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Bioprospecting of an Indigeneous *Bacillus thuringiensis* Strain G5-8-3T02 for Shrimp Culture System

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Authors' contributions

This work was carried out in collaboration between both authors. Author CNA conceived, designed and supervised the study. Author EUA participated during literature searches, sample collection, laboratory experiments, result analysis and interpretation of results. Author CNA also wrote the manuscript and contributed during literature searches and data analysis. Both the authors read and approved the final manuscript.

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ABSTRACT

Aim: An indigenous *Bacillus thuringiensis* strain G5-8-3T02 earlier isolated from healthy shrimp intestine and established to safeguard *Penaeus monodon* from *Vibrio mimicus* infection was assessed as a potential candidate bacterium for drug enhancement for shrimp culture systems.
Methodology: Optimal temperature, pH and salinity for growth and antibacterial activity of *Bacillus thuringiensis* against *Vibrio mimicus* were determined. The effects of enzymes (pepsin, catalase, lipase and α- amylase), pH (2-9), heat (37°C – 121°C) and surfactants (Sodium dodecyl sulphate and Tween 80) on antibacterial activity were also evaluated. Cell growth and antibacterial activity were monitored daily for 5 days via spectrophotometric method at 600 nm and agar well diffusion assay respectively. The metabolite extracted with methanol was analyzed for bioactive compounds using Gas Chromatography-Mass Spectrometry (GC-MS).
Results: The optimum temperature, pH and salinity for growth and metabolite production were 35°C, pH 6-7 and 10-15 ppt. respectively. The crude extract did not totally lose its activity when

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treated with enzymes, various pH values, heat and surfactants. The quantitative composition of the metabolite revealed 15 bioactive compounds. The main compound produced was 2, 6, 10-Trimethyltridecane with peak area of 14.58%.

Conclusion: The non-pathogenic, antagonistic, indigenous *Bacillus thuringiensis* strain G5-8-3T02 could be engaged in the management of vibriosis in shrimp culture since the use of antibiotics in aquaculture for disease control yields objectionable effects. The identified bioactive compounds might be valuable for drug development for shrimp culture system.

Keywords: Shrimp culture; antibacterial activity; bioactive compounds; metabolite.

1. INTRODUCTION

Sea food is one of the utmost vital source of animal protein. It is anticipated that aquaculture will meet the world's demand for sea food due to the decline and over exploitation of wild capture fisheries. Nevertheless, aquaculture business is affected by bacterial epidemic disease which leads to enormous financial loss [1]. *Vibrio* species are prevalent in marine locations and are often isolated in aquaculture environments [2]. They are implicated in major disease epidemics in aquaculture causing mass mortality [3]. They also cause several diseases in shrimp such as *Penaeus monodon* [4,5,6]. The unselective use of antibiotics in aquaculture has steered the expansion of drug- resistant bacteria which are becoming progressively tough to control and exterminate [7].

The use of probiotic bacteria is one of the approaches that has been recognized for disease control in aquaculture [8,9,10]. Probiotics have the competence to produce antimicrobial compounds and have been engaged in inactivation of several pathogens. Microbes particularly bacteria and fungi are promising sources of structurally different and effective bioactive compounds [11]. A lot of these bioactive compounds are employed as chemotherapeutic agents for the management of human and animal infections [12].

Bacillus species and their spores have been employed in shrimp culture production for management of vibriosis [13]. Fu et al. [14] conducted probiotic effect of *Bacillus pumilus* H2 to juvenile shrimp in aquaculture tanks. The probiotic potential of *Bacillus thuringiensis* strain G5-8-3T02 on shrimp (*Penaeus monodon*) culture infected with *Vibrio mimicus* has been reported [6].

However, there has not been any information on the bioactive compounds produced by this indigenous, non-pathogenic, antagonistic marine bacterium, *Bacillus thuringiensis*, which was earlier isolated from healthy shrimp intestine and established to shield the shrimp from *Vibrio mimicus* infection [6]. Therefore, this study was designed to evaluate the bio prospects of *Bacillus thuringiensis* strain G5-8-3T02 for shrimp culture systems.

2. MATERIALS AND METHODS

2.1 Source of Pathogenic *Vibrio mimicus* and Antagonistic *Bacillus thuringiensis*

Pathogenic *Vibrio mimicus* was earlier isolated from moribund shrimp (*Penaeus monodon*) intestine [15] and was identified molecularly as *Vibrio mimicus* XQ [5] while *Bacillus thuringiensis* was earlier isolated from healthy shrimp (*Penaeus monodon*) intestine [6]. They were preserved as part of the culture collection in Microbiology Laboratory, University of Port Harcourt, Nigeria.

2.2 Effects of Abiotic Factors on Growth and Antibacterial Activity of *Bacillus thuringiensis*

2.2.1 Effect of temperature

The optimum temperature for growth and antibacterial activity of *Bacillus thuringiensis* was evaluated. One hundred millilitres of sterile nutrient broth in 250 ml conical flasks were inoculated with 0.1 ml of a 24 h culture of *Bacillus thuringiensis* and exposed to a number of incubation temperatures (25, 30, 37 and 40°C) for 48 h. The antibacterial activity of *Bacillus thuringiensis* against *Vibrio mimicus* was measured at zero hour and every 24 h for 5 days using agar diffusion assay. Nutrient agar plates were inoculated with 0.1 ml of a 24 h broth culture of *Vibrio mimicus*. Wells were prepared on the plates using a sterile cork borer and 0.1 ml of *Bacillus thuringiensis* was put inside the wells and plates were incubated at different temperatures for 24 h. Clear zones of inhibition

were measured and documented. The optical density (OD) of *Bacillus thuringiensis* at different temperatures were measured every 24 h for 5 days via spectrophotometer at a wavelength of 600 nm.

2.2.2 Effect of pH

With a pH meter, the 100 ml nutrient broth in 250 ml conical flasks had their pH adjusted to pH of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using 0.1 M HCl and 0.1 M NaOH. Thereafter, the flasks were autoclaved at 121°C for 15 min. The flasks were inoculated with 0.1 ml of 24 h culture of *Bacillus thuringiensis* and incubated at 37°C. The antibacterial activity of *Bacillus thuringiensis* against *Vibrio mimicus* was assessed at zero hour and every 24 h for 5 days using agar diffusion assay. The growth of *Bacillus thuringiensis* at dissimilar pH levels were evaluated at zero hour and every 24 h for 5 days using spectrophotometer at a wavelength of 600 nm.

2.2.3 Effect of salinity

The 100 ml nutrient broth in 250 ml conical flasks were supplemented with diverse concentrations of sodium chloride (0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%) before autoclaving at 121^{o}C for 15 min at 15 psi. On cooling each flask was inoculated with 0.1 ml of 24 h culture of *Bacillus thuringiensis* and incubated at 37°C. The antibacterial activity of *Bacillus thuringiensis* against *Vibrio mimicus* was measured at zero hour and every 24 h for 5 days via agar diffusion assay. The growth of *Bacillus thuringiensis* at dissimilar salinities were measured at zero hour and every 24 h for 5 days with spectrophotometer at a wavelength of 600 nm.

2.3 Initial Characterization of the Antibacterial Constituents of Cell Free Supernatant of *Bacillus thuringiensis*

2.3.1 Effect of enzymes

The effect of enzymes (pepsin, catalase, lipase and α- amylase) on antibacterial activity of cell free supernatant (crude extract) of *Bacillus thuringiensis* were evaluated. The cell free supernatant of *Bacillus thuringiensis* having the antibacterial constituent was obtained after centrifuging a 48 h Mueller Hinton broth culture of *Bacillus thuringiensis* at 4000 rpm for 30 min. It was treated with the ensuing enzymes at a final concentration of 1 mg/ml pepsin, catalase, lipase and α- amylase. The test tubes containing supernatant and enzyme mixture were incubated at 37°C for 2 h and thereafter heated at 100°C for 5 min to discontinue enzyme activities. Untreated samples were used as controls. The antibacterial activities of enzyme treated and untreated cell free supernatant of *Bacillus thuringiensis* against *Vibrio mimicus* were evaluated via agar diffusion assay.

2.3.2 Effect of pH

The activity of antibacterial component of the cell free supernatant of *Bacillus thuringiensis* was assessed at dissimilar pH values (2-9). The pH of the cell free supernatant was adjusted with specific buffers. The pH of 2 and 3 were adjusted using glycine -HCl buffer while acetate buffer was employed for pH 4 and 5. Sodium phosphate buffer was employed for pH 6 and 7 while glycine -NaOH buffer was employed for pH 8 and 9 [16]. The antibacterial activities, at dissimilar pH levels, of cell free supernatant of *Bacillus thuringiensis* against *Vibrio mimicus* were evaluated via agar diffusion assay.

2.3.3 Effect of heat

The heat stability of antibacterial component of the cell free supernatant of *Bacillus thuringiensis* was evaluated by heating the cell free supernatant in a water bath at 37°C, 60°C, 80°C and 100°C for 30 min and at 121°C for 15 min. The antibacterial activities of heat treated and untreated cell free supernatant of *Bacillus thuringiensis* against *Vibrio mimicus* were evaluated via agar diffusion assay.

2.3.4 Effect of surfactants

The effect of surfactants (Tween 80 and SDS) on antibacterial component of the cell free supernatant of *Bacillus thuringiensis* was assessed. It was treated with 2% solutions of Tween 80 and SDS at a final concentration of 1.0% (v/v) of the surfactants. The mixtures were kept at 4°C for 24 h. The antibacterial activities of surfactant treated and untreated cell free supernatant of *Bacillus thuringiensis* against *Vibrio mimicus* were evaluated via agar diffusion assay.

2.4 Extraction of Metabolites

Bacillus thuringiensis strain G5-8-3T02 was cultivated on nutrient broth for 48 h. Twenty millilitres (20 ml) of cell free supernatant was obtained by centrifugation of the broth culture at 8000 rpm for 10 min. To extract the bioactive

compounds, equal volume of methanol was added and then the solvent was evaporated via a rotary evaporator. The extract was liquefied in Dimethyl sulfoxide (DMSO) and kept in tube [17].

2.5 Gas Chromatography- Mass Spectrometry (GC-MS) Analysis Using the Methanolic Extract

GC-MS analysis of the methanolic extract was carried out using Agilent 7890A-5975C GC-MS system employing the following conditions; operating in electron impact mode at 70eV, HP5-column (30 m X 0.25 mm X 0.25 μm), Carrier gas flow (a constant) = 1 ml/min, Split ratio = 10:1, Injection volume = 0.5μl, Injector temperature = 250°C, Oven temperature, Initial = 110°C (hold 2 mins), 110°C to 200°C at 5°C/min (hold 9 mins), Ion source temperature = 280°C and Mass spectra were taken at 70 eV.

2.6 Identification of Components

The interpretation of the mass spectra was carried out using the NIST (National Institute of Standards and Technology) Database. The spectra of the unknown components were matched with spectra of known components deposited in the NIST library. The name, molecular weight and structure of the components of the extract were established.

2.7 Statistical Analysis

Standard deviations for each of the experimental results were determined using Excel Spreadsheets, with Microsoft excel software. Differences between treatments were tested for significance by one-way ANOVA and P = 0.05 was well thought-out to be statistically significant.

3. RESULTS

3.1 Effects of Abiotic Factors

Temperature of 25-40°C sustained the growth (Fig. 1) and antibacterial activity (Fig. 2) of *Bacillus thuringiensis*. The optimum temperature for growth (Fig. 1) and inhibitory activity (Fig. 2) of *Bacillus thuringiensis* against *Vibrio mimicus* was observed at 35°C. The pH range of 4-10 sustained growth of the organism with optimum pH values observed at pH 6-7 on the 5th day (Fig. 3). There was no significant variation in the growth of *Bacillus thuringiensis* at pH values of 6-7. The pH of 4-10 sustained inhibition of *Vibrio mimicus* by the cell free supernatant of *Bacillus thuringiensis* with inhibitory zones ranging from 11 to 26 mm (Fig. 4). Salinity of 0-30 ppt. supported growth (Fig. 5) and inhibitory activity (Fig. 6) of *Bacillus thuringiensis* with optimum value observed at 10 and 15 ppt. There was no significant variation in the growth of *Bacillus thuringiensis* at salinity levels of 10-15 ppt.

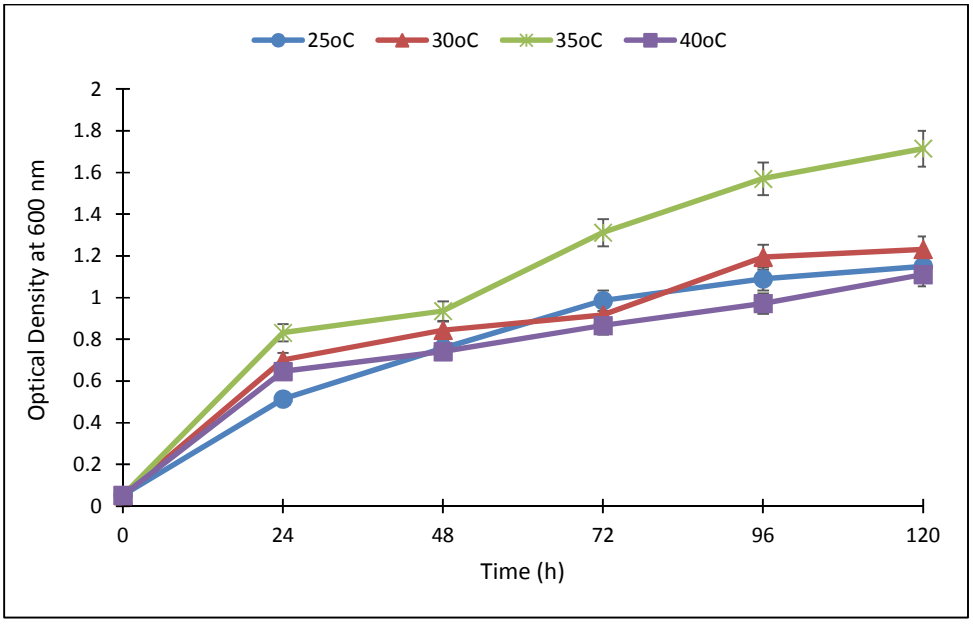

Fig. 1. Effect of temperature on growth of *Bacillus thuringiensis*

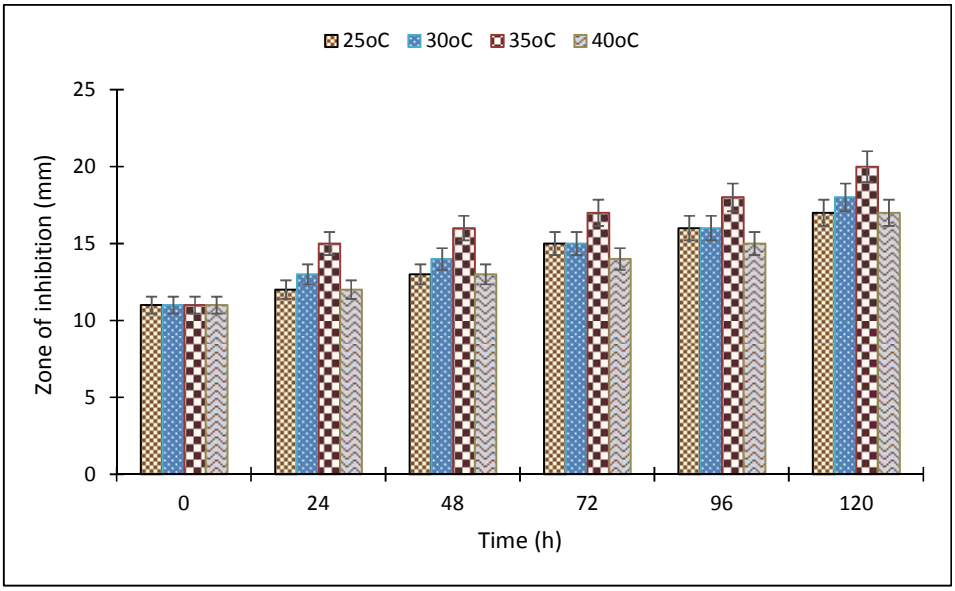

Fig. 2. Effect of temperature on antibacterial activity of *Bacillus thuringiensis* against *Vibrio mimicus*

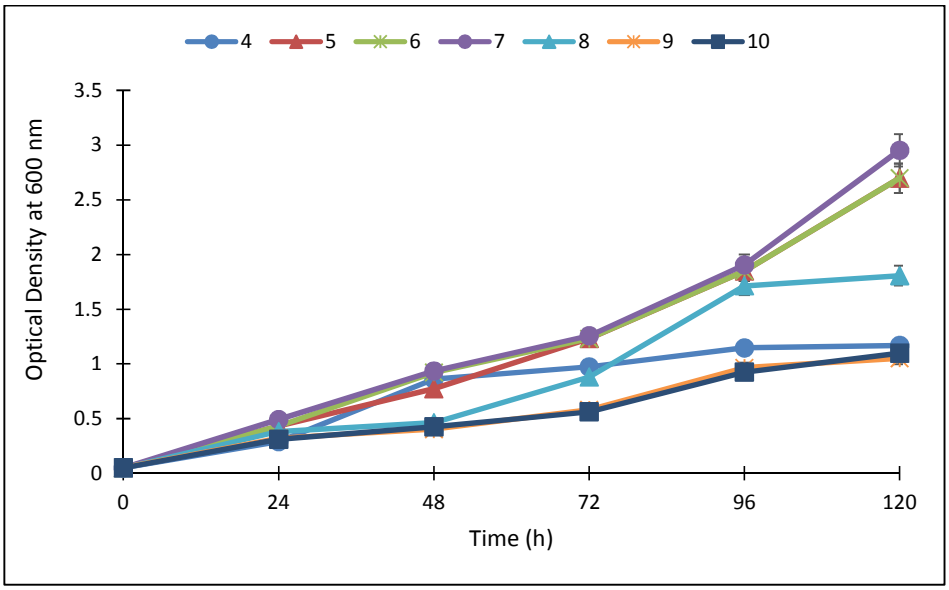

Fig. 3. Effect of pH on growth of *Bacillus thuringiensis*

Table 1. Effect of enzymes on antibacterial activity of cell free supernatant of *Bacillus thuringiensis*

Enzymes	Zone of inhibition (mm) mean ± SD
Pepsin	14.0 ± 0.06
Catalase	15.0 ± 0.07
Lipase	13.0 ± 0.05
α- amylase	13.0 ± 0.04
Control	16.0 ± 0.03

3.2 Effects of Enzymes, pH, Heat and Surfactants

The antibacterial component of *Bacillus thuringiensis* did not totally loose its activity after the crude extract was subjected to enzyme, pH, heat and surfactant treatments (Tables 1, 2, 3 and 4 respectively). None of the tested treatments caused total loss of activity.

Table 2. Effect of different pH levels on antibacterial activity of cell free supernatant of *Bacillus thuringiensis*

pH	Zone of inhibition (mm) mean ± SD
2	12.0 ± 0.12
3	12.0 ± 0.11
4	13.0 ± 0.01
5	14.0 ± 0.01
6	16.0 ± 0.1
7	16.5 ± 0.01
8	14.5 ± 0.10
9	13.0 ± 0.11

3.3 Gas Chromatogram of the Methanolic Extract

The gas chromatogram of methanolic extract of antimicrobial substance produced by *Bacillus thuringiensis* is presented in Fig. 7. Fifteen (15) peaks were observed.

3.4 Quantitative Composition of Metabolites

Table 5 reveals the quantitative composition of the *Bacillus thuringiensis* metabolites with 15 bioactive compounds. The major bioactive compound as revealed in Table 5 is 2, 6, 10-Trimethyltridecane ($C_{16}H_{34}$) with peak area of 14.58%.

Table 3. Effect of heat treatment on antibacterial activity of cell free supernatant of *Bacillus thuringiensis*

Temperature	Zone of inhibition (mm) mean ± SD
Untreated (Room temperature 28 ± 2°C)	16.0 ± 0.01
37°C	16.0 ± 0.01
60°C	10.75 ± 0.02
80°C	10.50 ± 0.01
100°C	9.0 ± 0.03
121°C	8.5 ± 0.05

4. DISCUSSION

The present study reports some bio prospects of an antagonistic marine *Bacillus thuringiensis* earlier isolated from healthy shrimp intestine and established to protect *Penaeus monodon* from *Vibrio mimicus* infection [6]. *Vibrio* and *Bacillus* are common bacterial genera often associated

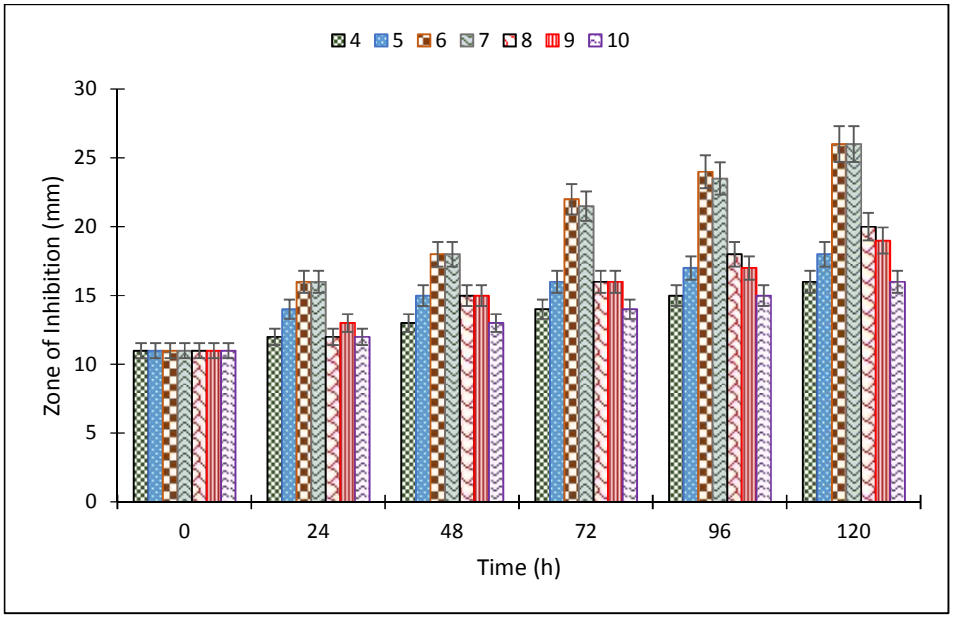

Fig. 4. Effect of pH on antibacterial activity of *Bacillus thuringiensis* against *Vibrio mimicus*

with shellfish and are identified as common inhabitants of aquatic environment such as shrimp culture ponds. *Bacillus* species are regarded as prospective probiotics in aquaculture while *Vibrios* are typically linked with vibriosis, a common aquaculture disease [18]. Other authors have also reported the inhibitory action of *Bacillus* species against shrimp and shellfish pathogens [19,13,6]. This is not unexpected since coastal marine environment habours diverse bacteria of antibacterial importance against fish and human pathogens [20].

Growth of antagonistic *Bacillus thuringiensis* over a broad array of pH is in agreement with the report of Rampelotto [21] who stated that isolates from marine location can tolerate hostile

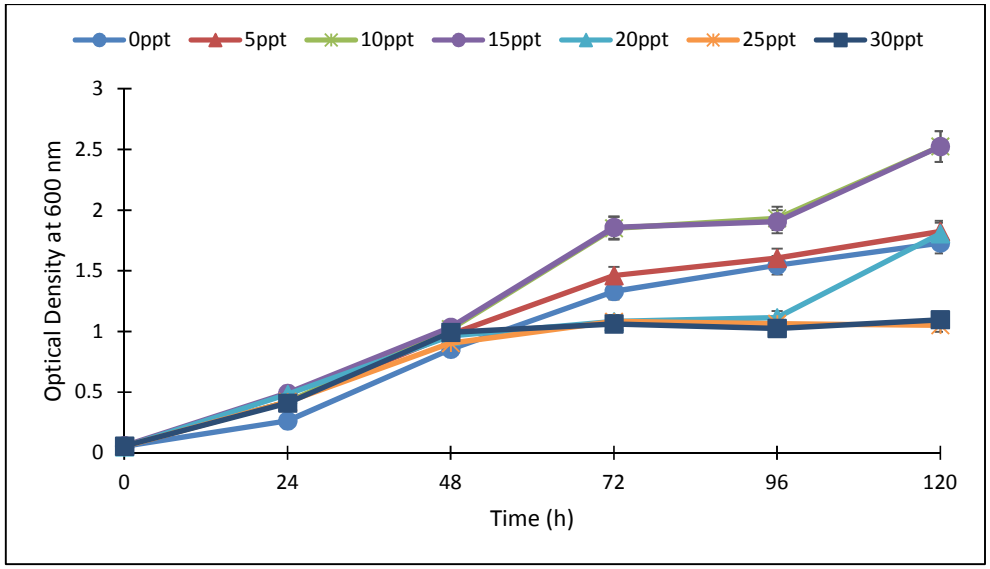

Fig. 5. Effect of salinity on growth of *Bacillus thuringiensis*

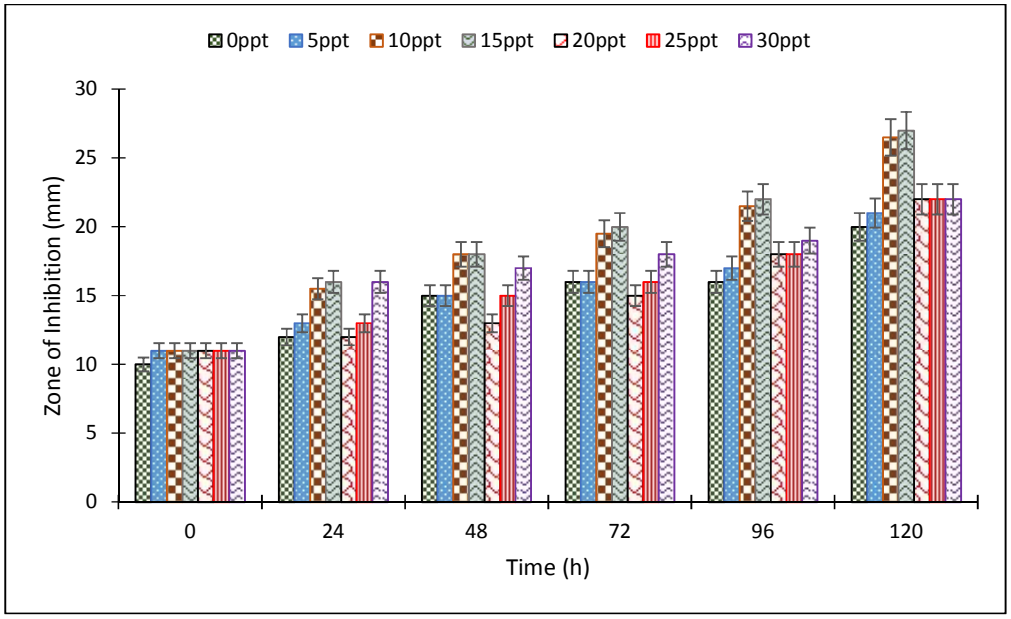

Fig. 6. Effect of salinity on antibacterial activity of *Bacillus thuringiensis* against *Vibrio mimicus*

Table 4. Effect of surfactant treatment on antibacterial activity of cell free supernatant of *Bacillus thuringiensis*

Surfactants	Zone of Inhibition (mm) mean ± SD
Tween 80	9.25 ± 0.01
Sodium dodecyl sulphate (SDS)	9.0 ± 0.02
Control (Untreated)	16 ± 0.05

Table 5. Quantitative composition of *Bacillus thuringiensis* metabolite

S/No.	RT	Compound Name	Formula	MW (g/mol)	Area%
1	6.071	Cyclopentane, 3-hexyl-1, 1-dimethyl	$C_{13}H_{26}$	182.35	0.49
2	9.127	Cyclododecane	$C_{12}H_{24}$	168.32	1.18
3	9.642	Heneicosane	$C_{21}H_{44}$	296.6	8.80
4	11.175	Undecane	$C_{11}H_{24}$	156.31	10.32
5	11.908	Pentadecane	$C_{15}H_{32}$	212.42	5.89
6	12.257	Naphthalene, 2, 3, 6-trimethyl	$C_{13}H_{14}$	170.25	5.71
7	12.652	Hexadecane	$C_{16}H_{34}$	226.41	10.82
8	13.641	Tridecane	$C_{13}H_{28}$	184.37	10.73
9	13.910	Heptadecane	$C_{17}H_{36}$	240.48	4.62
10	14.013	2, 6, 10-Trimethyltridecane	$C_{16}H_{34}$	226.44	14.58
11	15.072	Tetradecane	$C_{14}H_{30}$	198.39	7.86
12	15.146	Pentadecane, 2, 6, 10, 14-tetramethyl	$C_{19}H_{40}$	268.53	10.73
13	16.331	Methoxyacetic acid, 2-pentadecyl ester	$C_{18}H_{36}O_3$	300.5	5.66
14	17.464	Nonadecane	$C_{19}H_{40}$	268.5	1.62
15	18.614	Octadecane	$C_{18}H_{38}$	254.5	0.99

environments such as high pressure, temperature, pH and salinity. Activity was sustained over a varied pH levels (2-9). Tolerance to an extensive array of abiotic factors that affect shrimp culture makes this strain a prospective probiotics for shrimp rearing system. The best conditions for growth and production of antibacterial substance by *Bacillus thuringiensis* stated in this study, may be useful for industrial manufacturing of this strain as the growth of microorganisms is intensely affected by medium components as well as abiotic conditions like temperature and pH [22].

The ability of the cell free supernatant of *Bacillus thuringiensis* not to lose its activity totally when treatment at elevated temperatures (100°C and 121°C) reveals the stability of the antagonistic constituent. Chythanya et al. [23] and Vijayan et al. [24] documented similar reports that the antibacterial activity of a cell free supernatant of *Pseudomonas* was still active at 100°C. The heat stability property of the metabolite will be useful during industrial manufacturing of the probiotics to be employed as feed supplement.

Compounds such as Cyclopentane, Cyclododecane, Undecane, Pentadecane, Naphthalene, Hexadecane, Tridecane, Heptadecane, Tetrdaecane, Pentadecane and Octadecane produced by *Bacillus thuringiensis* could be employed as raw materials in production of valuable agricultural chemicals or used in drug enhancement. These compounds may be accountable for its antagonism as the main mechanism of anti-*Vibrio* action seemed to consist of cell lysis as a result of cell membranes disruption [25]. Baruzzi et al. [26] stated that *Bacillus* isolated from marine environment are recognized to produce structurally different classes of secondary metabolites, such as polypeptides, lipopeptides, macro-lactones, polyketides, fatty acids, isocoumarins and lipoamides which displayed an array of biological actions such as anticancer, antimicrobial, anti-peronosporomycetal and antialgal. The fifteen (15) identified metabolic compounds produced by *Bacillus thuringiensis* may be useful in petroleum, cosmetics and pharmaceutical industries and can be examined further for bio-therapeutics in management of definite diseases.

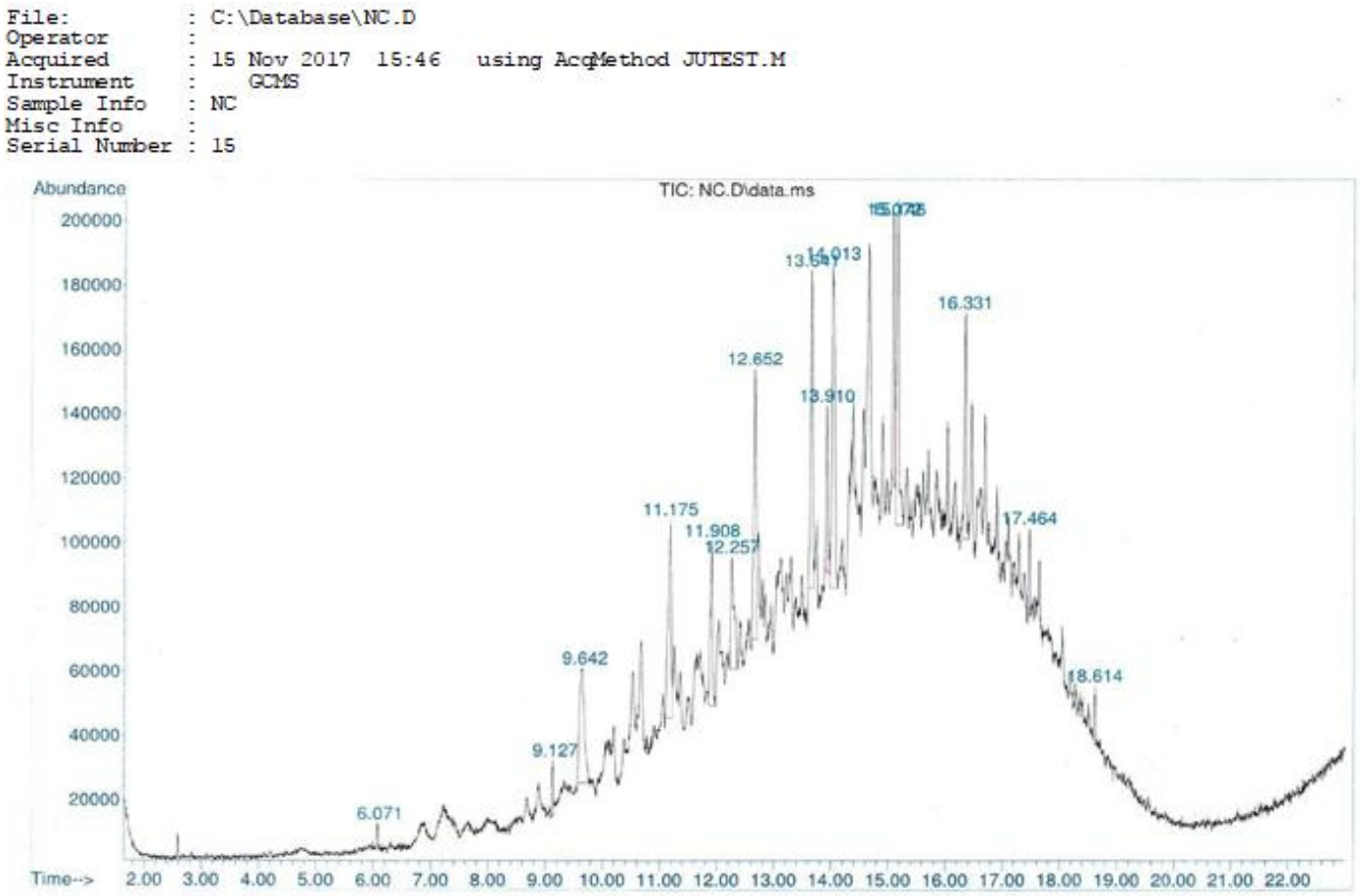

Fig. 7. Gas chromatogram of extracted metabolite of *Bacillus thuringiensis*

5. CONCLUSION

In this present study, the bio prospect of an indigenous *Bacillus thuringiensis* strain G5-8-3T02 earlier isolated from healthy shrimp (*Penaeus monodon*) intestine and established to shield the shrimp from *Vibrio mimicus* infection was investigated. The strain tolerated a variety of temperatures (25-40°C), pH (4-10) and salinities (0-30 ppt.) for shrimp culture. The antibacterial constituent produced did not entirely lose its activity after treatment with enzymes (pepsin, catalase, lipase and α- amylase), different pH values (2-9), heat (37°C – 121°C) and surfactants (Sodium dodecyl sulphate and Tween 80). Fifteen compounds were found in the cell free supernatant. The identified bioactive compounds might be beneficial for drug enhancement and the strain has healthy prospect for control of vibriosis in shrimp culture systems.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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