

Isolation, Identification and Media Optimization of Gut Bacteria Species from *Danio Rerio* (Zebra Fish) against the *Spodoptera Litura* Larvae

Salem Sundarrajan Maithili*,
Muthu Ganesh Boopathi and Kandhasamy Lalitha

Department of Microbiology, AVS College of Arts and Science (Autonomous), Tamil Nadu, India.

<http://dx.doi.org/10.13005/bbra/3474>

(Received: 28 November 2025; accepted: 18 December 2025)

Spodoptera litura control is genuinely expected to replace the deadly compound pesticides; the disadvantages of the invention pesticides are issues, an effect on human and animals, environmental pollution and killing the non-target insect and highly resistance response to an insect pest. This situation used as ecologically agreeable, moved nearer to the bio pesticide. In this study, four strains were isolated from the zebra fish gut, among the two strains the highest bacterial growth was observed in ZBF-2 *Bacillus subtilis*, ZBF-4 *Bacillus cereus*. In view of biochemical, physiological and molecular characterization the isolated zebra fish gut was distinguished as having a place with Bacillaceae family. Phylogenetic investigation of the 16S rDNA sequence of zebra fish gut bacteria are ZBF-2 *Bacillus subtilis*, ZBF-4 *Bacillus cereus*. In *Danio rerio* gut bacterial optimal growth conditions were tested with different media optimal growth level with ZBF-2 and ZBF-4 showed the highest growth rates under optimized conditions, including glucose as the key carbon source, peptone as the nitrogen source, pH levels of 4 to 7 and a temperature of 35°C at 72hrs. The two strains, ZBF-2 *Bacillus subtilis* and ZBF-4 *Bacillus cereus*, were selected for 16S rRNA sequencing, and the resulting sequences identified as and get the accession number of *Bacillus subtilis* (PV297978) and *Bacillus cereus* (PV300533). These optimized conditions fostered the growth of gut microbes that are important for the secondary metabolites enhance for the bioactive compounds may be responsible for the insecticidal properties. And we further tested, with the isolated bacterial cell free supernatant of ZBF-2 and ZBF-4 produced 76-80% mortality at 48hrs in *Spodopteralitura* larvae. This study shows that, the symbiotic bacteria ZBF-2 *Bacillus subtilis*, ZBF-4 *Bacillus cereus* are virulent, and their secondary metabolites are also toxic and can be utilized in pest control programs.

Keywords: *Bacillus cereus*; *Bacillus subtilis*; Gut bacteria; Larval mortality; Optimization; Secondary metabolites.

Trillions of bacteria make up the vertebrate gut microbiome, which is essential for digestion, energy acquisition, nutrient absorption, and host health.¹ Fish development, reproduction, and ecological interactions are all greatly impacted by gut

microbes.² Since fish larvae are initially sterile and pick up microorganisms from their surroundings, the microbiome is especially crucial during the early stages of development.³ Environmental factors such as salinity and temperature largely shape gut

*Corresponding author E-mail: ssmmicro@gmail.com



microbial communities.⁴ Additionally, variations in gut microbiota among individuals of the same species often reflect differences in environmental conditions.⁵ Exploring microbiome changes in wild hosts can provide insights into ecological adaptability. Fish gut microflora includes aerobic, facultative anaerobic and obligate anaerobic bacteria. Similar to humans, microbial composition changes with age, nutrition, and environmental conditions.⁶ Bacterial colonization occurs on the skin, gills, and intestine with zebrafish (*Danio rerio*) actively ingesting water as early as four days post-fertilization (dpf), allowing microbes to enter their digestive tract.⁷ By adulthood, zebrafish gut microbiomes are well-established, with high bacterial abundance. Zebrafish, a freshwater omnivorous teleost fish native to South Asia, has become a widely used model organism in biomedical research.⁸ *Bacillus* species are known to produce a wide range of bioactive compounds with potential applications in medicine, agriculture, and industry. *Bacillus megaterium* produces megacin, a bacteriocin with activity against Gram-positive bacteria.⁹ *Bacillus cereus* produces cereulide, a toxin that can cause food poisoning,¹⁰ bacilysin, an antibiotic with activity against Gram-positive bacteria.¹¹

Bacillus subtilis produce's bacitracin, an antibiotic with activity against Gram-positive bacteria¹² and surfactin, a lipopeptide with antimicrobial and antiviral activities¹³. *Bacillus* spp. Produced by bacillomycin, a lipopeptide with antimicrobial and antifungal activities of *bacillus sps*¹⁴ and fengycin, a lipopeptide with antimicrobial and antifungal activities¹⁵. These bioactive compounds have potential applications in various fields, including medicine, agriculture, and industry. Only a minor fraction of bacterial populations can grow on standard laboratory media,¹⁵ In fish, skin microbiota is estimated to have less than 0.01% culturable bacteria.¹⁶ Similar discrepancies have been observed in human gut microbiota, where cultural methods overestimated to bacteria populations.¹⁷ Despite extensive studies on fish gut microbiota, research has predominantly relied on culturable bacteria, limiting the understanding of total microbial diversity.¹⁸ Traditional microbial identification methods based on phenotypic and biochemical characteristics are time-consuming and may

not accurately reflect the actual composition of microbial communities.¹⁹

Spodoptera litura, commonly known as the tobacco caterpillar or armyworm, is a significant agricultural pest that causes substantial damage to various crops, including tobacco, cotton, and vegetables.²⁰ This polyphagous pest is widely distributed across Asia, Africa, and Australia, and its management is a major challenge due to its rapid adaptation to insecticides and resistant crop varieties.²¹ The larvae of *S. litura* feed on plant leaves, causing defoliation and reducing crop yields.²² Effective management of *S. litura* requires an integrated approach, including cultural, biological, and chemical controls. Chemical insecticides, such as pyrethroids, organophosphates, and carbamates, have been widely used to control *S. litura* populations.²¹ However, the excessive and indiscriminate use of these insecticides has led to the development of resistance, environmental pollution, and harm to human health.²² Some of the chemical insecticides commonly used against *S. litura* include Cypermethrin, Chlorpyrifos, Carbofuran. *S. litura*, is a devastating agricultural pest that causes significant damage to various crops, including tobacco, cotton and vegetables. To manage this pest, chemical insecticides have been widely used, providing quick and effective control. However, the excessive and indiscriminate use of chemical insecticides has raised concerns about their environmental impact, human health risks, and the development of pesticide-resistant to *S. litura* populations. In recent years, researchers have explored alternative approaches, including the use of beneficial microorganisms, such as gut bacteria from zebrafish (*Danio rerio*), to control insect pests. Studies have shown that zebrafish gut bacteria, including *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis*, and other *Bacillus species*, possess insecticidal properties against various insect pests, including *S. litura*.²³ These bacteria produce bioactive compounds, such as lipopeptides, bacteriocins, and enzymes, which can be used to control insect pests.^{24,25} Injecting *S. litura* with these beneficial bacteria or their bioactive compounds offers a promising alternative to chemical insecticides. This approach has been shown to be effective in controlling *S. litura* populations while minimizing harm to the environment and human health.²⁶ Therefore, the

full investigation and exploitation of gut bacteria can be significant for biological control of insect pests. The present study focuses on the isolation, identification and culturing of two strains of gut bacteria from *Danio rerio* against insecticidal efficacy on *S. litura* larvae.

MATERIALS AND METHOD

Sample Collection, Isolation of gut microbes

Zebrafish (*Danio rerio*) were collected from Edward Aquarium lies on the latitude of 11.6607636°, and longitude of 78.1504985° Salem, and acclimated for 24 hours before sample collection. They were anesthetized using tricaine methane sulfonate (MS-222) and humanely sacrificed. The gastrointestinal tract (GIT) was carefully dissected, and gut samples were stored at -80°C for further analysis. Gut microbes were isolated by serial dilution in sterile double-distilled water and cultured on Nutrient Agar at 28 °C for 24-48 hours. Pure cultures were obtained using the streak-plate method and stored at -80 °C. Further, these colonies were collected and sub-cultured in 10ml of Nutrient broth. Description of samples used in this study as shown in (Table.1).

Morphological analysis of gut microbes

Bacterial isolates were sub-cultured on nutrient agar plates. After 24 hrs of incubation, the characteristic gut bacterial isolates were screened. Selected bacterial colonies were identified as Gram negative or Gram-positive microscopes organisms and biochemical characteristics of four gut bacteria were performed using KB001- HiLMViC biochemical test kit.

Phylogenetic analysis of Bacterial isolates

Genomic DNA was isolated from the bacterial sample using PAR Life science and research bacterial genomic DNA isolation kit (PAR Life science and research PVT Ltd. Trichy India). 16S rRNA primers used in PCR reaction. PCR mixture containing 1µl DNA, 400 ng forward primer, 400ng reverse primer, 4µl dNTPs, 10µl 10X Taq DNA polymerase assay buffer, 1µl Taq DNA polymerase enzyme and distilled water. The total reaction volume was 100 µl. PCR amplification was carried out under following conditions step-I initial denaturing for 5mins at 94^R°C, denaturing at 94^R°C for the 30sec, step-ii annealing at 55^U°C for 30s and step-iii. extension at 72^U°C for 40 sec

for 35 cycles. PCR product was obtained and gel was eluted using agarose gel extraction kit. After elution, the PCR products were sequenced using ABI sequencing machine (ABI 3500 XL Genetic Analyzer). 10µl of sequencing mixture contains 4µl Big Dye Terminator Ready Reaction Mix, 1µl Template (100 ng/µl), 2µl primer (10pmol/ē) and 3µl Milli Q water. PCR amplification of sequencing mixture was carried out with the following conditions. 25 cycles of amplification were started after initial denaturing at 96^U°C for 1min, denaturing at 96^U°C for 10sec, extension at 50^U°C for 5sec and elongation at 60^U°C for 4mins. DNA concentration can be determined by both UV spectrophotometer and quantitative analysis on Agarose gel. The gel documentation system with UV- light and images were captured. Data analysis was performed by sequencing Analysis_ version 5.4 software. Phylogenetic tree was constructed using MEGA 6 software, according to the Neighbour-joining tree method.

Growth of symbiotic bacteria at various culture media

The growth conditions of recovered isolates four bacteria strains were determined at different culture media: nutrient broth, LB broth, Tryptic soy broth, Muller Hinton broth and MacConkey broth. A single primary colony was inoculated in 150 ml conical flask containing 100ml of various culture media and incubated between 0 and 96 hrs. After incubation, the growth of the bacterial culture was determined at optical density (OD) of 600 nm²⁷.

Growth of symbiotic bacteria at various pH

The growth conditions of recovered isolates four bacterial strains were determined at different pH. A single primary colony was inoculated in 100 ml conical flask containing 50ml of nutrient broth and incubated at the pH range of 5.0, 6.0, 7.0, 8.0 and 9.0 from 0 to 96 hrs. After incubation, the growth of the bacterial culture was determined at optical density of 600 nm²⁷.

Growth of symbiotic bacteria at various Temperatures

The growth conditions of recovered isolates four bacterial strains were determined at different temperatures. A single primary colony was inoculated in 100 ml conical flask containing 50ml of nutrient broth and incubated at the temperature 10 °C, 20 °C, 25°C, 30 °C, 35°C and

40°C for 48 hrs. After incubation, the growth of the bacterial culture was determined by optical density at 600 nm²⁷.

Growth of symbiotic bacteria at various Carbon source

The growth conditions of recovered isolates four bacterial strains were determined at different carbon source: Glucose, Sucrose, Lactose, Starch and Fructose. As for the control, the commercialized media were used. A single primary colony was inoculated in 150 ml conical flask containing 100ml of nutrient broth. To the nutrient broth, 0.5g of above-mentioned carbon source was added separately and incubated from 0 to 96 hrs. After incubation, the growth of the bacterial culture was determined by optical density at 600 nm.

Growth of symbiotic bacteria at various Nitrogen source

The growth conditions of recovered isolates four bacterial strains were determined at different nitrogen source: beef extract, peptone, yeast extract, soyabean and potassium nitrate. A single primary colony was inoculated in 150 ml conical flask containing 100ml of nutrient broth. To the nutrient broth, 0.5g of above-mentioned nitrogen source were added and incubated between 0 to 96 hrs. After incubation, the growth of the bacterial culture was determined by optical density at 600 nm.

Extraction of secondary metabolites from bacterial supernatant

The grown cultures were centrifuged at 10,000 rpm for 15 min after incubation, and the collected supernatants were filtered through 0.22 µm membrane filter to remove the debris from pellets. The extract was concentrated, suspended in DMSO (Dimethyl sulfoxide), and stored at -20°C²⁸.

Larval bioassay using crude bacterial metabolite of ZBF-2 and ZBF-4

From the broth culture, the bacterial culture of *Bacillus subtilis* (ZBF-2) and *Bacillus cereus* (ZBF-4) 1 mg of secondary metabolite were extracted and dissolved in 1 ml of (DMSO) dimethyl sulfoxide (1mg/ml). The bacterial secondary metabolites were diluted to various concentrations like as 10⁻⁴, 10⁻⁵ and 10⁻⁶ ranging from 5, 10, and 20 µl/mL were injected into proleg of *S. litura* larvae. PBS (Phosphate Buffer Saline)

were used as control. Mortality was recorded after 24 hrs of infection²⁹.

Percentage of larval motility = Number of dead larvae / Number of total larvae × 100

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism (version 5.1 San Diego, CA). The data are expressed as Mean ± SD. The significant difference between the Mean replicates was determined using Two-Way ANOVA.

RESULTS

Sample Collection, Isolation of gut microbes

In order to isolate the *bacillus* species, four different cultures were isolated from the gut of *danio rerio* (Zebra fish) as shown in (Figure.1). These sample of gut region of *Danio rerio* are crushed using sterile motor and pestle and then serial dilution should be performed and then streaked on nutrient agar medium (NA). The different types of bacterial colonies on nutrient agar medium should be streaked on another plate to isolate pure colonies of *bacillus* species (Table.1).

Identification of gut microbes

The bacterial isolates on nutrient agar medium (NA) are suspected as *bacillus* species and these were identified according to their morphological and cultural characteristics and bio-chemical test

Morphological and cultural characteristics

Gut Bacterial isolates from *Danio rerio* (zebra fish) such as ZBF-1 *Bacillus megatorium*, ZBF-2 *Bacillus subtilis*, ZBF-3 *Bacillus sps*, ZBF-4 *Bacillus cereus* strains were isolated from nutrient agar plate and their morphology and characteristics should be identified by their color, odor, Growth condition etc.

Identification of gut bacterial microbes

Bacteria were isolated from the gut of Zebra Fish (*Danio rerio*) using NA plates and bromothymol blue stain as an indicator. Four strains, ZBF-1 *Bacillus megatorium*, ZBF-2 *Bacillus subtilis*, ZBF-3 *Bacillus sps*, ZBF-4 *Bacillus cereus*, were identified as Gram-positive rod-shaped bacteria based on morphological as shown in (Fig. 2, 3, 4,5). The gut bacterial isolates of *Danio rerio* results that the isolates of bacterial culture are gram positive *bacillus*. Colonies of these

isolates are highly convex, yellow color colony, Opaque, frosted glass appearance with either rough or smooth surface. The phenotypic characteristics of gut bacterial isolates were shown in (Table.2)

Biochemical identification

The bacterial isolates of gut bacteria of four different strains, ZBF-1 *Bacillus megatorium*, ZBF-2 *Bacillus subtilis*, ZBF-3 *Bacillus sps* and ZBF-4 *Bacillus cereus* were examined for biochemical characterization as shown in (Figure.6). The results of gut bacteria of four different strains

were showed as gram positive rod-shaped bacteria. The results are shown in (Table.3).

Optimization of isolation of bacterial species
Carbon source

The growth of isolates ZBF-1, ZBF-2, ZBF-3 and ZBF-4 was tested with various carbon sources like as (glucose, sucrose, lactose, starch, fructose) in nutrient broth. After incubation for 0 to 96 hours, glucose was found to be the most effective carbon source, promoting the highest bacterial growth levels at 72 hours compared to

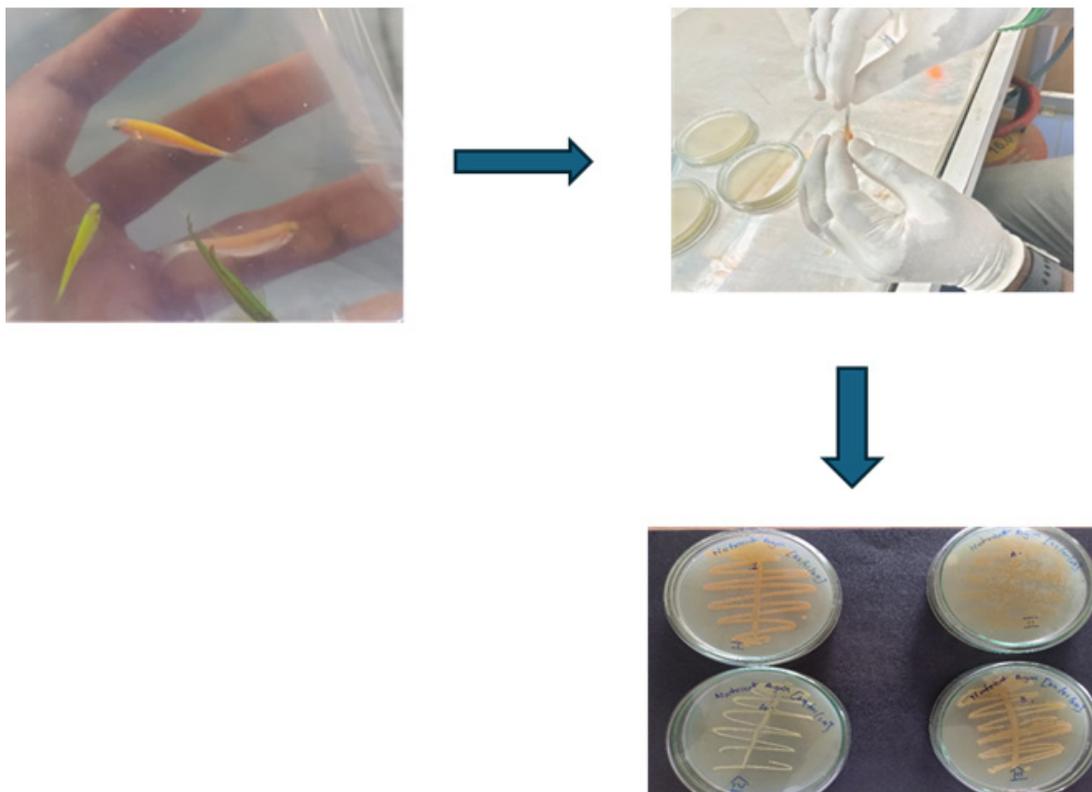


Fig. 1. Sample collection and isolation of gut microbes form *danio rerio* (zebra fish)

Table 1. Description of samples used in this study

Sample name	Common or host name	Environment	Sample Site	Water habitat	Collection date
ZBF2	Zebrafish (<i>Danio rerio</i> , superorder Ostariophysii)	Conventional lab aquaculture	Whole intestinal contents	F	20-Jan-25
ZBF4	Zebrafish (<i>Danio rerio</i> , superorder Ostariophysii)	Conventional lab aquaculture	Whole intestinal contents	F	20-Jan-25

*F- Fresh Water Abbreviations: F, freshwater

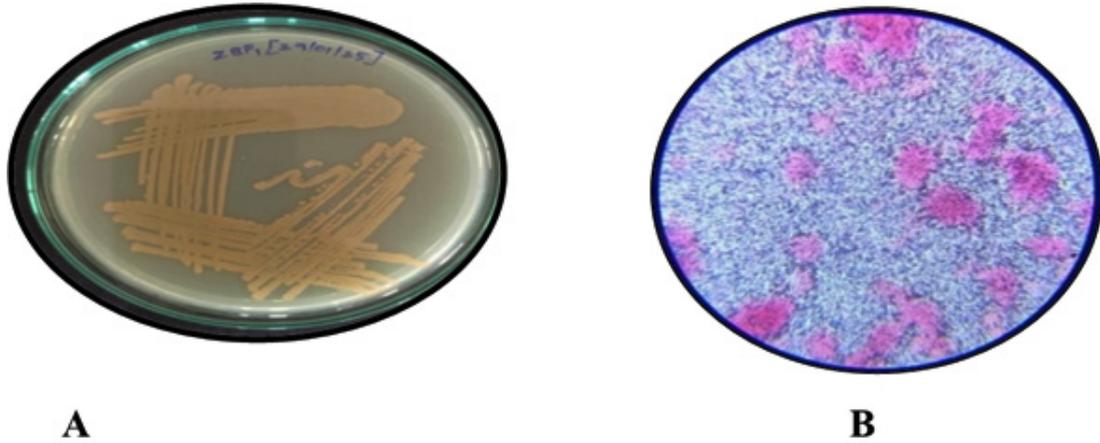


Fig. 2. The *Bacillus megaterium* culture plate with yellow and round colonies (morphology) on Nutrient Agar plate. (B) Colonies of gram-positive rod-shaped bacteria under a light microscope at 40 × magnification.

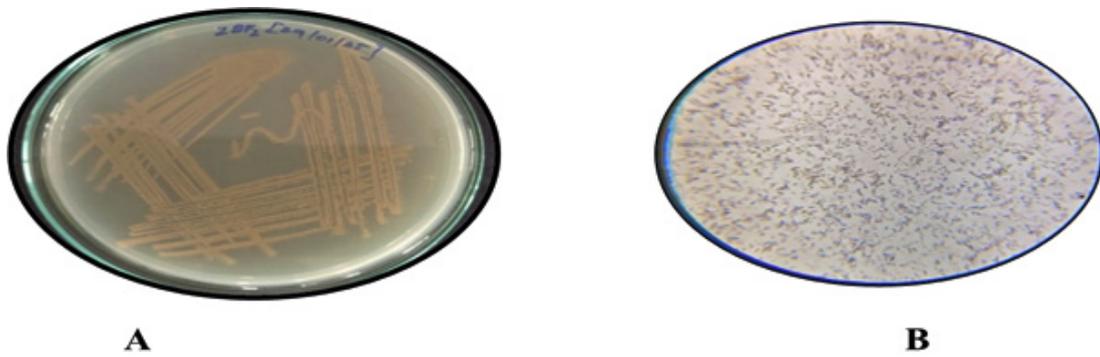


Fig. 3. (A) The *Bacillus subtilis* culture plate with yellow and round colonies (morphology) on Nutrient Agar plate. (B) Colonies of gram-positive rod-shaped bacteria under a light microscope at 40 × magnification.



Fig. 4. (A) The *Bacillus subtilis* culture plate with yellow and round colonies (morphology) on Nutrient Agar plate. (B) Colonies of gram-positive rod-shaped bacteria under a light microscope at 40 × magnification



Fig. 5. (A) The *Bacillus cereus* culture plate with white and round colonies (morphology) on Nutrient Agar plate. (B) Colonies of gram-positive rod-shaped bacteria under a light microscope at 40 × magnification

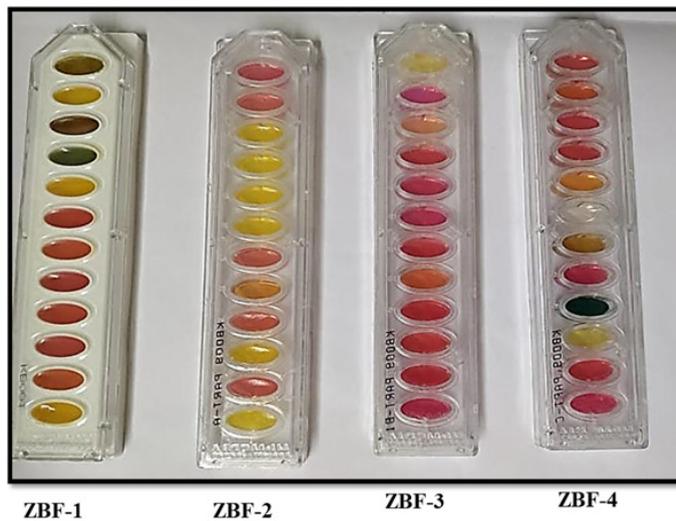


Fig. 6. Biochemical Test Kit - KB001 Hi – HI media. The kit contains 12 wells with corresponding medium and reagents for 12 biochemical tests and this kit is used to identify the biochemical characteristics of *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus sps* and *Bacillus cereus* bacterial species

Table 2. Phenotypic characteristics of isolated bacteria

Characteristics	ZBF1 (<i>Bacillus megaterium</i>)	ZBF2 (<i>Bacillus subtilis</i>)	ZBF3 (<i>Bacillus sps</i>)	ZBF4 (<i>Bacillus Cereus</i>)
Morphology	G (+) rods	G (+) rods	G (+) rods	G (+) rods
Bioluminescence	-	-	-	-
Bacterial pathogenicity	+	+	+	+
Growth on 28!	+	+	+	+
Growth on 37	+	+	+	+
Absorption of bromothymol blue	+	+	+	+
Catalase	+	+	+	+
Spore Formation	+	+	+	+
Colony Morphology	Yellow-colour	Yellow-colour	Yellow-colour	White-colony

the control. Glucose was identified as the key component for enhancing secondary metabolite production in these gut bacteria. Effect of various carbon sources on the growth condition of gut bacterial culture shown in (Figure.7)

Nitrogen source

The growth of isolates ZBF-1, ZBF-2, ZBF-3, and ZBF-4 was tested with different nitrogen sources like (beef extract, peptone, yeast extract, and soybean) in nutrient broth. After incubation for 0 to 96 hours, promoting the highest

Table 3. Biochemical tests for the bacterial strains of ZBF1, ZBF2, ZBF3 and ZBF4

S.No.	Biochemical test	ZBF1	ZBF2	ZBF3	ZBF4
1.	Indole test	-	-	+	+
2.	Methyl red	-	-	-	-
3.	Voges-Proskauer	-	-	-	-
4.	L-arabitol	-	-	-	-
5.	D-Cellobiose	-	-	-	-
6.	Beta-Galactosidase	-	-	-	-
7.	H ₂ S Production	-	-	-	-
8.	D- methyl –D- Glycosides	-	-	-	-
9.	Glutaryl ArylamidasepNa	+	+	+	+
10.	D-Glucose	+	-	+	-
11.	Dextrose	+	+	-	-
12.	Glucose	+	-	+	-
13.	Beta-Glucosidase	+	-	+	-
14.	D-Maltose	-	-	-	-
15.	D-Mannitol	-	-	-	-
16.	D-Mannose	+	-	+	-
17.	Beta-Xylosidase	-	-	-	-
18.	Alpha- Methyl-D- Glycosides	-	-	-	-
19.	L-Proline Aryl amidase	+	+	+	+
23.	Urease	+	-	+	-
24.	D-Sorbitol	+	-	+	-
25.	Sucrose	-	+	+	-
27.	D-Trehalose	-	-	-	-
28.	Citrate-sodium	-	-	-	-
29.	Malonate	-	-	+	+
30.	Alpha-Glucosidase	+	+	-	-
31.	Succinate Alkalisiation	+	-	+	-
32.	L- Arabinose	-	-	-	-
33.	Galactosaminidase	+	+	-	-
34.	Alpha-Galactosidase	-	+	-	+
35.	Phosphatase	+	-	+	-
36.	Glycine Aryl amidase	+	+	-	-
37.	Ornithine Decarboxylase	+	+	-	-
38.	Lysine Decarboxylase	-	-	-	-
39.	L-Histidine assimilation	+	-	+	+
40.	Esculin	-	+	-	+
41.	Beta-Glucuronidase	+	-	+	-
42.	Rhamnose	-	-	-	-
43.	Adonitol	+	+	-	-
44.	Salicin	-	+	+	-
46.	L-Lactate assimilation	+	+	+	-

‘+’ positive and ‘-’ negative

bacterial growth at 72 hours compared to the control. peptone showed the highest growth in the gut bacterial cultures ZBF-2, ZBF-4 as determined by optical density at 600 nm, making it the most effective nitrogen source for enhancing bacterial growth. Effect of various nitrogen source on the growth condition of gut bacteria culture as shown in (Figure.8)

Growth of gut bacteria at various Temperature

The growth of gut bacteria from isolates ZBF1, ZBF2, ZBF3 and ZBF4 was assessed at different temperatures (10°C, 20°C, 25°C, 30°C, 35°C and 40°C) using nutrient broth. After 24-48 hours of incubation, optical density at 600 nm was measured, with the highest growth observed

at 35°C, indicating it as the optimal temperature for maximum growth ZBF and ZBF4 . Effect of various temperature on the growth condition of gut bacteria culture shown in (Figure.9)

Growth of gut bacteria at various pH

The growth of recovered isolates ZBF1, ZBF2, ZBF3, and ZBF4 was evaluated at different pH levels (5.0, 6.0, 7.0, 8.0, and 9.0) in nutrient broth. After incubating for 0 to 96 hours, the growth was measured by optical density at 600 nm. The highest growth was observed at pH 7, indicating it as the optimal pH for maximum bacterial growth. Effect of various pH on the growth condition of gut bacteria culture shown as (Figure.10)

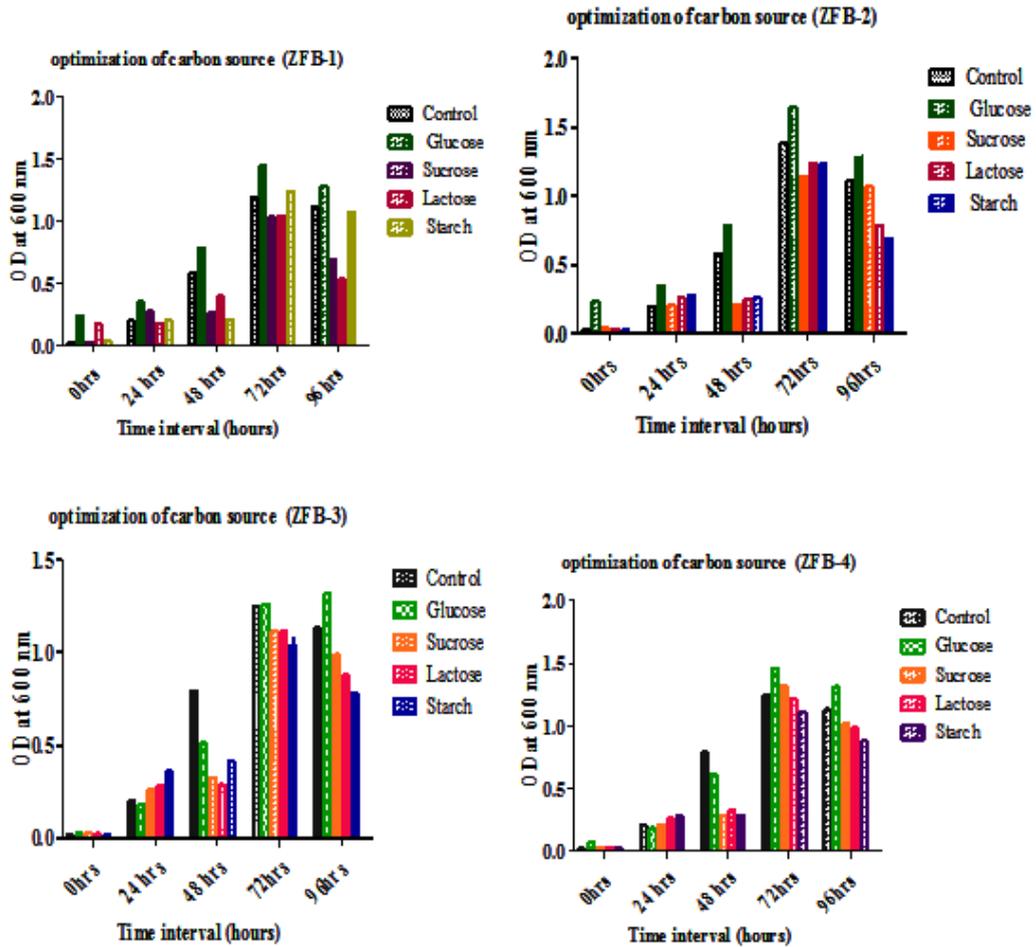


Fig. 7. Effect of various carbon source on the growth condition of gut bacterial culture (Values are expressed by mean ± SD)

Growth of gut bacteria at various culture media

The growth conditions of isolates ZBF1, ZBF2, ZBF3, and ZBF4 were tested in different culture media: nutrient broth, LB broth, Tryptic soy broth, Muller Hinton broth, and MacConkey broth. After incubating for 0 to 96 hours, the growth was measured by optical density at 600 nm. The highest growth was observed in nutrient broth, indicating it as the optimal medium for maximum bacterial growth. Gut bacterial growth on various media at different time intervals shown in (Figure.11)

Cell free supernatant extraction of ZBF-2 & ZBF-4 of gut bacteria

The cell-free supernatants from *Bacillus subtilis* ZBF-2 and *Bacillus cereus* ZBF-4, grown in nutrient broth, were extracted after centrifugation and sterile filtration. Bioactivity tests showed that *Bacillus subtilis* ZBF-2 exhibited strong

antimicrobial activity against both Gram-positive and Gram-negative bacteria, while *Bacillus cereus* ZBF-4 showed moderate antibacterial effects, though less potent. Summarizing the cell-free supernatant extraction of *Bacillus subtilis* ZBF-2 and *Bacillus cereus* ZBF-4 (gut bacteria strains) and their biological activities as shown in (Table.4)

16S rRNA Sequencing Identification

Genomic DNA of two isolated gut bacterial strains was used for PCR amplification with 16S rDNA primers. The amplified fragments (1260-1500 bp) were analyzed on an agarose gel as shown in (Figure.12). BLAST analysis revealed 98% sequence similarity with *Bacillus subtilis* for ZBF-2 and *Bacillus cereus* for ZBF-4. A phylogenetic tree was constructed using the Neighbour-Joining method in Mega 6 software as shown in (Figure.13A, B). The 16S rDNA

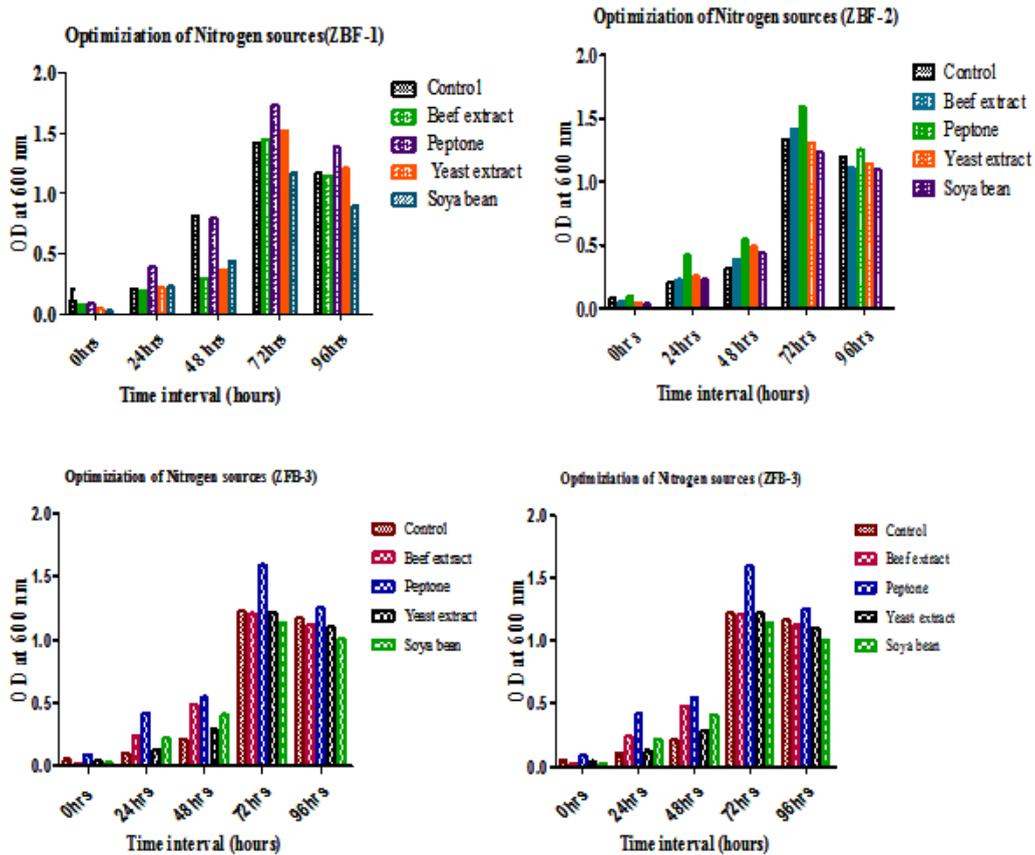


Fig. 8. Effect of various nitrogen source on the growth condition of gut bacteria (Values are expressed by mean ± SD).

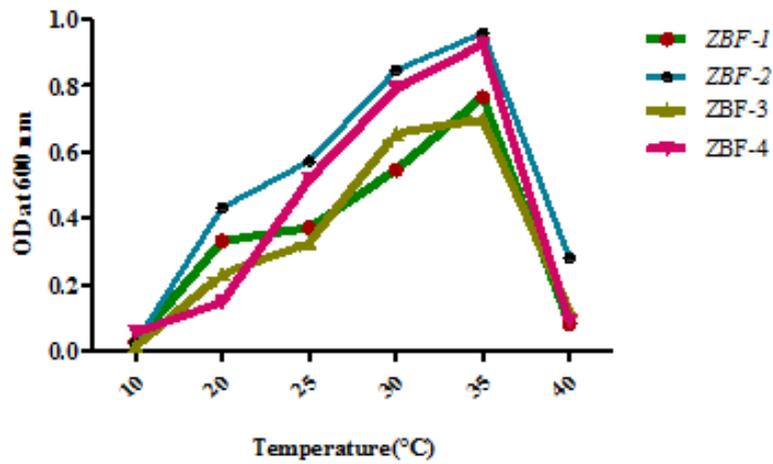


Fig. 9. Effect of various Temperature on the growth condition of gut bacteria (Values are expressed by mean ± SD).

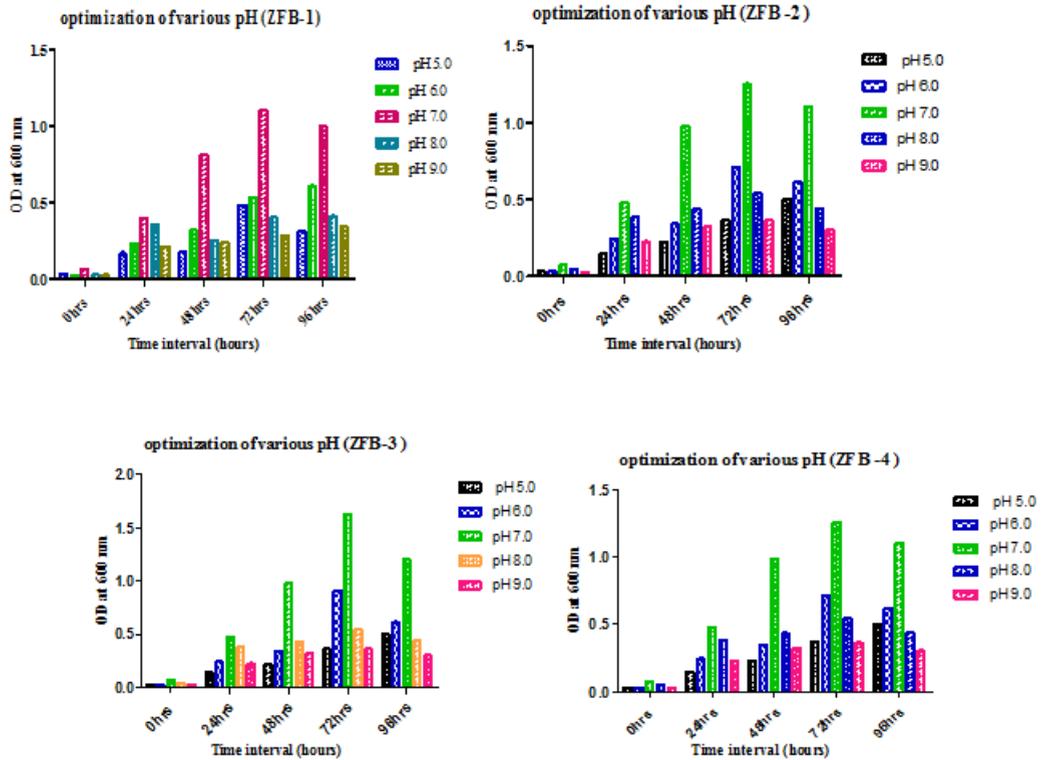


Fig. 10. Effect of various pH on the growth condition of gut bacteria (Values are expressed by mean ± SD).

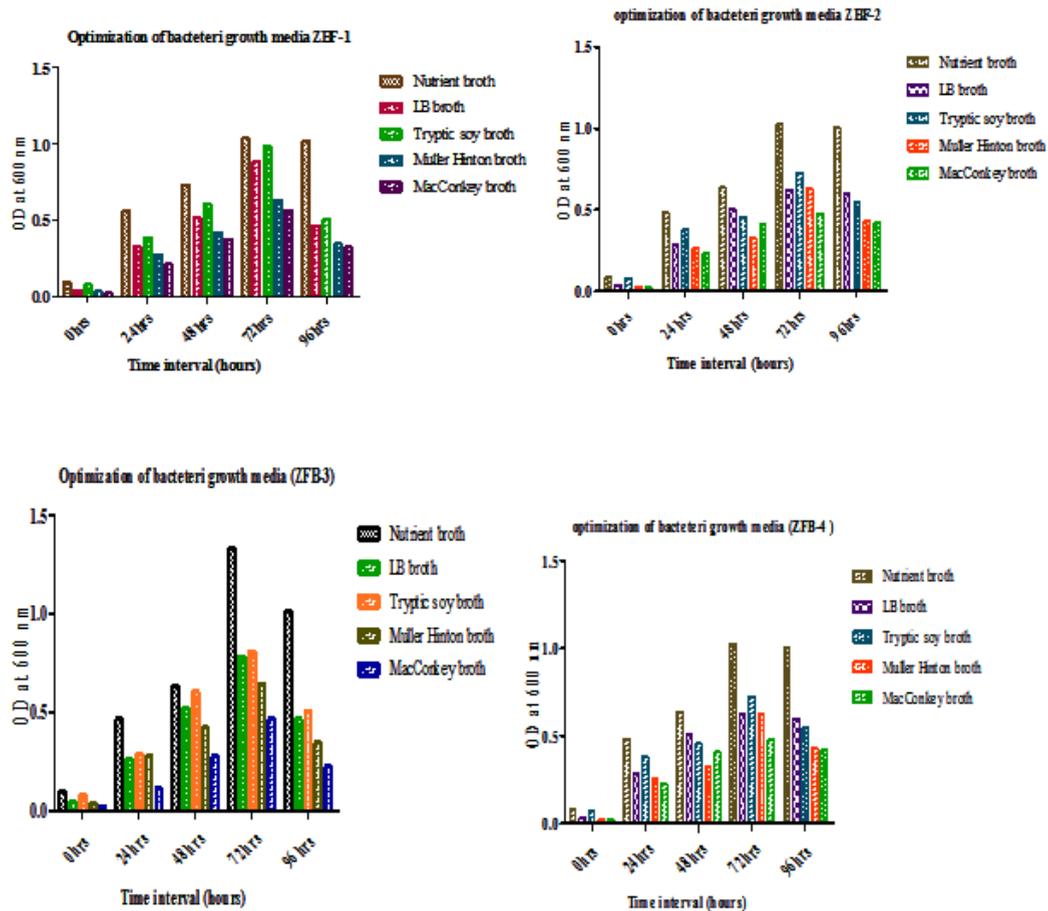


Fig. 11. Gut bacterial growth on various media at different time intervals.

sequences were submitted to GenBank, with accession numbers PV297978 (*Bacillus subtilis*) and PV300533 (*Bacillus cereus*).

Secondary Metabolites from gut bacteria and larval toxicity

Virulence of gut bacteria *Bacillus subtilis* (ZBF-2) and *Bacillus cereus* (ZBF-4) and larval toxicity. From the broth culture, the extracted secondary metabolite of *Bacillus subtilis* (ZBF-2) and *Bacillus cereus* (ZBF-4) 1mg was dissolved in 1 ml of (DMSO)dimethyl sulfoxide(1 mg/ml). The larvicidal efficacy of secondary metabolite on *S. litura* was studied based on various concentration of secondary metabolite. After 24hrs of treatment the highest mortality was recorded in 10⁻⁴ concentration in all treatments. Mortality of *S. litura* depends on the concentration of secondary metabolites injected

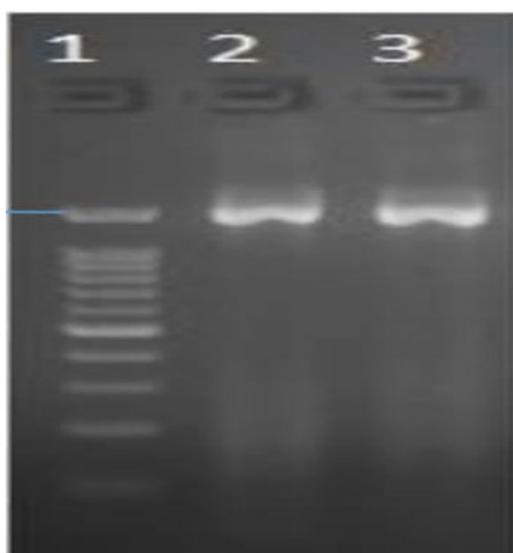
into larvae remaining concentration low levels mortality was observed (Figure.14,15).

DISCUSSION

Ray et al.³⁰ study reported that explored the influence of enzymatic activity and gut microbiota on fish digestion, emphasizing the role of factors like food type, digestive enzyme function, and exposure time. Their research highlighted that gut microbiota, including genera such as *Bacillus*, *Enterobacteriaceae*, and *Acinetobacter*, positively affects digestion by producing enzymes that aid in food breakdown. However, the varying digestive tract composition across fish species complicates determining the exact impact of this microbiota. In our study, we identified several *Bacillus* species,

Table 4. Summarizing the cell-free supernatant extraction of *Bacillus subtilis* ZBF-2 and *Bacillus cereus* ZBF-4 (gut bacteria strains) and their biological activities

Bacterial Strain	Incubation Time	Supernatant Collection Method	Bioactivity Assay	Larvicidal Activity	Feeding Inhibition
ZBF-2 (Gut Bacteria)	24-48 hours	Centrifugation followed by sterile filtration (0.22 µm filter)	<i>Spodoptera litura</i> larvicidal bioassay	65-75% mortality at high concentration	Reduced feeding observed
ZBF-4 (Gut Bacteria)	24-48 hours	Centrifugation followed by sterile filtration (0.22 µm filter)	<i>Spodoptera litura</i> larvicidal bioassay	50-60% mortality at high concentration	Mild feeding inhibition

**Fig. 12.** PCR amplification of 16s rDNA fragment from ZBF-1 and ZBF-4 strains. The size of PCR amplified product is ~1500bp.

including *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus spp.*, and *Bacillus cereus*, which aligns with previous study of the ³⁰ findings on the significance of *Bacillus* in fish digestion. Despite this, both studies face challenges in understanding the interactions of these species within the complex gut microbiota of different fish species. The gut microbiota is producing varieties of bioactive compounds such as proteins, enzymes, antibacterial and antifungal compounds were more responsible to the pharmaceutical and insecticidal agent of the microbiota organisms.

Dhayalan *et al.* ³¹ focused on isolating *Bacillus thuringiensis* from the gut of *Systemussarana* and optimizing culture parameters

for maximal protease enzyme production using response surface methodology (RSM) and Box-Behnken design (BBD). Their study emphasized optimizing culture conditions to enhance enzyme yield, with a focus on protease production and its peptide profile. In contrast, our study evaluated factors such as carbon and nitrogen sources, temperature, pH, and different broth cultures to optimize bacterial growth rate of the parameters. The findings in our result suggest the main role of carbon, nitrogen, minerals, vitamin, temperature and pH source in promoting for the bacterial growth, with optimal growth at room temperature in especially in nutrient broth when compare to other broth. Our study, offers a broader examination of growth conditions. Despite the differences, both studies underscore the significance of tailored conditions for enhancing microbial activity, with implications for fish nutrition and enzymatic processes.

Mansooreh Jami *et al.* ³² studied the diversity of Actinobacteria in the gut microbiota of two freshwater fish species, *Schizothorax zarudnyi* and *Schizocypris altidorsalis*. They cultured 277 isolates using three different agar media, identifying a range of Actinobacteria, primarily from the *Streptomycetaceae* family, along with other genera such as *Saccharomonospora*, *Micromonospora*, *Nocardioopsis*, and *Arthrobacter*. Their focus was on the diversity of secondary-metabolite biosynthetic genes (e.g., PKS I, NRPS, PhzE) and their antimicrobial activity against human and fish pathogens. In contrast, our study investigates the bacterial genus *Bacillus*, using PCR-based 16S rRNA gene sequencing and partial sequencing, which differs in both the bacterial genus and the methodological approach to the

sequencing were identified and similarity to the BLAST tool alignment base pair of the nucleotide.

In present study, the identification of *Bacillus subtilis* and *Bacillus cereus* through 16S rRNA gene sequencing aligns with the method used by Jami *et al.*³³ for taxonomic identification. However, previous study, focuses more specifically on *Bacillus* species, known for their antimicrobial properties and insecticidal activity. Whereas, Jami *et al.*³² explored the broader diversity of Actinobacteria. Both studies highlight

the potential of freshwater fish gut microbiota as a source of bacteria with antimicrobial and bioactive compound-producing capabilities of these organisms. While Jami *et al.*³³ examined a wide array of Actinobacteria with potential for secondary metabolite production, our study's submission to the GenBank further supports the genetic characterization and global sharing of *Bacillus* isolates. In conclusion, while both studies focus on microbiota from freshwater fish, your research is more narrowly focused on *Bacillus*

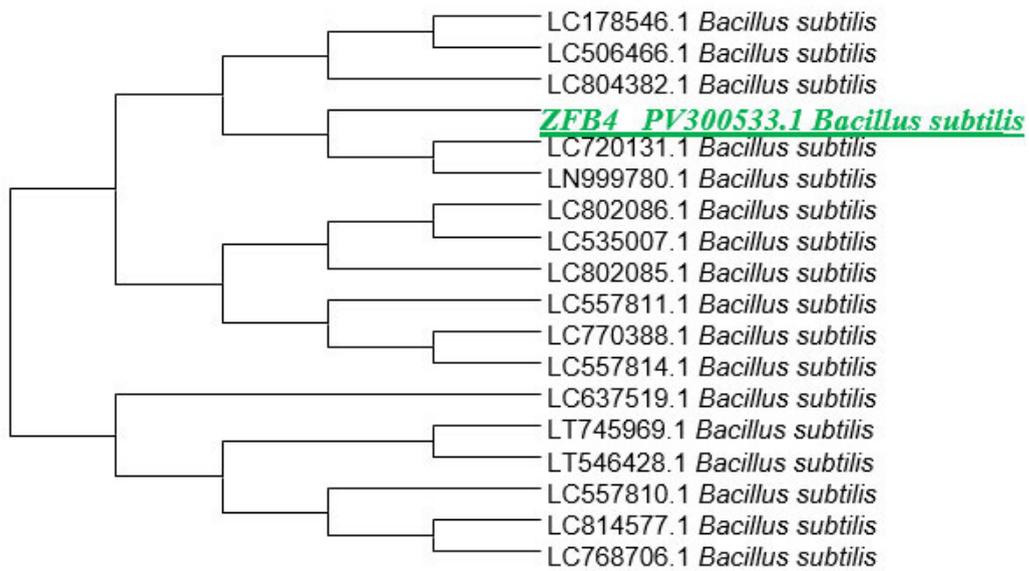


Fig.13. (A) gut bacteria isolated organisms identified and phylogentic tree was construction by mega 6 software

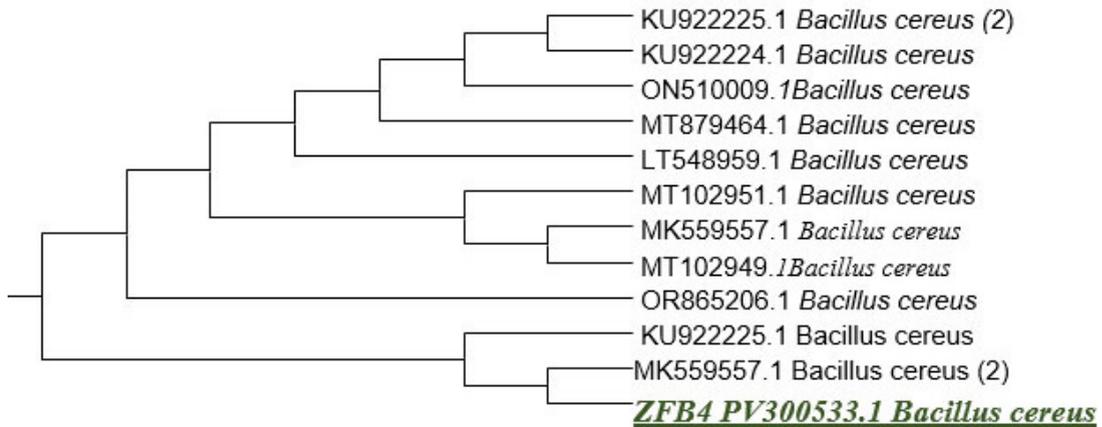


Fig. 13. (B) gut bacteria isolated organisms identified and phylogentic tree was construction by mega 6 software

species, whereas Jami *et al.*³³ emphasized a wider range of Actinobacteria and their secondary metabolite gene diversity.

Park *et al.*³⁴ study reported that *X. hominickii* isolated from *S. monticolum* caused significant larval mortality when injected into different larvae of *S. exigua*, *P. xylostella*, *H. assulta*, *P. brassicae*, and *D. melanogaster*. Vicente-Diez *et al.*³⁵ found that the symbiotic bacterial supernatant from *P. laumondii* resulted in

64% mortality in *P. spumarius* nymphs. Ngugi *et al.*³⁶ study observed that the bacterial supernatant of *Bacillus subtilis* at a concentration of 3.6×10^{-9} induced 68-88% larval mortality in *Spodoptera litura* within 24 and 48 hours. In our present study, the supernatant of isolated gut bacteria *Bacillus subtilis* and *Bacillus cereus*, injected at a concentration of 40 il/ml, produced 76-80% mortality at 48 hours, confirming the pathogenicity of the gut bacteria secondary metabolites.

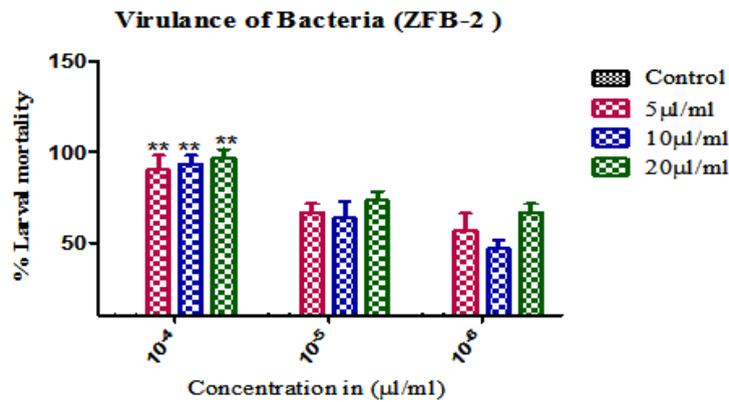


Fig. 14. Mortality in 3rd instar *S. litura* following exposure to gut bacteria in different concentration. Treatments were conducted in triplicates of 10 larvae. The values are expressed as mean ± SD and analysed by One-Way Analysis of Variance (ANOVA). Asterisk (**) (*) with indicates significant difference among respect to control (p<0.05) (p<0.01).

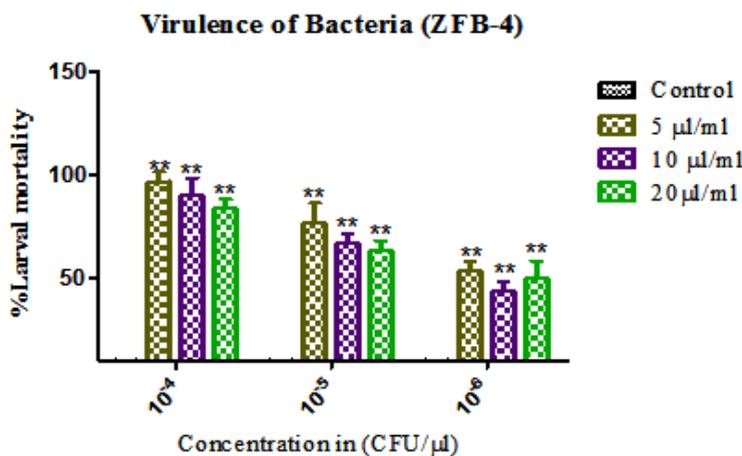


Fig. 15. Mortality in 3rd instar *S. litura* following exposure to gut bacteria in different concentration. Treatments were conducted in triplicates of 10 larvae. The values are expressed as mean ± SD and analysed by One-Way Analysis of Variance (ANOVA). Asterisk (**) (*) with indicates significant difference among respect to control (p<0.05) (p<0.01).

This present study reported that, the gut bacteria *Bacillus subtilis* ZBF-2 and *Bacillus cereus*, ZBF-4 showed optimal growth levels with the help of the added carbon (glucose) and nitrogen (peptone) sources to commercialized media. In addition, optimal temperature for *Bacillus subtilis* and *Bacillus cereus*, was found to be 30 and 35°C. Among the tested pH at 7.0 showed the best bacterial growth rate for the bacterial species *Bacillus subtilis* and *Bacillus cereus*, through enhancing for the secondary metabolites production. Isolated gut bacterial cell free supernatant shows the higher concentration in *Bacillus subtilis* and *Bacillus cereus*, produced 76- 80% larval mortality at 48hrs. When compared to the control groups.

CONCLUSION

In conclusion, this study reported that, the zebrafish gut microbes ZBF-2 *Bacillus subtilis*, ZBF-4 *Bacillus cereus* showed optimal growth level supplemented with carbon (glucose) and nitrogen (peptone) source to commercialized media. In addition, optimal temperature for ZBF-2 *Bacillus subtilis*, ZBF-4 *Bacillus cereus* was found to be 30 and 35°C. Among the tested pH at 7.0 showed the best bacterial growth for ZBF-2 *Bacillus subtilis*, ZBF-4 *Bacillus cereus* through enhancing the secondary metabolites production of the gut bacteria. Cell free supernatant of isolated gut bacteria ZBF-2 *Bacillus subtilis*, ZBF-4 *Bacillus cereus* with 10⁻⁴ µl/ml concentration produced 76- 80% larval mortality at 48hrs. The bioassays revealed a dose- and time-dependent effect, with higher concentrations causing larval mortality at 48 hours. This confirms the pathogenicity of ZBF-2 *Bacillus subtilis*, ZBF-4 *Bacillus cereus* strains. Our studies suggest that the larvicidal activity from the cell free supernatant extract could be used for production of novel class of insecticides properties in future.

ACKNOWLEDGEMENT

We would like to thank the Department of Microbiology, AVS College of Arts and Science (Autonomous), Salem, Tamil Nadu, India for providing the infrastructural facility for carrying out this research work.

Funding Sources

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Permission to reproduce material from other sources

Not Applicable

Author contributions

Salem Sundarrajan Maithili: Visualization, Supervision, Writing – Review and Editing. Kandhasamy Lalitha: Conceptualization, Methodology, Writing – Original Draft. Muthu Ganesh boopathi: Data Collection, Analysis, Writing – Review and Editing and authors gave approval for publication.

REFERENCES

1. Budd, K. Effects of diet, habitat, and phylogeny on the fecal microbiome of wild African savanna (*Loxodonta africana*) and forest elephants (*L. cyclotis*). *Ecol. Evol.* 2020;10(12); 5637-5650. doi: 10.1002/ece3.6305
2. Cui, X. Research progress of the gut microbiome in hybrid fish." *Microorganisms*.2022;10(5);891. <https://doi.org/10.3390/microorganisms10050891>
3. Stephens, W. Z et al. The composition of the zebrafish intestinal microbial community varies across development." *The ISME journal*. 2016;10(3): 644-654. <https://doi.org/10.1038/ismej.2015.140>
4. Fortunato, Elvira, Pedro B, and Rodrigo M, et al. Oxide semiconductor thin film transistors: a review of recent advances. *Adv. Mater.* 2012; 24(22): 2945-2986.

5. Su, L. The different clinical characteristics of corona virus disease cases between children and their families in China—the character of children with COVID-19. *Emerg. Microbes Infect.* 2020;9(1): 707-713. doi: 10.1080/22221751.2020.1744483
6. Ringø, E, Electron microscopy of the intestinal microflora of fish. *Aquaculture* 2003;227(1-4):395-415. <https://doi.org/10.1016/j.aquaculture.2003.05.001>
7. Bates A, Two zebras and a cardiac arrest: a case report of concomitant Brugada syndrome and an anomalous coronary artery. *Eur. Heart J. Case Rep.* (2020); 4(6) :1-6. doi: 10.1093/ehjcr/ytaa425
8. Engeszer R, Patterson Andrew A. Rao and David M. Parichy et al. Zebrafish in the wild: a review of natural history and new notes from the field.” *Zebrafish*. 2007;41: 21-40.
9. Kumar D, Vinod Kumar AK, Verma Prashant Singh, and Ashutosh Dubey, et al., Characterization and immobilization of partially purified alkaline protease extracted from rhizospheric soil bacterium, *Bacillus megaterium* strain EN-2 and *Bacillus subtilis* strain EN-3. *Research Afr. J. Microbiol. Res* 2014;8(1): 33-39. <https://doi.org/10.5897/AJMR2013.6167>
10. Ehling-S, Monika, E F, and Michel G, et al. Food–bacteria interplay: pathometabolism of *Bacillus cereus*. *Front. Microbiol.* 2015;(6):704 doi: 10.3389/fmicb.2015.00704
11. Abriouel, H, Charles MAP Franz, Nabil Ben O, and Antonio G. “Diversity and applications of *Bacillus bacteriocins*. *FEMS Microbiol. Rev.* 2011; 35(1):201-232. doi: 10.1111/j.1574-6976.2010.00244
12. Luise D, Micol B, Vincenzo M, Chiara S, Paolo Bosi, Andrea L, Flaminia F et al. “*Bacillus* sp. probiotic supplementation diminish the *Escherichia coli* F4ac infection in susceptible weaned pigs by influencing the intestinal immune response, intestinal microbiota and blood metabolomics. *J. Anim. Sci. Biotechnol.* 2019; 10(1): 74. doi: 10.1186/s40104-019-0380-3
13. Yakimov AP. Seregina TA. Kholodnyak AA. Kreneva RA. Mironov AS. Perumov DA and Timkovskii, AL, et al. Possible function of the ribT gene of *Bacillus subtilis*: theoretical prediction, cloning, and expression. *Acta Naturae (äiäëÿçü÷-îäÿääðñëÿ)*, 2014;6(3):106-109: <https://doi.org/10.32607/20758251-2014-6-3-106-109>.
14. Kim -J, Jae-KH Jong-SP, Jin-SL, Soon-HL, Joon-II C, and Keun-S K. et al. Various enterotoxin and other virulence factor genes widespread among *Bacillus cereus* and *Bacillus thuringiensis* strains. *J Microbiol Biotechnol.* 2015;25(6):872-879. doi: 10.4014/jmb.1502.02003.
15. Wang XQDL, Zhao LL, Shen CL Jing and CS, Zhang et al. Application and mechanisms of *Bacillus subtilis* in biological control of plant disease. In *Role of rhizospheric microbes in soil: Volume 1: Stress management and agricultural sustainability*, Singapore: *Springer Singapore*, 2018:225-250. DOI:10.1007/978-981-10-8402-7_9
16. Bernadsky, G, and Rosenberg E et al. Drag-reducing properties of bacteria from the skin mucus of the cornetfish (*Fistularia commersonii*). *Microbial Ecol.* 1992; 24(1): 63-76. <https://doi.org/10.1007/BF00171971>.
17. Langendijk, Petra S, Frits Schut Gijsbert J, Jansen, Gerwin C, Raangs, Ger R., Kamphuis, MH, Wilkinson and Gjalte W. Welling, et al. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl. Environ. Microbiol.* 1995; 61(8):3069-3075.
18. Munro P O A, Barbour and T H Blrkbeck, Comparison of the gut bacterial flora of start feeding larval turbot reared under different conditions. *J. Appl. Microbiol.* 1994; 77, no. 5 560-566.
19. Cahill Marian M. Bacterial flora of fishes: a review. *Microbiol ecol.* 1990; 19(1) 21-41. doi: 10.1007/BF02015051.
20. Gandhi K, and R H. Patil. Field resistance of *Spodoptera litura* (Fab.) to conventional insecticides in India. *Crop Prot.* 2016; 88:103-108. <https://doi.org/10.1016/j.cropro.2016.06.009>
21. Armes Nigel J, John A. Wightman Deepak R. Jadhav and Gangavalli V. Ranga R et al. Status of insecticide resistance in *Spodoptera litura* in Andhra Pradesh, India. *Pestic. Sci.* 1997; 50(3) 240-248.
22. Sharma, S and Tejinder S B, et al. Effects of elevated temperature and carbon dioxide on food consumption and growth of *Spodoptera litura* Fabricius on cauliflower. *J. Agrometeorol.* 2018;20(4)305-310. DOI: <https://doi.org/10.54386/jam.v20i4.571>
23. Singh A, Singh S, Singh, R, Kumar S, Singh, SK. and Singh I.K, et al. Dynamics of *Zea mays* transcriptome in response to a polyphagous herbivore, *Spodoptera litura*. *Funct Integr Genomics* 2021; 21(5): 571-592. doi: 10.1007/s10142-021-00796-7
24. Yakimov, A.P. S. Afanasev M. A. Khodorkovskiy, and M.G Petukhov et al. Design of stable α -helical peptides and thermostable proteins in

- biotechnology and biomedicine. *Acta Naturae*. 2016; 8,4 (31):70-81.
25. Wang, Yutang, Lixia Li, Hua Liu, Tong Zhao, Chenshuo Meng, Zhande Liu, and Xuebo Liu et al. Bioactive compounds and in vitro antioxidant activities of peel, flesh and seed powder of kiwi fruit." *Int. J. Food Sci. Technol.*2018;53(9): 2239-2245. <https://doi.org/10.1111/ijfs.13812>
 26. Kaur, M, Chadha P, KaurS, Kaur A, Kaur R, YadavAK, Kaur R et al.*Schizophyllum* commune induced genotoxic and cytotoxic effects in *Spodoptera litura*." *Sci Rep.*2018: 8(1): 4693. <https://doi.org/10.1038/s41598-018-22919-0>
 27. Vijayakumari Nadaraja, A, Jayakumaran Nair, A, Prameela M, Hari N, BalakrishnanN et al. Evaluation of currently employed food preservation conditions to tackle biofilm forming food pathogens. *J. Food Saf.* 2018: 38(1);12407. <https://doi.org/10.1111/jfs.12407>
 28. Park J, Park BS, Wang P, Patidar SK, Kim JH, Kim SH, Han MS, et al. Phycospheric native bacteria *Pelagibacabermudensis* and *Stappia sp. ameliorate* biomass productivity of *Tetraselmis striata* (KCTC1432BP) in co-cultivation system through mutualistic interaction." *Front. Plant Sci.* 2017: 8; 289. <https://doi.org/10.3389/fpls.2017.00289>
 29. Lalitha, K., Kalaimurgan, D, Nithya K, Venkatesan S. and Shivakumar MS. Et al. Antibacterial, antifungal and mosquitocidal efficacy of copper nanoparticles synthesized from entomopathogenic nematode: Insect–host relationship of bacteria in secondary metabolites of *Morganella morganiisp.**PMAI Arab. J. Sci. Eng.*.2020: 45(6); 4489-4501. DOI:10.1007/s13369-020-04487-6
 30. Ray AK, K. Ghosh and EJA N, Ringø et al. Enzyme producing bacteria isolated from fish gut: a review. *Aquac. Nutr.* 2012: 18(5); 465-492. DOI:10.1111/j.1365-2095.2012.00943.x
 31. Dhayalan A, Velramar B, Govindasamy B, Ramalingam KR, Dilipkumar A, and Pachiappan P et al. Isolation of a bacterial strain from the gut of the fish, *Systomussarana*, identification of the isolated strain, optimized production of its protease, the enzyme purification, and partial structural characterization *J. Genet. Eng. & Biotechnol.*(2022): 20(1);24. <https://doi.org/10.1186/s43141-022-00299-3>
 32. Jami SA, Ajamhassani M, and Naveh VH, et al. Biochemical characteristics of digestive enzymes of codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). *Phytoparasitica*. 2025: 53(2); 30.
 33. Jami M, Lai Q, Ghanbari M., Moghadam MS, Kneifel W, and Domig, KJ, et al. Phylogenetic diversity and biological activity of culturable Actinobacteria isolated from freshwater fish gut microbiota. *Microbiol. Res.* .2015: 175;6-15. <https://doi.org/10.3389/fmicb.2015.00737>
 34. Park Y, Kang S, Sadekuzzaman M, Kim H, Jung J.K and Kim Y, et al. Identification and bacterial characteristics of *Xenorhabdushominickii* ANU101 from an entomopathogenic nematode, *Steinernema monticolum*. *J. Invertebr. Pathol* 2017;144: 74-87. doi: 10.1016/j.jip.2017.02.002.
 35. Vicente-Díez I, Blanco-PérezR, ChelkhaM, PuellesM, Pou A, and Campos-Herrera R et al. Exploring the use of entomopathogenic nematodes and the natural products derived from their symbiotic bacteria to control the grapevine moth, *Lobesia botrana* (Lepidoptera: Tortricidae). *Insects*. (2021);12,(11)1033. <https://doi.org/10.3390/insects12111033>
 36. Ngugi Cecilian N, et al. Characterization and evaluation of entomopathogenic nematodes for the management of tomato leafminer (*Tuta absoluta* Meyrick)." PhD diss., University of Nairobi, 2021. <http://erepository.uonbi.ac.ke/handle/11295/157047>