted to isolate and characterize proteinase producing gut bacterial species in eri silkworm larvae.

MATERIALS AND METHODS

Insect Rearing

Eri silkworm larvae were reared from newly hatch first instar till fifth instar mature stage on castor leaves till 3rd generation until they fully attain the homogenicity.

Harvesting of larvae and extraction of gut

Fully matured larvae were picked randomly and were kept on starvation for 24hrs before extraction. The larvae were then brought into laminar airflow cabinet and then surface stabilized to remove any dust and contamination from larvae. Finally the whole gut was then extracted from the larvae by making incision through abdomen from mouth part till anus.

Gut Sample preparation

The extracted gut was then taken for homogenization and then homogenate prepared was then centrifuged at 14000 rpm for 10 min under 4^0C condition. The bacterial cells win clear supernatant was taken for further process.

Inoculation and culture

The supernatant was collected and subjected to serial dilution based on the appropriate dilution factor. From each dilution, 0.1ml of the sample was inoculated onto nutrient agar plates. These plates were then incubated inside a BOD incubator at 37^0C for 36-48 hours. The plates showing no contamination and clear, non-overlapping colonies were selected for further screening.

Isolation of pure culture

From the culture plate seven independent bacterial colonies were carefully isolated for pure culture to obtain isolated and uncontaminated colonies of bacterial isolates. These pure colonies can then be used for subsequent characterization and enzyme screening processes.

Qualitative Screening of proteinase activity

Skim milk agar media was used for screening of proteinase producing bacterial isolates (15g/L agar, 5g/L cesein, 1g/L Dextrose, 28g/L skim milk powder, 2.5g/L yeast extract; pH-7.) Incubation at 37^0C for 48 h, the halo/clear zone around colonies indicates the presence of enzyme activity. All the seven pure isolates were subjected into screening and the isolates showing higher proteinase activity was selected for quantitative enzyme assay and their phenotypical and molecular characterization.

Proteinase enzyme assay

The enzyme sample was prepared from the bacterial cells in the nutrient broth medium containing 1% substrate (casein). The culture cells were separated by centrifuging the broth medium at 12000 rpm for 3 min under 4^0C condition. The supernatant was then taken as enzyme source for assay. proteinase enzyme activity was assayed according to the protocol described by [7] by using Casein as a substrate and TCA as stopper of the enzyme activity. Absorbance of the samples was recorded using UV-VIS spectrophotometer. The sample absorbance was measured at 660 nm. Proteinase enzyme activity was determined using a tyrosine standard curve, where one unit of activity corresponds to the amount needed to produce 1 µmol of tyrosine equivalents per ml of TCA filtrate under the assay conditions.

PHENOTYPICAL CHARACTERIZATION OF PROTEINASE PRODUCING BACTERIA

Morphological characterization

The isolates were examined under a microscope to observe their colony morphology, including characteristics such as shape, size, color, consistency, margin, elevation, and opacity. The observations were recorded following the guidelines described in the manual by [8].

Physiological characterization

The isolates were subjected to various characterization tests, including the Catalase test, Oxidase test, and Gram nature determination, using freshly grown bacterial cultures following the procedures outlined in [8]. To assess their tolerance to pH, salt, and temperature, the isolates were grown on nutrient agar plates for 96 hours. For pH optimization, nutrient agar plates with pH ranging from 7 to 11 were prepared, with increments of 0.5 pH units using KH_2PO_4/K_2HPO_4 or $Na_2CO_3/NaHCO_3$ buffer systems, and the plates were incubated at 37 ºC. Salt tolerance was evaluated by exposing the isolates to NaCl concentrations of 0 to 10% (w/v), with increments of 2%, at 37 °C on nutrient agar plates. Furthermore, API 20E (analytical profile index) strips were used to perform various substrate utilization tests and assess the ability of the isolates to ferment or oxidase different carbohydrate sources.

MOLECULAR IDENTIFICATION

DNA isolation and PCR amplification

DNA was isolated from bacterial sample using commercial DNA isolation kit (Himedia). The quality of the isolated DNA was checked on agarose gel electrophoresis and the concentration was checked on nanodrop. After getting desired quality of the DNA was amplified using 16S universal primers namely as 27F and

1492R respectively as forward and reverse primers for amplification And Primer 1 (907R) and Primer 2(785F) sequencing primers in Thermocycler PCR

Gene sequencing and Data analysis

The PCR amplicons were purified and 2 µl volumes of samples were taken for the sequencing per reaction using BDT V3.1 chemistry. After sequencing data were generated in the form of FSTA file which were taken further for their Quality checking and further bioinformatics analysis. Poor contaminated bases of sequencing data was trimmed from both the end forward and reverse sequencing data. Then Trimmed forward and reverse bases were aligned together using bioinformatics software Bioedit. Alligned bases were then blast together in NCBI to find the sequence similarity; sequence having maximum sequence similarity was then selected in the result.

RESULTS

Qualitative and Quantitative proteinase assay

Among the seven bacterial isolates tested on skim milk agar, four of them demonstrated positive proteinase activity. Specifically, one of these four isolates, designated as C1, displayed the highest enzyme activity level, evidenced by a substantial clearance zone of 6 mm surrounding the colony (Figure 1a). Due to its notable proteinase activity, isolate C1 was selected for further quantitative enzyme assay. To quantify the enzyme activity, a standard curve was established using L-tryptophan, and based on this curve, the quantitative enzyme activity of isolate C1 was calculated to be 0.115 ± 0.003 U/ml. This result highlights the significant proteolytic capability of the C1 isolate.

Morphological and Physiological characteristics of isolate

Morphologically C1 exhibited the following characteristics: the size was pinpoint, the shape was round, the margin was entire, the color was creamish white, the opacity was opaque, the elevation was convex, and the consistency was smooth. The C1 isolate exhibited the characteristics of Gram-positive cocci in bunch. It demonstrated positive catalase activity but was negative for oxidase and KOH tests. The organism displayed tolerance to a wide pH range (pH 7, 6, 9, 10, and 11) as well as growth in varying NaCl concentrations (0%, 2%, 4%, 6%, 8%, and 10%). In terms of temperature tolerance, growth was observed at 20°C, 25°C, 37°C, and 45°C, while growth was inhibited at 5°C and 10°C. Substrate utilization tests revealed the organism's inability to utilize ß-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, and citrate. Additionally, it did not produce H2S, urease, tryptophane deaminase, or indole. However, weak positive acetoin production was observed. The organism exhibited gelatinase activity and was capable of fermenting/oxidizing glucose but not mannitol, inositol, sorbitol, rhamnose, saccharose, or melibiose (Table 2). Result of molecular identification through 16 s rRNa sequencing NCBI database result the bacterial isolate was identified as *Mammaliicoccus sciuri* with NCBI accession Id Isolate ERC6 OR211560.

DISCUSSION

Eri silkworm *Samia ricini* is an economically important insect that primarily feeds on *Ricinus communis* leaves. Insects have evolved diverse biological and chemical mechanisms, including defence molecules, lytic and stabilizing proteins, to thrive in various ecological niches. Enzymes produced either by insects themselves or by symbiotic microorganisms, play a central role in these systems and aid in digestion [9]. In this study, we identified a gut bacterial isolate, *M. sciuri*, which exhibited protease activity and various physiological activities. Gut bacterial symbionts are known to influence the physiology of their host organisms. Protease enzymes are essential for the breakdown of complex proteins into simpler forms, and the presence of protease activity in the gut bacterial isolate from eri silkworms confirms the functional involvement of gut bacteria in the digestive processes of these insects. These findings provide valuable insights into the physiological and biochemical traits of the organism, highlighting its metabolic potential and ecological contributions. Our results suggest that the *M. sciuri* gut bacterial isolate may be an essential symbiont, aiding eri silkworm larvae in protein digestion. Although there is no previous record of protease activity in *M. sciuri* isolated from the gut of eri silkworms, other researchers have isolated various bacteria from insect guts capable of producing key digestive enzymes such as amylase, lipase, cellulose, xylynase, pectinase, and gelatinase. These reports provide evidence that gut bacteria not only inhabit the gut of the host insect but also establish intrinsic symbiotic relationships with their hosts. Phenotypic characterization methods have provided physiological and morphological information about the *M. sciuri* bacterial isolate. Similar methods have been employed by [5, 6, 10] and others to identify phenotypic characteristics of important enzyme-producing gut bacteria in silkworms. However, phenotypic characterization based on culture-dependent methods alone is insufficient for species-level identification. Therefore, in our investigation, we utilized 16S rRNA gene sequencing, a molecular technique widely recognized for its

reliability and acceptability in organism identification, to identify the isolate at the species level. The gut of insects harbors a diverse microbial community, and the isolation of protease-producing gut bacteria in eri silkworms holds the potential to offer valuable insights into both ecological and industrial applications of *M. sciuri* gut bacteria. Numerous studies have been published on bacteria found in the digestive tracts of insects, and the presence of other enzymatic activities, such as cellulase and pectinase, in *M. sciuri* has been previously reported in eri silkworms [11]. Our findings further highlight the important function of protease enzyme activity in *M. sciuri*. Eri silkworms consume castor plants, which contain essential nutritional components such as carbohydrates, proteins, and lipids that need to be broken down into simpler forms. The presence of proteinase activity in *M. sciuri* may be associated with aiding the digestion of protein components in the food plants. Silkworms rely on enzymes derived from gut microflora for their nutrition, growth, and development [5, 6, 12, 13]. Additionally, the probitic potential of *Mammliicoccus sciuri* was reported by [14] isolated from goat milk indicating import potential roles of *M. sciuri* in overall physiologic processes as well as industrial processes.

Table 1.Morphological characteristics of Mammaliicoccus sciuri

Morphological characters	**Isolate C1**
Size	pinpoint
Shape	round
Margin	Entire
Color	Creamish white
Opacity	Opaque
Elevation	Convex
Consistency	Smooth

Table 2.Physiological Characteristics

Physiological characters	**Isolate C1**
Gram nature	Gram positive cocci in bunch
Catalase	positive
Oxidase	negative
KoH	negative
Tolerance to pH	
7	positive
6	positive
9	positive
10	positive
11	positive
Nacl Conc.	
0%	positive
2%	positive
4%	positive
6%	positive
8%	positive
10%	positive
Temperature	
5^0C	Negative
10^0C	-negative
20^0C	+positive
25^0C	+positive
37^0C	+positive
45^0C	+positive
Substrate utilization	
ß-galactosidase	negative

Arginine DiHydrolase	- negative
Lysine Decarboxylase	- negative
Ornithine Decarboxylase	negative
Citrate utilization	negative
H2S production	negative
UREase	negative
Tryptophane Deaminase	negative
Indole production	negative
acetoin production (Voges Proskauer)	Weak positive
GELatinase	+positive
Fermentation/ Oxidation of	
Glucose	+positive
Mannitol	-negative
Inositol	-negative
Sorbitol	-negative
Rhamnose	negative
Saccharose	negative
Melibiose	negative

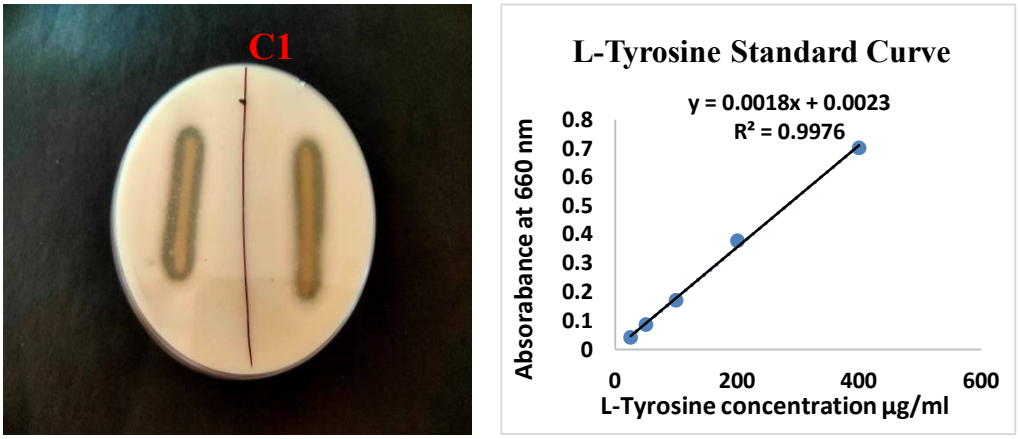

Figure 1. a. Proteinase activity of *M. sciuri* showing clear zone of hydrolysis. b. L-Tyrosine standard curve for protease assay

Table 3.Molecular identification of isolate

Isolate	Accession ID	NCBI blast result
Isolate C1	IsolateERC6 OR211560	*Mammaliicoccus sciuri*

CONCLUSION

The findings from this study offered valuable insight into the physiological and biochemical characteristics of the gut isolate *M. sciuri*, which contributed to a deeper understanding of its metabolic capabilities and potential ecological significance. This study provided evidence of the crucial role played by gut bacteria in the host organism, particularly highlighting the presence of the *M. sciuri* gut bacterial isolate and its involvement in digestive enzyme activity through the production of extracellular proteinases in the gut. These findings have promising implications for enhancing the health and growth of silkworms, as well as other animal species, and they hold potential for applications in the food industry and other biotechnological fields.

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Conflict of Interest: Authors declares no conflict of interest of this work.

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