Original Research Article

Isolation and characterization of antibiotic producing Actinomycetes from mud nest of wasps

ABSTRACT

The recent increase in antibiotic resistance demands the discovery of novel antibiotics. Hence, this project was designed to explore novel antibiotic producing Actinomycetes from mud nest of wasps. For this, 9 types of active mud nests of wasp available in Rajshahi, Bangladesh were collected. For each nest, nest material was aseptically homogenized with a 1X saline solution and then diluted homogenate was plated in Actinomycetes Isolation Agar medium to isolate Actinomycetes. Total 27 purified cultures of bacteria were isolated from 9 collected mud nests of wasp. To collect the extract of mud nest, homogenate was filtered and centrifuged. Then, the extracts were assessed for their efficacy to inhibit bacterial growth with disc diffusion method. However, only extract of nest number 9 (N9) showed antimicrobial efficacy against tested bacteria, *E. coli.* Then antimicrobial efficacy of the 27 isolates was assessed using an agar cross-streak method and disc diffusion method. It was found that among the 27 isolates, only the isolate N9C2 was able to inhibit the growth of studied bacteria, *E. coli*. Then, 16S rDNA was isolated, amplified and sequenced from the isolate N9C2 for its identification. According to NCBI blast, the highest similarity of sequence (99%) of 16S rDNA of the isolate N9C2 was shown to that of *Streptomyces coelescens* strain AS 4.1594. Then, the isolate N9C2 was characterized. It was found that the isolate was a gram positive filamentous bacterium. It was found that the isolate N9C2 was resistant to Amoxicillin, Ampicillin and Cephalexin while it was sensitive to Tetracycline, Erythromycin and Ciprofloxacin. It was also found that the isolate N9C2 can grow optimally at pH 7 and at 37 ^{0}C. Finally, it can be concluded that mud nests of wasp is a vital source of antibiotic producing Actinomycetes such as *Streptomyces coelescens* strain AS 4.1594.

Keywords: Actinomycetes, Antibiotic, Mud nest, Wasp

1. INTRODUCTION

Since the introduction of antibiotics more than 100 years ago, for the first time in human history we were able to combat life-threatening diseases caused by bacteria, fungi, viruses, and protozoa [1]. At least in industrialized countries we are now so familiar with antibiotic, especially antibiotics, that almost everybody has used them at least once to treat various bacterial infections, which otherwise would have at least taken longer to cure and would have been more painful and severe. However, the golden age of antibiotics that started in 1950 is coming to an end: Resistance against new antibiotics, including those representing our last line of defense (e.g. for antibiotics), is emerging dramatically, leading to a return of almost eradicated diseases such as tuberculosis even in industrialized countries[2, 3]. As antibiotic research is very expensive and time consuming, [1] several pharmaceutical companies have reduced activity in this important area. Combined effect of this factor and emergence of antibiotic resistant pathogens may lead to a dramatic situation in the very near future similar to that 100 years ago. Our only chance is to continue to fight the microorganisms in order to at least maintain the current status quo. Therefore, discovery of new antibiotic is essential. As natural products, their derivatives, and compounds inspired by natural products have been by far the major source for clinically used antibiotic[4]. Insects are known as a rich source of bacteria that produce interesting natural products, [5] as highlighted by Paederus bettle symbionts that produce pederin [6]. Because most clinically available antibiotics are originate from actinomycetes and

fungi. Hence the current epidemic of antibiotic resistant pathogens has produced a renewed interest in identifying uncharacterized habitats from which to isolate unique actinomycetes that produce novel antibiotics. Insect nest associates are consequently being investigated as a likely source of such novelty. Despite the potential for microbial or chemical novelty, investigations of nest material have been conducted to relatively few wood excavating or soil-nest/constructing insects. Hence, this project was designed to explore novel antibiotic producing Actinomycetes from mud nest of insects especially wasps available in Rajshahi, Bangladesh.

2. MATERIAL AND METHODS

2.1 Nest Collection

Mature, active nests of wasps were aseptically collected in polyethylene bag and stored at 4^0C in refrigerator for further use in research work. Nests were used for identification of wasp genus. Nests were determined to be active if at the time of collection they were found to have live wasp residents on them at night. In the laboratory, all active individuals, larvae, pupae, and eggs were aseptically removed from the nest. Nests were visually inspected and any found to be parasitized by brood parasitoids [7] were removed from the study. All nests were collected from Rajshahi city, Bangladesh.

2.2 Microorganism Isolation

For each nest, nest material (including paper, pedicel, meconium) was aseptically mechanically homogenized with forceps, and placed in a conical tube with a 1X saline solution. Tubes were vortexed and the resulting homogenate was diluted in the same solvent and plated in Actinomycete Isolation Agar medium. Plates were incubated at ambient room temperature (28^0C) and bacterial colonies were streaked for purification as they grew (continuously after 72 h). Colonies exhibiting morphologies indicative of Actinomycetes were selected for further purification.

2.3 Antimicrobial Production Assay

The ability of the Actinomycete isolates to inhibit bacterial growth was assessed using an agar cross-streak method and disc diffusion method. For agar cross-streak method, the selected isolates were inoculated onto LB agar plates by continuous streak on one side (3 cm width). The plates were incubated at 30 °C for 3 days. Gram-negative bacterium *Escherichia coli* and gram-positive bacterium *Bacillus cereus* were streaked perpendicular to the antagonist on the agar medium. The plates were incubated at 37 °C for 24 h. The microbial inhibitions were observed by determining the diameter of the inhibition zones [8].

For disc diffusion method, the selected isolate was inoculated into LB broth and incubated at 30°C in a shaker at 200 rpm for seven days. After incubation the broth was collected and filtered through what man No. 1 filter paper. Further the filtrates were centrifuged at 5000 rpm for 10 min. The supernatant was transferred aseptically into a screw capped bottles and stored at 4 °C for further assay. Similarly, nest of wasp was crushed and refined with pestle and mortar. Then, refined nest material was mixed with water. Mixture was centrifuged at 5000 rpm for 10 min. The supernatant was transferred aseptically into a screw capped bottles and stored at 4 °C for further assay. For each test, 100ml Luria Bartini broth in each flask was inoculated with few cells of *Escherichia coli* and *Bacillus cereus* separately and incubated at 37°C for 24 hours in rotary sacker rotate at 120 rpm. After incubation, 1ml of broth culture was spread uniformly on a nutrient agar plate with a sterile glass spreader. The plate was air-dried for few minutes. Sterile filter paper discs were soaked with 100% concentration of collected supernatant (preserved in a screw capped bottles as mentioned above). Then these discs were placed on inoculated nutrient agar plates which were incubated at 37°C for 24 hours. After incubation, clear zones indicated inhibition of growth of the microorganisms. The zones around the discs were measured and recorded.

2.4 Identification of antibiotic producing bacteria with 16S rDNA sequencing

Genomic DNA was extracted from antimicrobial agent producing bacterium using CTAB method [9]. A universal PCR primer was used for amplification of 16S rDNA fragments. The protocol as previously described [10]. Briefly, the PCR amplification was performed by Swift™ Minipro Thermal Cycler (Model: SWT-MIP-0.2-2, Singapore) using the following program: Denaturing at 95°C for 5 minutes, followed by 40 cycles of 40 seconds of denaturing at 95°C, 60 seconds of annealing at 65°C and 2 minutes of elongation at 72°C with a final extension at 72°C for 10 minutes. Then, the PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 1500 bp PCR products. PCR amplified 16s rDNA of the selected isolate was sent for automated sequencing (Applied Biosystem 3130). The sequence generated from automated sequencing of PCR amplified DNA was analyzed through NCBI BLAST (http://www.ncbi.nlm.nih.gov) program to find out possible similar organism through alignment of homologous sequences. Finally, the isolate was identified based on alignment of partial sequence of 16S rDNA with the existing sequences available in the database.

2.5 Antibiotic sensitivity test

Antibiotic Sensitivity test was done by disc diffusion method as previously described[2, 11]. Briefly, the isolate N9C2 collected from nest of wasp was grown overnight in nutrient broth that were placed in a shaker at 35°C temperature and

120 rpm for antibiotic sensitivity test. 1ml of overnight culture was transferred and gently spread on the nutrient agar plate and dried. Antibiotic disks were placed on the respective plates and incubated overnight at 35°C.

2.6 Determination of optimum growth conditions

To determine the optimum pH of bacterial growth, culture medium was adjusted to pH 6.5, 7.0, 7.5 and 8.0. Then, each 50 mL of culture media were inoculated with 1 mL of culture of isolated bacteria. The growths of bacteria at different condition were determined at different time intervals (4 hourly) by measuring optical density at 660 nm with photoelectric colorimeter.

For determination of optimum temperatures, culture medium was adjusted to pH 7.0. Then, medium was distributed in four different conical flasks, 50 mL in each flask. Each 50 mL of culture media were inoculated with 1 mL of culture of isolated bacteria. Then, inoculated media were incubated at 25^0C, 30^0C, 35^0C and 40^0C temperature in incubator. The growths of bacteria at different condition were determined at different time intervals (4 hourly) by measuring optical density at 660 nm with photoelectric colorimeter.

3. RESULTS AND DISCUSSION

3.1 Muddy nests of wasp available in Rajshahi, Bangladesh

Nine types of nest of wasp (N1, N2, N3, N4, N5, N6, N7, N8 and N9) were collected from different location in Rajshahi, Bangladesh (Fig. 1). Collected nests were diverse in shape, structure and size. Nest N1 was a cluster of short vase like structure which was made of mud. Nest N2 was a single long vase like muddy structure. Nest N3 and N6 were collection of few pipes like muddy structure. Nest N4 was long irregular shaped muddy structure with several opening. Nest N5 was short multi-chambered muddy structure. Nest N7 was long single chambered nest surrounded with multiple shields like structure. Nest N8 was collection of few capsules like short muddy chambers while N9 was collection of small vase like structure which was made of some resin like sticky material mixed with mud, sand particles and sticky material (Fig 1).

In this study, diversity of nests of wasp was found in shape, size and structure which was reported to be related to taxa of wasps [12]. It is established from the data that intensities of antimicrobial defenses vary among different wasp taxa and that these differences are strongly linked to ranks of social complexity. When examined together, the phylogenetic and antimicrobial data recommend that the production of antimicrobial agents may have first arisen in solitary wasps as a response to environmental, probably soil-borne, pathogens. Burrowing wasps, exposed to soil-based pathogens, may have developed broad-scale antimicrobial defenses in response. These may have evolved into stronger compounds in the social lineages [13].

N1

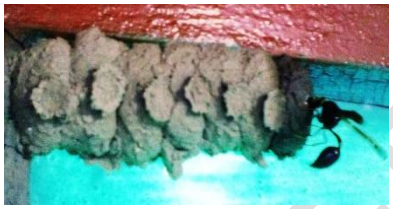
N2

N3

N4

N5

N6



N7

N8

N9

Fig. 1. Nests of wasp available in Rajshahi, Bangladesh

3.2 Bacterial colonies isolated from collected nests of wasp

Colonies exhibiting morphologies indicative of Actinomycetes were selected for further purification. Total 27 colonies were selected for purification which were tagged with code number according to serial of collection and number of the nest, viz. N1C1, N1C2, N1C3, N2C1, N2C2, N3C1, N3C2, N3C3, N4C1, N4C2, N4C3, N5C1, N5C2, N5C3, N5C4, N6C1, N6C2, N6C3, N7C1, N7C2, N8C1, N8C2, N8C3, N9C1, N9C2, N9C3 and N9C4. All the isolated colonies were large, irregular in margin and whitish in colour like fungus. After purification of collected bacterial colonies by repeated streaking, the bacterial isolates were preserved at 4 ^{0}C for further study.

3.3 Antimicrobial efficacy of extract of nests of wasp

The disc diffusion method was used to test the antimicrobial efficacy of nest extract against *E. coli* and *Bacillus cereus* bacteria. The result of this experiment revealed that extract of nest N9 was only able to yield a moderate zone of inhibition around the disc indicating that extract contained antimicrobial agent to inhibit the growth of *E. coli.*

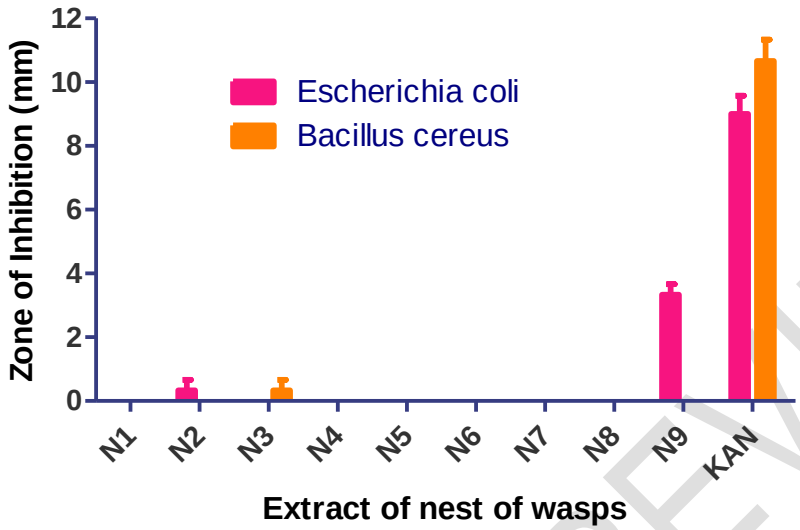


Fig. 2. Antimicrobial efficacy of extract of nest of wasps assessed with disc diffusion method against *E. coli* and *B. cereus* bacteria. Data are mean+SEM from three independent experiments. Antibiotic kanamycin (KAN) was used as control.

In this study, the most of the extract of nest of wasps were unable to inhibit the growth of tested bacteria. Unviability of active compound in nest may be because of construction of mud nest above-ground by solitary species where there is less risk of disease. Otherwise, individual species may have developed specific compounds to combat niche pathogens which are ineffective against tested bacteria [13].

3.4 Antimicrobial efficacy of bacterial isolates collected from nests of wasp

Total 27 isolates were collected from nine different nests of wasp. The efficacies of these bacterial isolates were assessed with agar cross-streak and disc diffusion method. It was found that antimicrobial compound was produced by the isolate N9C2 only to inhibit the growth of the tested gram-negative bacteria (*E. coli.*) remarkably (Fig. 3). But, the isolate N9C2 failed to inhibit the growth of the tested gram-positive bacteria (*Bacillus cereus*). However, no antimicrobial compound was produced by remaining 26 isolates against tested bacteria or their production was insignificant (Fig. 3). The result of this test was similar to that of agar cross-streak method. However, zone of inhibition produced by the isolate N9C2 was comparative lower than that produced by Control antibiotic Kanamycin. It might be resulted from lower concentration of antimicrobial agents in supernatant produced by the isolate N9C2 or from their lower efficacy as compared with Kanamycin.

The isolate N9C2 was capable of producing antibiotic to inhibit the growth of gram-negative *E. coli* bacterium used in this study. Though, it is not clear if this activity arose from one or multiple antimicrobial compounds. *Streptomyces* spp. are able to produce over 100,000 different antibiotics [14], with some strains generating multiple antimicrobials [15]. This is true even under in vivo conditions, where a cocktail of antimicrobials is expected to assist in nest hygiene [16].

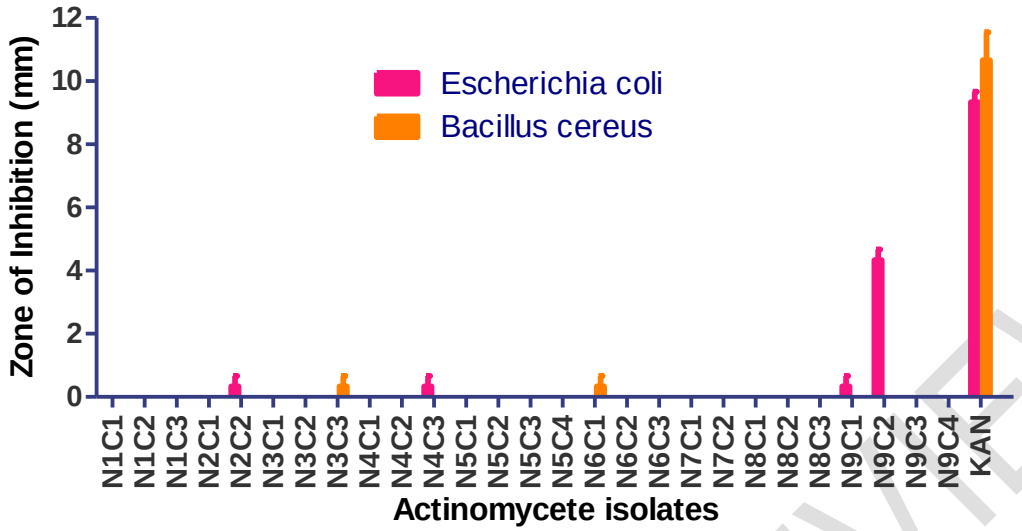


Fig. 3. Antimicrobial efficacy of the compound produced by Actinomycete isolates assessed with disc diffusion method against *E. coli* and *B. cereus* bacteria. Data are mean+SEM from three independent experiments. Antibiotic kanamycin (KAN) was used as control.

3.5 Identification of bacterial isolate N9C2

The isolate N9C2 was selected for 16S rDNA sequence based identification. The sequence submitted to NCBI revealed that the highest similarity of sequence (99%) of 16S rDNA of the isolate N9C2 was shown to that of *Streptomyces coelescens* strain AS 4.1594. Thus, the sequence analysis indicated that the isolate N9C2 was *Streptomyces coelescens* strain AS 4.1594 or member of same cluster. Similar results were revealed by other studies. For example, the 30 sequenced actinomycetes isolated from these nests belong to the two most bioactively-rich actinomycete families: Streptomycetaceae and Micromonosporaceae [17], including the three genera, *Streptomyces, Micromonospora,* and *Actinoplanes*. While *Streptomyces,* is common soil microbes [18, 19].

The strain N9C2 was isolated from mud nest of wasp belong to the genus *Streptomyces,* reliable with similar studies investigating nest-associated insect material. This includes those studies linking to leaf-cutter ants [20, 21], wood boring beetles [22, 23], honey and stingless bees [24], solitary bees [25], digger wasps [26], mud dauber wasps [27], and termites [28]. Furthermore, a study by Ruddick and Williams (1972), proposes that spores of *Streptomyces* spp. were associated with the cuticle of many arthropods [29]. Therefore, it is not surprising that strepomycetes are often found in nest material.

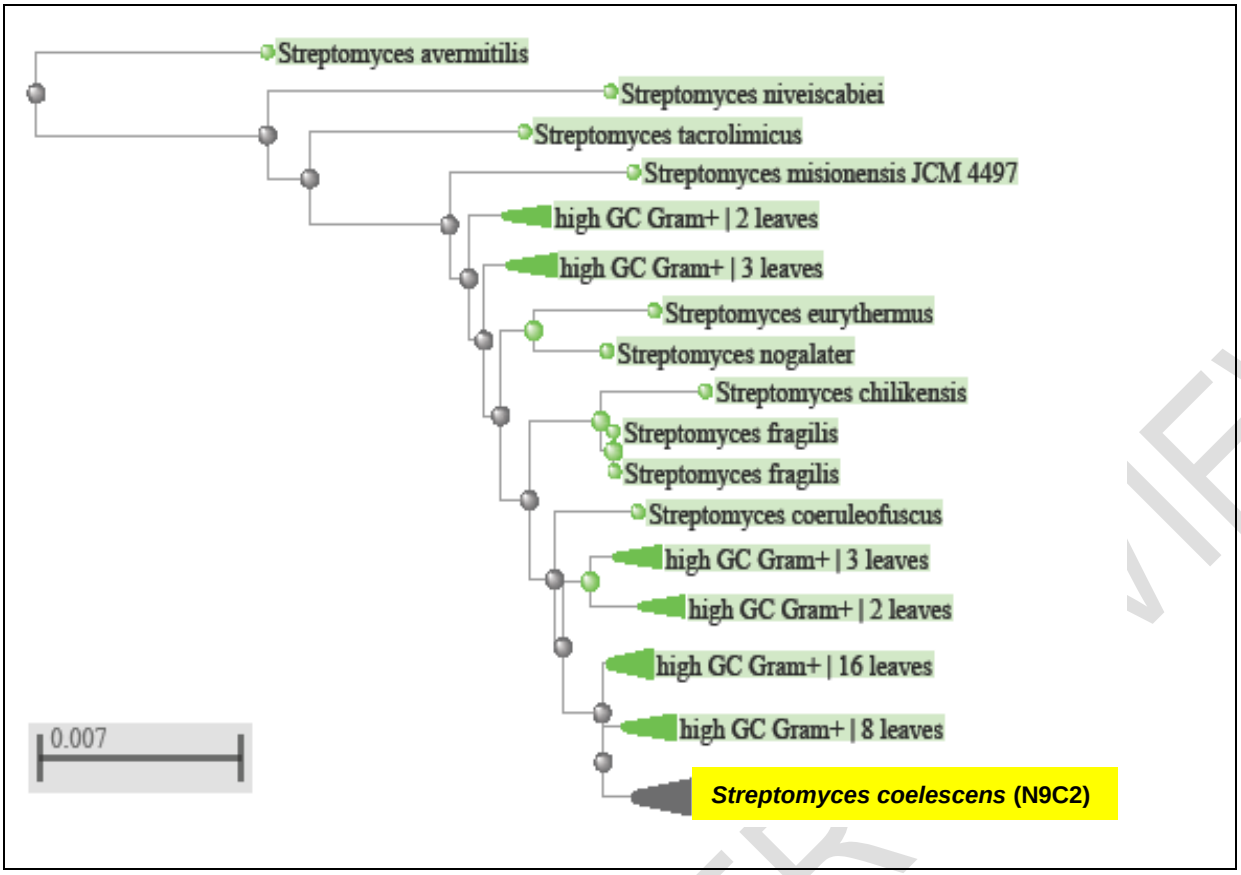


Fig. 4. Unrooted Phylogenetic tree showing the genetic relationship between the isolate N9C2 collected from Nest N9 of wasp and reference 16S rRNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences.

3.6 Antibiotic sensitivity pattern of bacterial isolate N9C2

Study of antibiotic sensitivity pattern is vital to maintain pure culture of a bacterial isolate as well as to take a decision for using it safely in any environmental application. The patterns of antibiotic sensitivity of bacterial isolate N9C2 to 8 different antibiotics was tested by disk diffusion method using nutrient agar medium. After incubation overnight at 35°C, the diameter of inhibition zone was measured. It was found that the isolate N9C2 was resistant to 3 antibiotics viz. Amoxicillin, Ampicillin and Cephalexin while it was sensitive to 3 other antibiotics viz Tetracycline, Erythromycin and Ciprofloxacin. However, the isolate showed intermediate sensitive to Kanamycin and Neomycin (Table 1).

Table 1 Antibiotic sensitivity pattern of bacterial isolate N9C2

Name of Antibiotic	Zone of Inhibition	Comment
Tetracycline	20 mm	S
Amoxicillin	No zone	R
Erythromycin	26 mm	S
Kanamycin	15 mm	I
Ampicillin	No zone	R
Neomycin	15 mm	I
Ciprofloxacin	30 mm	S
Cephalexin	No zone	R

S= sensitive, R= Resistant, I= Intermediate

3.7 Optimum temperature for growth of bacterial isolate N9C2

Optimum temperature for growth of bacterial isolate N9C2 was determined at pH 7 in nutrient broth medium. The optimum temperature for growth of the isolate N9C2 was 37 ^{0}C (Fig. 5). The maximum growth rate of the isolate N9C2 (OD 1.3)

was observed at 35 ^{0}C at 32 hours while the minimum growth rate (OD 1.1) was observed at 28 ^{0}C and 33 ^{0}C at that time (Fig. 5).

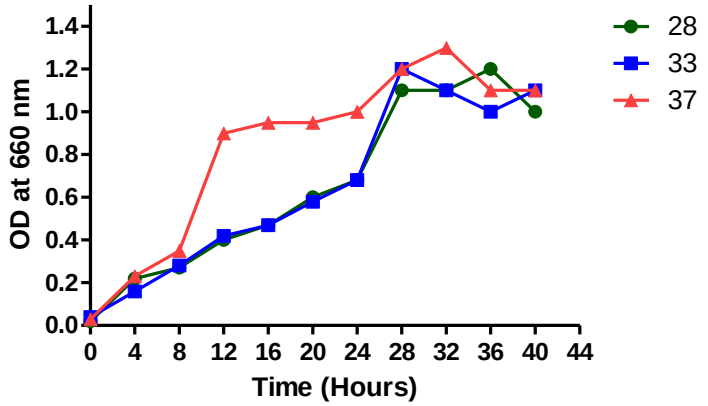


Fig. 5. Optimum temperatures for growth of bacterial isolate N9C2.

3.8 Optimum pH for growth of bacterial isolate N9C2

Optimum pH for growth of bacterial isolate N9C2 was determined at 37^{0}C temperature in liquid broth medium at pH 6, pH 7 and pH 8. As shown in Fig. 6, the isolate N9C2 exhibited maximum growth (OD 1.5) at pH 7 after 40 hours of incubation while the minimum growth (OD 0.97) was observed at pH 6 after that time of incubation (Fig. 6).

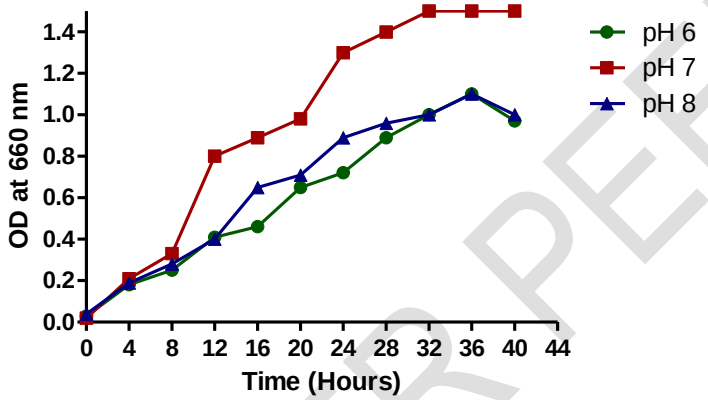


Fig. 6. Optimum pH for growth of bacterial isolate N9C2.

4. CONCLUSION

In this study, the isolate N9C2 which was identified as *Streptomyces coelescens* strain AS 4.1594 can produce antimicrobial compound to inhibit the growth of studied gram-negative bacteria. However, the compound responsible for inhibition of growth was not separated from other compounds produced by the isolate N9C2. Hence, future studies should be focused on separating and characterizing the antibiotic producing by the isolate N9C2 to determine if the actinomycetes isolated within this study produce novel chemistry. By further targeting the full diversity of the microbial community associated with these wasps, we will be able to better understand how these wasps maintain nest hygiene, and what microbes may impact their fitness.

REFERENCES

1. Bode, H.B., *Insects: True Pioneers in Anti-Infective Therapy and What We Can Learn from Them.* Angewandte Chemie International Edition, 2009. 48(35): 6394-6396.
2. Mohanta, M.K., et al., *Status of antibiotic sensitivity pattern of clinically isolated bacteria collected from Rajshahi City, Bangladesh.* Univ. j. zool. Rajshahi. Univ, 2015. 34: 1-5.

3. Haque, M.F., et al., *Emergence of multidrug resistant Escherichia coli as a common causative agent in urinary tract infection in Bangladesh.* Univ. j. zool. Rajshahi. Univ., 2018. 37: 8-13.
4. Newman, D.J. and G.M. Cragg, *Natural products as sources of new drugs over the last 25 years⊥.* Journal of natural products, 2007. 70(3): 461-477.
5. Piel, J., *Metabolites from symbiotic bacteria.* Natural product reports, 2004. 21(4): 519-538.
6. Piel, J., *A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of Paederus beetles.* Proceedings of the National Academy of Sciences, 2002. 99(22): 14002-14007.
7. Madden, A., M. Davis, and P. Starks, *First detailed report of brood parasitoidism in the invasive population of the paper wasp Polistes dominulus (Hymenoptera, Vespidae) in North America.* Insectes sociaux, 2010. 57(3): 257-260.
8. Lemos, M.L., A.E. Toranzo, and J.L. Barja, *Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds.* Microbial Ecology, 1985. 11(2): 149-163.
9. Smith, G., S. Socransky, and C. Smith, *Rapid method for the purification of DNA from subgingival microorganisms.* Oral microbiology and immunology, 1989. 4(1): 47-51.
10. Saha, A.K., et al., *Identification and Characterization of Azo Dye Decolourizing Bacterial Strains, Alcaligenes faecalis E5. Cd and A. faecalis Fal. 3 Isolated from Textile Effluents.* American Scientific Research Journal for Engineering, Technology, and Sciences (ASRJETS), 2017. 31(1): 163-175.
11. Saha, A., et al., *Antibacterial effects of some antiseptics and disinfectants.* Journal of Life and Earth Science, 2009. 3(4): 19-21.
12. Subathra, D., et al., *Screening of actinomycetes isolated from soil samples for antibacterial and antioxidant activity.* International Journal of Pharmacy and Pharmaceutical Sciences, 2013. 5(4): 483-489.
13. Hoggard, S.J., et al., *Social complexity and nesting habits are factors in the evolution of antimicrobial defences in wasps.* PLoS One, 2011. 6(7): e21763.
14. Watve, M.G., et al., *How many antibiotics are produced by the genus Streptomyces?* Archives of microbiology, 2001. 176(5): 386-390.
15. Seipke, R.F., M. Kaltenpoth, and M.I. Hutchings, *Streptomyces as symbionts: an emerging and widespread theme?* FEMS microbiology reviews, 2012. 36(4): 862-876.
16. Kroiss, J., et al., *Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring.* Nature Chemical Biology, 2010. 6(4): 261-263.
17. Berdy, J., *Bioactive microbial metabolites.* The Journal of antibiotics, 2005. 58(1): 1-26.
18. Lazzarini, A., et al., *Rare genera of actinomycetes as potential producers of new antibiotics.* Antonie van Leeuwenhoek, 2000. 78(3): 399-405.
19. El-Tarabily, K.A. and K. Sivasithamparam, *Non-streptomycete actinomycetes as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters.* Soil Biology and Biochemistry, 2006. 38(7): 1505-1520.
20. Haeder, S., et al., *Candicidin-producing Streptomyces support leaf-cutting ants to protect their fungus garden against the pathogenic fungus Escovopsis.* Proceedings of the National Academy of Sciences, 2009. 106(12): 4742-4746.
21. Zucchi, T.D., A.S. Guidolin, and F.L. Cônsoli, *Isolation and characterization of actinobacteria ectosymbionts from Acromyrmex subterraneus brunneus (Hymenoptera, Formicidae).* Microbiological research, 2011. 166(1): 68-76.
22. Hulcr, J., et al., *Presence and diversity of Streptomyces in Dendroctonus and sympatric bark beetle galleries across North America.* Microbial ecology, 2011. 61(4): 759-768.
23. Grubbs, K.J., et al., *Genome sequence of Streptomyces griseus strain XylebKG-1, an ambrosia beetle-associated actinomycete.* Journal of bacteriology, 2011. 193(11): 2890-2891.
24. Promnuan, Y., T. Kudo, and P. Chantawannakul, *Actinomycetes isolated from beehives in Thailand.* World Journal of Microbiology and Biotechnology, 2009. 25(9): 1685-1689.
25. Inglis, G.D., L. Sigler, and M.S. Goette, *Aerobic microorganisms associated with alfalfa leafcutter bees (Megachile rotundata).* Microbial ecology, 1993. 26(2): 125-143.
26. Kaltenpoth, M., et al., *'Candidatus Streptomyces philanthi', an endosymbiotic streptomycete in the antennae of Philanthus digger wasps.* International Journal of Systematic and Evolutionary Microbiology, 2006. 56(6): 1403-1411.
27. Kumar, V., et al., *Actinomycetes from solitary wasp mud nest and swallow bird mud nest: isolation and screening for their antibacterial activity.* World Journal of Microbiology and Biotechnology, 2012. 28(3): 871-880.
28. Visser, A.A., et al., *Exploring the potential for Actinobacteria as defensive symbionts in fungus-growing termites.* Microbial ecology, 2012. 63(4): 975-985.
29. Ruddick, S. and S. Williams, *Studies on the ecology of actinomycetes in soil V. Some factors influencing the dispersal and adsorption of spores in soil.* Soil Biology and Biochemistry, 1972. 4(1): 93IN7101-100IN10103.