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Identification and *In vitro* Antimicrobial Activity of a Compound Isolated from *Streptomyces* Species

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Abstract: A pure antimicrobial compound was isolated from the chloroform extract of the broth culture of *Streptomyces* species by preparative thin layer chromatography (PTLC) and was characterized as 4-hydroxy nitrobenzene (ZS-3) on the basis of spectral data (UV, IR, ^{1}H-NMR, HMBC and HMQC). The compound showed significant antimicrobial activity against six gram positive bacteria, ten gram negative bacteria and three pathogenic fungi. The minimum inhibitory concentration (MIC) of the compound determined against ten pathogenic organisms was found to be between 32-64 g/ml.

Key words: Antimicrobial activity, *Streptomyces* sp., 4-hydroxy nitrobenzene

Introduction

The problems of drug resistance, patient sensitivity and inability to control certain infectious diseases have given an impetus for continuous search of new antibiotics all over the world. The first systematic search for antibiotics made by Gratia and Dath (1924), resulted in the discovery of Actinomycetin from *Actinomycete* bacteria. In United States and Japan between 1953 to 1970 approximately 85% of the antibiotics were produced by *Actinomycetes*, 11% by fungi and 4 % by bacteria (Reiner, 1982).

As a part of our continuous search for novel microbial metabolites from soil samples collected from different parts of Bangladesh, we isolated an A*ctinomycetes* strain that was identified as *Streptomyces* species (John *et al.*, 1994). From the chloroform extract of the yeast-extract glucose broth culture filtrate of the organism, an antimicrobial agent was isolated by PTLC (Egon and Stahl, 1969) and was identified as 4-hydroxy nitrobenzene by its spectral analysis (Sathi, 2000). We herein, report the optimum conditions for the production of antibiotic from the organism and *in vitro* antimicrobial activity of the isolated compound ZS-3.

Materials and Methods

Isolation and identification of antagonistic organisms: For screening, soil samples were collected from a wide range of places like cultivated lands, road sides, construction sites, graveyards, food wastage, sewage, play ground and under medicinal plant of different parts of Bangladesh. Soils of depth ranging from 0.25 to 1.5 meter were collected. Finally, a strain of *Actinomycetes* was isolated by crowded plate technique (Hammond & Lambert 1978). The organism was identified on the basis of its morphological and biochemical study according to Bergay's Manual of Determinative Bacteriology, 9th edition.

Selection of suitable broth medium and correct culture conditions for the production of antibiotic: For optimum production of antibiotic from the selected organism, a number of broth culture media such as Czapek-Dox broth (acidic), Czapek-Dox broth (alkaline), yeast-extract glucose broth, Bonner Addicot broth, Oat broth etc. were tried. The liquid culture was tested for its antimicrobial activity against *Bacillus subtilis* and *Shigella shiga* by disc diffusion assay method (Baeur *et al.*, 1966).

The effect of physical parameters such as carbon sources (Sucrose, D-glucose, D-fructose, D-galactose, maltose, D-mannose, lactose, D-mannitol, L-arabinose, D-xylose, and rhamnose) incubation period (up to 20 days), temperature (30, 32.5, 35, 37.5, 40, 42.5 and 45°C), pH values (1, 2, 3, 4, 5, 6, 7, 8 and 9) and salt concentration (0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0% w/v) on the production of antibiotic were studied against *Bacillus subtilis* and *Shigella shiga* by disc diffusion assay method (Bauer *et al.*, 1966).

Extraction, isolation and characterization of the antimicrobial compound: The antimicrobial compound was isolated from the culture filtrate of the *Streptomyces* species after incubation in yeast extract glucose broth media at 37.5 °C . The culture filtrate was extracted with chloroform and the extract was resolved on TLC. The compound ZS-3 having R_f value 0.75 ($CHCl_3$: CH_3OH, 10:1), possesses antimicrobial activity and was isolated by PTLC technique. The isolated antimicrobial compound was characterized on the basis of its spectral data.

Antimicrobial screening: The antimicrobial activity of the compound was tested at a concentration of 30 g/disc and 20 g/disc against nineteen test pathogenic organisms by the standard disc diffusion method (Baeur *et al.*, 1966). Kanamycin (K-30, 30g/disc) and Griseofulvin (G-20, 20g/disc) were used as standard. The minimum inhibitory concentration (MIC) was determined against *Bacillus subtilis, Bacillus cereus, Bacillus megaterium, Streptococcus hemolyticus, Staphylococcus aureus, Shigella dyscenteriae, Shigella shiga, E. coli, klebsiella* sp. and *Pseudomonas aeruginosa* by serial dilution technique (Hammond & Lambert, 1978).

Results and Discussion

Identification of the organism: The organism was identified as genus of *Streptomyces* on the basis of the Bergey's Manual of Determinative Bacteriology (9th edition) due to, spore chain morphology, spore wall ornamentation, ability to utilize particular carbon containing compounds for growth, ability to produce antimicrobial principle, colour of the matured sporulated aerial mycelium, absence of sporangia like vesicle, non-motile aerial spore produced and presence of well developed mycelia.

Suitable broth medium and correct culture condition: Different broth media, carbon sources and different physical parameters were tested for the best production of antibiotic. It was found that yeast extract glucose broth media after 15 days incubation (Fig. 2), at pH 7 (Fig. 4), and 37.5°C temperature (Fig. 6), using sucrose as a carbon source (Fig. 5) at 3 % salt (NaCl) concentration (Fig. 3) was the most suitable for large scale production of antibiotics.

Fig. 1: Spore Chain morphology of organism

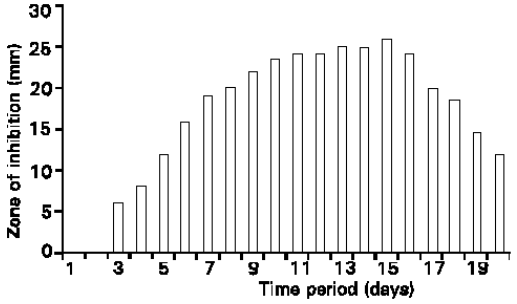

Fig. 2: Production of antibiotic at different days from *Streptomyces* sp.

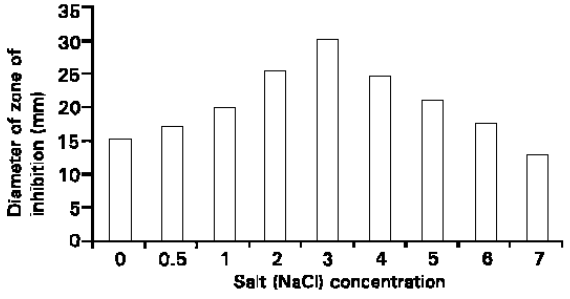

Fig. 3: Effect of salt conc. on antibiotic production from *Streptomyces* sp.

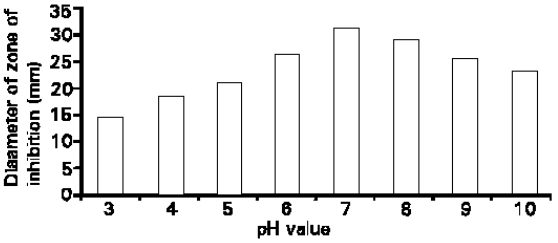

Fig. 4: Effect of pH on antibiotic production from *Streptomyces* sp.

Isolation and chemical characterization of the compound: The crude chloroform extract of the culture filtrate of *Streptomyces* species has lead to the isolation of an

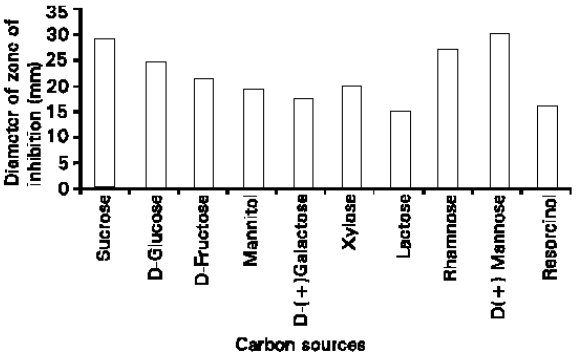

Fig. 5: Effect of carbon source on antibiotic production from *Streptomyces* sp.

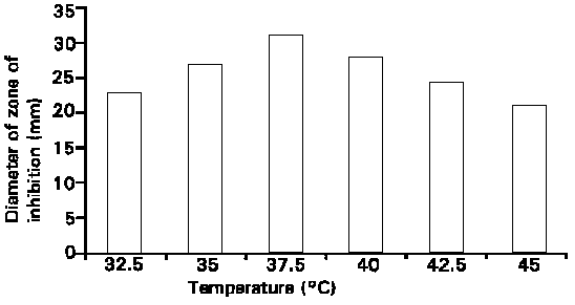

Fig. 6: Effect of temperature on antibiotic production from *Streptomyces* sp

Table 1: Antibacterial activity of the compound

Test Bacteria / Compound	Zone of inhibition (mm)	
	Kanamycin (K-30)	
	30 g/disc	30 g/disc
Gram positive		
Bacillus subtilis	21	28
Bacillus cereus	20	27
Bacillus megaterium	18	27
Sarcina lutea	24	28
Staphylococcus aureus	19	27
Streptococcus haemolyticus	21	28
Gram negative		
Salmonella typhi-A	27	30
Salmonella typhi-B56	29	32
Shigella flexneri	27	34
Shigella boydii	23	30
Shigella sonnei	25	30
Shigella dysenteriae	27	33
Shigella shiga	26	33
E. coli	26	31
Pseudomonas aeruginosa	24	32
Klebsiella sp.	22	26

Table 2: Antifungal activity of compound (antibiotic)

Test Fungus / Compound	Zone of inhibition (mm)	
100 g/disc	Griseofulvin	20 g/disc
Tinea pedis	18	16
Tinea corporis	20	17
Candida species	16	15

Table 3: Minimum Inhibitory Concentration of the compound (antibiotic)

Test organism	Concentration of the compound (g/ml)									
	512	256	128	64	32	16	8	4	2	1
Gram positive										
Bacillus subtilis	-	-	-	-	+	+	+	+	+	+
Bacillus cereus	-	-	-	-	-	+	+	+	+	+
Bacillus megaterium	-	-	-	-	-	+	+	+	+	+
Streptococcus haemolyticus	-	-	-	-	+	+	+	+	+	+
Staphylococcus aureus	-	-	-	-	+	+	+	+	+	+
Gram negative										
Shigella dysenteric	-	-	-	-	-	+	+	+	+	+
Shigella shiga	-	-	-	-	-	+	+	+	+	+
E. coli	-	-	-	-	-	+	+	+	+	+
Klebsiella sp.	-	-	-	-	+	+	+	+	+	+
Pseudomonas aeruginosa	-	-	-	-	+	+	+	+	+	+

antimicrobial compound, ZS-3 which was isolated and purified by preparative thin layer chromatographic technique (Egon & Sathl, 1969) and was characterized as 4-hydroxy nitrobenzene on the basis of spectral data UV, IR, ^{1}H-NMR, ^{13}C-NMR, HMBC, and HMQC (Sathi, 200).

The elemental composition was C=51.80, H=3.62, N=10.07 and O= 34.50, obtained from the elemental analysis.

The proton peak at 6.93 each (H, d, J= 8.7 Hz, 2H) gives long range (HMBC) correlation, with carbon peak at 141.6 and the proton peak at 8.18 (H, d, J=8.7 Hz, 2H) gives long range coupling with carbon peak at 161.4. This indicates that the carbon of 141.6 and 161.4 are ortho-para to each other (Pretsch, 1983). Therefore the structure of the compound ZS-3 is determined as 4-hydroxy nitro benzene.

Antibacterial and antifungal activity of the compound: Antibacterial activity of the compound (antibiotic), ZS-3 was shown in Table 1. The compound exhibit moderate to strong activity against the pathogenic test bacteria. The antibacterial activity of the compound against *Salmonella typhi A.* and *Salmonella typhi-B 56* was approximately similar to that of the standard kanamycin whereas the test pathogenic bacteria showed three fourth to four fifth than that of the standard kanamycin. The compound also showed antifungal activity (Table 2) against *Tinea pedies, Tinea corporis* and *Candida* species. But the activity was not so prominent.

Minimum inhibitory concentration: The Minimum inhibitory concentration (MIC) of the compound is shown in Table 3. The minimum inhibitory concentration (MIC) of the compound against *Bacillus subtilis, Bacillus cereus, Bacillus megaterium, Streptococcus haemolyticus, Staphylococcus aureus, Shigella dyscenteriae, Shigella shiga, E. coli, klebsiella* sp. and *Pseudomonas aeruginosa* were 64 g /ml, 32g/ml, 32g/ml, 64g/ml, 64g /ml, 32g/ml, 32g/ml, 32g/ml, 64 g /ml, 64 g /ml, respectively.

Acknowledgments

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