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Antibiotic sensitivity and antibacterial activity of *Micrococcus* sp SCS1

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ABSTRACT

The xylanase producing bacterial strains were isolated from bamboo garden waste. The strains were isolated on xylan agar media and screening was carried out by xylanolysis method. To test the sensitivity of the isolates, seven different antibiotics were used. The strains showed sensitivity to Ampicillin, Amoxycillin, Bacitracin, Chloramphenical, Doxycyclin, Erythromycin and Kanamycin when they were tested by disc diffusion method on nutrient agar plate and confirmed by antibiotic spread plate method. Ethyl acetate was used for solvent extraction of antimicrobial principals from the culture filtrate. The ethyl acetate extract of Micrococcus sp SCS1 showed promising antibacterial activity against a number of gram positive *(Bacillus subtilis* and *Staphylococcus aureus)* and gram negative (*Escherichia coli, Shigella dysenteriae, Shigella sonnei, Shigella shiga, Salmonella typhi* and *Klebsiella Pneumonia*) pathogenic bacteria. The zone of inhibition against pathogenic bacteria ranged from 08 – 22 mm. The minimum inhibitory concentration (MIC) of the extract against *Escherichia coli, Salmonella typhi, Salmonella dysenteriae, Shigella dysenteriae, Shigella shiga, Bacillus subtilis and Staphylococcus aureus* were 128,256, 256, 64, 128, 256 and 256μg/ml respectively. The extract exhibited cytotoxic effects in brine shrimp lethality bioassay with LC_{50} value of 30 μg/ml.

KEYWORDS

Antibacterial activity;
Cytotoxic effect;
Micrococcus sp SCS1.

INTRODUCTION

The frequency of life threatening infections such as diarrhea, acute respiratory tract, infections, tuberculosis, cancer and recently AIDS caused by various pathogenic microorganism[1,2] is increasing world wide (especially in the developing countries) and is becoming an important cause of morbidity and mortality in immunocompromized patients. But a number of microorganisms are also known to produce antimicrobial substances that are used for the treatment of human diseases. Currently more than 10,000 antibiotics have been discovered from microorganism but only a few of them are clinically useful and rests of them are toxic to human

or not highly effective against microorganisms. The reason for the demand of new antibiotics is appearance of antibiotic resistance pathogenic microorganisms. This situation is usually overcome by applying antibiotics more frequently with enhanced doses. This practice is certainly injurious to all living beings. To overcome this alarming situation, the discovery of novel antibiotics or chemical agents that are much more effective against resistant microorganism is essential. For this reason, the isolation of a variety of antimicrobial agents from microorganism has been major contribution of scientific and research community. Antimicrobial agents have been discovered by screening many natural products or chemically synthesized derivatives of natural products for their efficacy against pathogenic microorganisms. Still now, research is going on to discover new and more effective antimicrobial agents from natural source like microbes which may combat against a number of infections to fatal disease like AIDS, cancer etc. due to lack of optimal antibiotic for therapeutic application. Microorganisms producing antimicrobial agents can be isolated from various sources but soil is the richest source of antimicrobial compound producing microorganism. A number of antibiotics have been isolated from microorganisms such as actenomycetes (eg. *Streptomyces*), fungi (e.g *penicillum*) and bacteria (eg *Bacillus subtilis*) of the soil origin. Most of the antibiotics have been isolated through screening of soil samples because antibiotic producing microorganisms are common component of most soil communities[3,4]. The aim of this work was to investigate the bioactive component produced by bacteria and their possible relation to antibiotic susceptibility as well as resistance to antimicrobial agents.

MATERIALS AND METHODS

Sample collection

For the screening of xylanase producing *Micrococcus* sp. strains the soil samples were collected using pre-sterilized sample bottles and sterile spatula from different areas of the bamboos garden wastes, Bangladesh.

Media and culture conditions

Nutrient agar media, Yeast extract xylan agar media and Czapek-dox agar media were used as a solid medium throughout the work. Yeast extract xylan agar plates were used for the isolation and identification of the bacteria and the bacteria were cultured at 37°C.

Isolation and characterization of bacteria

In a preliminary experiment of this research, the sample containing bacterial strains were spread on the different agar plates for isolation and rapid identification of the xylanase-producing bacteria and xylanolytic properties. The plates were then incubated at 37°C for 48 h, the colonies which formed clear zone on the xylan agar plates were picked, and then further purified by pure colonies producing clear zone on the xylan agar plate. All the xylanase producing bacterial strains which were isolated by their growth on xylan agar media as clear zones and xylanolytic properties were characterized according to the biochemical tests using the criteria of Bergey's Manual of Systematic Bacteriology[5].

Antibiotic resistance and sensitivity

The antibiotic resistance and sensitivity test to *Micrococcus* sp strains containing xylanolytic activity were carried out using the disc diffusion method[6,7]. A 16 hours broth culture of the isolated strains was spread on nutrient agar plate using sterilized glass spreader. Then Ampicillin (10 μg $disc^{-1}$), Amoxycillin (10 μg $disc^{-1}$), Chloramphenical (30 μg $disc^{1}$), Kanamycin (30 μg $disc^{-1}$), Doxycycline (30 μg $disc^{-1}$), Erythromycin (15 μg $disc^{-1}$), and Bacitracin (10 μg $disc^{-1}$) antibiotics were distributed on plates and kept the plates at 4°C for 6-8 hours, so that the antibiotic can diffuse on the agar media. The plates were then incubated at 37°C for 16 hours and the growth of the bacteria was observed. The presence of a clear zone around the disc was the index of sensitivity to the antibiotic. The test results of antibiotic sensitivity were determined according to the inhibition zone diameter[6,8]. The absence of such a clear zone or the presence of some colonies within the clear zone indicated that the collected strains were resistant to that antibiotic. The drug resistance bacteria tested by disc diffusion method were again confirmed by spreading its culture on the selected antibiotic plates of different concentrations.

Preliminary screening of antibacterial activities

Preliminary screening for antibacterial activity of the isolates was checked by cross streaking technique. Pure isolate was streaked on nutrient agar plate in a single line and then incubated at 37ºC for two days to allow

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the isolate to secrete antibiotics into the medium. After incubation period the over night growth culture of test organisms were cross streaked perpendicularly along the line of fully grown isolate. Each streaking was started near the edge of the plate and streaked toward the isolate growth line. The plates were then incubated at 37ºC for 24 hours. Absence of growth of pathogenic bacteria adjacent to *Micrococcus* sp SCS1 growth indicated inhibition of target culture and the zone of inhibition was measured using a millimeter[9].

Production of antibacterial substance

The production of antimicrobial substance was carried out using *Micrococcus* sp SCS1 cultured in 500 ml Erlenmeyer flasks containing 100 ml of broth medium [0.5% wheat bran xylan, 0.5% yeast extract, 1% peptone, 0.5% NaCl] at 30^{0}C for 4 days in an incubator on a rotary shaker. After this period of time the culture was centrifuged at 10000 rpm for 10 min and the cell free supernatant was used as the crude fermentation product.

Extraction of antibacterial substance

The crude fermentation product obtained after centrifugation was mixed with ethyl acetate solvent. After shaking the mixture gently for several hours, the solvent layer was separated by separating funnel and then evaporated at room temperature. As a result the crude extract was obtained.

Study of antibacterial activity

The ethyl acetate extract was assessed for antibacterial activity against six gram-negative *(Escherichia coli, Shigella dysenteriae, Shigella sonnei, Shigella shiga, Salmonella typhi* and *Klebsiella Pneumoniae*) and two gram-positive *(Bacillus subtilis* and *Staphylococcus aureus*) bacterial strains using disc diffusion method[6,10]. 2 mg of the isolated extract was dissolved in 1 ml ethyl acetate as a result the concentration was 2 mg/ml. Now the sample solution of desired concentration (30 µg/disc and 60 µg/disc) was applied on paper disc. Standard *Doxycyclin* (30 µg/disc) was used as positive control. Both experimental and control discs were then placed in petridishes seeded with organism in nutrient agar medium. The petridishes were kept in a refrigerator at 4°C for 6-8 hours to ensure diffusion of the test materials. Finally they were incubated at 37°C for 24 hours. The antibacterial activity was determined by measuring the diameter of zone of inhibition in millimeter.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of ethyl acetate extract and their fractions were determined by serial dilution technique[11] against *Escherichia coli, Salmonella typhi, Salmonella dysenteriae, Shigella dysenteriae, Shigella shiga, Klebsiella pneumonia, Bacillus subtilis and Staphylococcus aureus*

Brine shrimp lethality bioassay

The cytotoxic effect of the test extract was studied by Brine shrimp lethality bioassay[12]. Brine shrimp (*Artemia salina*) eggs were hatched in artificial seawater (prepared by dissolving 38 gms NaCl in 1000 ml distilled water) at room temperature under constant aeration for 48 hours. 10 mg of ethyl acetate extract was dissolved in 1 ml of DMSO and the resulting solution was used as Stock solution. The appropriate amount of stock solution was added to each vial, so that the final concentration of the extract became 0, 10, 20, 30, 40, 60, 80 and 100 µg/ml after diluting them up to 5 ml with seawater. To each vials, 10 living shrimps were added and allowed to stay them for 24 hours. The survived nauplii in each vial were counted and the results were recorded.

Figure 1 : Bacterial colonies (SCS1, SCS2, SCS3) producing clear zone on master plate.

RESULTS

Isolation and characterization of bacteria

Bacterial strains were isolated from bamboo garden waste, which was described in materials and methods. All bacterial strains produce clear zone on xylan agar plate around the colony shown in Figure 1 and 2.

Thus they were xylanase producing bacteria and may also have antibacterial activity. From morphological and biochemical tests these three strains were identified as *Micrococcus* sp (TABLE 1)

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Antibiotic resistance and sensitivity test

The results of antibiotic resistance and sensitivity pattern on xylan agar plate, it was observed that three isolated strains SCS1, SCS2 and SCS3 were sensitive to antibiotics Ampicillin, Amoxycillin, Chloramphenical, Doxycyclin, Bacitracin, Kanamyci and Erythromycin (TABLE 2).

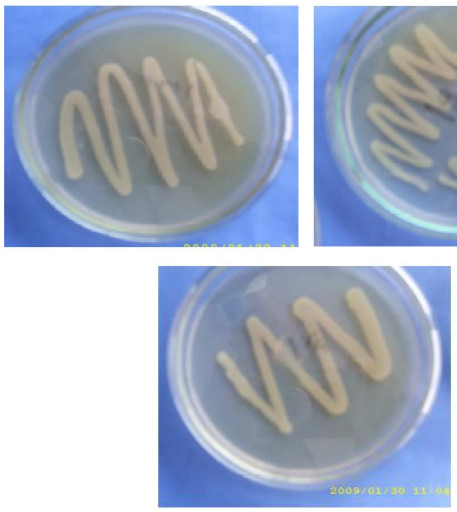

Figure 2 : Photograph of pure strains (SCS1, SCS2, SCS3)

Preliminary screening of antibacterial activities

Although three bacterial strains were isolated from soil sample, but one strain (*Micrococcus* sp SCS1) of them showed best antibacterial activity against pathogenic bacteria in primary screening by cross streaking method (Figure 3) and selected for further study. The antibacterial activity was subsequently confirmed by agar disc diffusion method.

Antibacterial activity

The antibacterial activity of ethyl acetate extract of *Micrococcus* sp SCS1 was determined at doses 30 μg/disc and 60 μg/disc. The ethyl acetate extract of *Micrococcus* sp SCS1 showed good antibacterial activity at 60 μg/disc and a moderate activity at dose of 30 μg/disc against both gram-negative *(Escherichia coli, Shigella dysenteriae, Shigella sonnei, Shigella shiga, Salmonella typhi* and *Klebsiella Pneumonia*) and gram-positive *(Bacillus subtilis* and *Staphylococcus aureus*) pathogenic bacteria (Figure 4).

The results of the antibacterial activity were measured in terms of zone of inhibition and were compared with standard antibiotic *Doxycycline* (TABLE 3).

The ethyl acetate extract showed antibacterial activity against the tested pathogenic bacteria with inhibition zone in the range of 08-22 mm. The strain (*Micrococcus* sp SCS1) was tested against pathogenic bacteria to determine MIC and the results were cited in the TABLE 4.

TABLE 1 : Morphological and biochemical characteristic of isolated bacterial strains.

Test	*Micrococcus* sp SCS1	*Micrococcus* sp SCS2	*Micrococcus* sp SCS3
Gram Staining	Positive	Positive	Positive
Shape	Small circular	Small circular	Small circular
Motility	Non-Motile	Non-Motile	Non-Motile
Indole production	-	-	-
Methyl red	-	-	-
Voges proskauer	+	+	+
Citrate utilization	+	+	-
H2 S Production	-	-	-
Urea hydrolysis	-	-	-
Oxidase test	+	-	-
Catalase test	+	+	+
Nitrate reduction	+	+	+
Gelatinase	+	+	+
Glucose	+	+	+
Sucrose	+	-	-
Maltose	+	-	-
Fructose	+	+	+
Galactose	-	-	-
Lactose	-	-	+

The minimum inhibitory concentrations of the ethyl acetate extract of *Micrococcus* sp SCS1 were within the values of 64-256 μg/ml against those pathogenic bacteria.

Brine shrimp lethality bioassay

In the brine shrimp lethality bioassay, the lethality of the crude extract of ethyl acetate extract of *Micrococcus* sp SCS1 to brine shrimp was determined on *Artemia salina* after 24 hours of exposure of the sample. The median lethal concentration (LC_{50}) of brine shrimp lethality was measured from the plot of

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TABLE 2: Antibiotic sensitivity & resistance test

Bacteria (Strains)	Antibiotics (Diameter of the clear zone of inhibition in mm)						
	Ampicillin (10µg/disc)	Amoxycillin (10µg/disc)	Chloramphenical (30µg/disc)	Doxycycline (30µg/disc)	Erythromycin (15µg/disc)	Kanamycin (30µg/disc)	Bacitracin (10µg/disc)
SCS1	16	14	32	40	22	27	34
SCS2	10	12	30	32	36	30	25
SCS3	14	10	38	36	46	35	22

percentage of mortality versus concentration of the sample (Figure 5) and it was found to be 30 µg/ml.

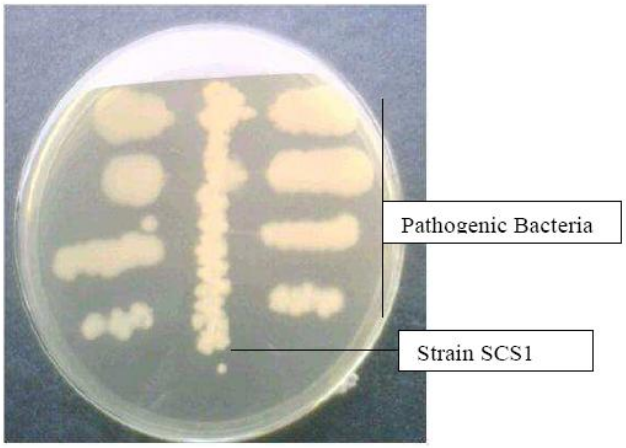

Figure 3 : Preliminary screening for antibacterial properties of strain SCS1 by cross streak method.

DISCUSSION

In this study, bacterial strains were isolated from bamboo garden waste, which degraded β-1, 4 xylan and to belong *Micrococcus* sp. It was observed that, the strains were sensitive to antibiotics Ampicillin, Amoxycillin, Chloramphenical, Doxycyclin, Bacitracin,Kanamycin and Erythromycin. All of the *B. thuringiensis* isolates were resistant to Amoxicillin, Ampicillin, Ceftriaxone, Penicillin and Oxacillin while susceptible to the remaining antimicrobials[13]. Xylanase producing *Aeromonas* strains were resistant to Amoxicillin, Ampicillin, Cotrimazole, and sensitive to Erythromycin, Tetracycline and Doxycyline[14,15].

Present investigation suggests that the ethyl acetate extract of the cultural broth of *Micrococcus* sp SCS1 contained some antibacterial components, which have activity against both gram positive and gram-negative bacteria (TABLE 3). Strains of *B. thuringiensis*, *B. subtilis*, *B. stearothermophilus*, *B. liceniformis*, *B. megaterium* and *B. cereus* have been reported to produce substances like bacteriocin. Bacteriocins are

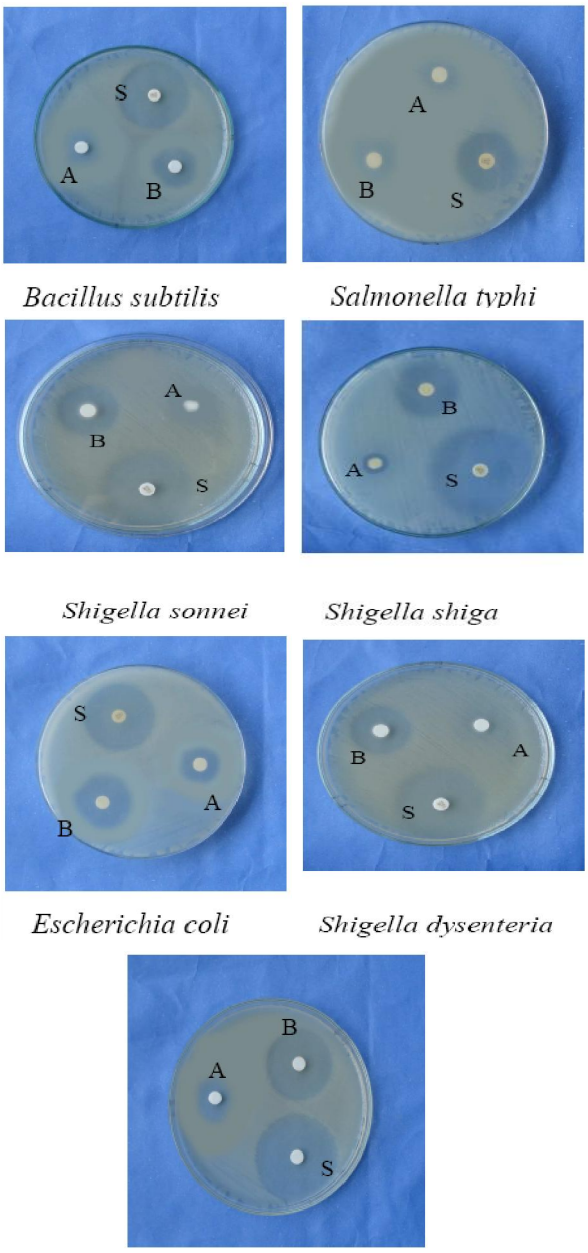

Figure 4 : Antibacterial activity of the ethyl acetate extract of *Micrococcus* sp SCS1; where A= 30µg/disc, B= 60µg/disc and S= Standard Doxycycline (30µg/disc)

ribosomally synthesized antimicrobial peptides produced by a number of different bacteria[16]. It was observed

TABLE 3 : Antibacterial activity of ethyl acetate extract of *Micrococcus* sp SCS1

Test bacteria		Diameter of zone of inhibition in mm of the ethyl acetate extract		Standard antibiotic *Doxycycline* (30 µg/disc)
		30 µg/disc	60 µg/disc	
Gram negative bacteria	*Escherichia coli*	12	20	24
	Shigella dysenteriae	15	20	28
	Shigella sonnei	14	18	33
	Shigella shiga	8	14	28
	Salmonella typhi	8	12	37
	Klebsiella Pneumonia	14	22	32
Gram positive bacteria	*Bacillus subtilis*	8	14	30
	Staphylococcus aureus	12	16	30

TABLE 4 : MIC values of ethyl acetate extract of *Micrococcus* sp SCS1

Test Bacteria	MIC (µg/ml)
Escherichia coli	128
Shigella shiga	256
Shigella dysenteriae	128
Shigella sonnei	128
Klebsiella pneumoniae	64
Salmonella typhi	256
Bacillus subtilis	256
Staphylococcus aureus	128

that *B. thuringiensis* D1, *B. thuringiensis* D3, had an inhibitory effect on all of the above tested bacteria[17].

The Brine shrimp lethality bioassay is a recent development in the bioassay for the bioactive compounds, which indicates cytotoxicity of the compound. The ethyl acetate extract showed positive results in brine shrimp lethality bioassay indicating that the extract was biologically active. In this bioassay, the mortality rate of brine shrimp was observed to be increased with the increase in concentration of the test sample. There were many reports in where the mortality rate increased with increasing concentration of sample.

CONCLUSION

From the results discussed above it is clear that the ethyl acetate extract from *Micrococcus* sp SCS1 is biologically active and possesses good antibacterial activity against both gram positive and gram negative pathogenic bacteria. Thus the ethyl acetate extract is a broad-spectrum antibacterial agent with moderate cytotoxicity.

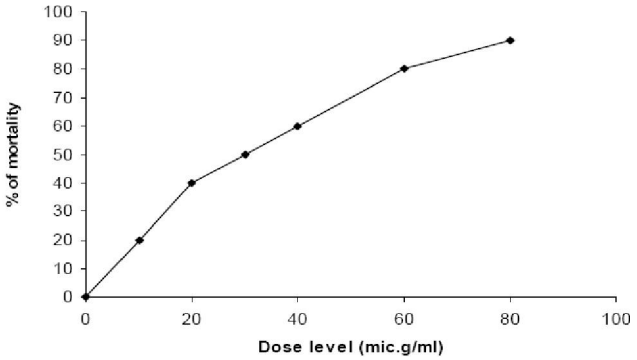

Figure 5 : Brine shrimp lethality bioassay of ethyl acetate extract of *Micrococcus* sp SCS1

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