Physico-chemical Characterization of Indigenous *Streptomyces* and Influence of pH on Antimicrobial Activity

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Abstract

Background. Emergence of multi-drug resistant pathogens has afflicted the population of developing countries like Bangladesh in recent years for which a sustainable holistic combating approach is required. Since *Streptomyces* is a source of numerous bioactive molecules, the study was aimed at physico-chemical characterization of 8 indigenous *Streptomyces* isolates of Bangladesh. **Methods.** Tolerance of *Streptomyces* isolates to different growth conditions was assessed at temperature range 4 °C to 60 °C, pH range 3 to 11 and salinity up to 15% of NaCl concentration. Ability of isolates in utilizing different carbohydrates was checked through media of single sugar as sole carbon and energy source. The antimicrobial activity of the isolates against four pathogens was assayed with culture supernatant obtained from 5 different pH levels. The data was analyzed statistically by software R version 3.4.1. **Results.** All the isolates grew optimally in the temperature range of 20- 40 °C, pH range of 5- 9 and salinity of 1% NaCl concentration although certain isolates tolerated up to 60 °C and 10% of salinity. Based on the sugar profiles, the isolates were allocated into different biotypes and their relatedness was found with *S. mutabilis* (C_a), *S. subrutilis* (C_{b1}) and *S. mirabilis* (C_{b2}) as positioned at same clusters in the dendrogram. The antimicrobial molecules produced by the *Streptomyces* isolates were not heat stable and denatured by ethanol, hence presumed as protein. The maximum antagonism was recorded against *E. coli* by isolate B-5 at pH 6 (18 mm), against *S. typhimurium* by A-9 at pH 9 (20 mm), against *S. aureus* by B-7 at pH 5 (18 mm) and against *B. cereus* by B-7 at pH 8 and 9 (18 mm) as well as by D-5 at pH 7 (18 mm). It was also deduced that the pH as a growth condition significantly influenced the production of pathogen specific antimicrobial compounds by the *Streptomyces* isolates. **Conclusion.** The ability of the *Streptomyces* isolates in tolerating wide range of growth conditions would be of special advantage and the influence of pH in pathogen specific antimicrobial production could enhance the chances of obtaining diverse antimicrobials.

Keywords: *Streptomyces*, Multi-drug resistance, Antimicrobial activity, Growth parameters, Biotyping, pH.

Abbreviations: ISP- International Streptomyces Project, ATCC- American Type Culture Collection, spp- species, SCDA- Soybean Casein Digest agar, ZOI- Zone of Inhibition, MDR-Multi drug resistant.

Significance | Importance of the quality assurance of the service of the suburb diagnostic facilities to reduce the misuse of antibiotics.

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Introduction

The emergence of Multi Drug Resistant (MDR) pathogens is a serious threat to the public health, especially in the developing countries like Bangladesh (Faiz & Ariful, 2011; MacGowan & Macnaughton, 2017; Rahman & Huda, 2014). Although the records lack precision and verification, the people usually suffer heavily from several infectious diseases connected to MDR (Barai et al., 2017). The emergence of MDR strains could be attributed to several factors including frequent and improper use of antibiotics, prescribing antibiotics without antimicrobial susceptibility

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testing, presence of MDR strains in food, feed and environment, emergence of multidrug resistance in common pathogens, horizontal and vertical transfer of antibiotic resistance genes, and improper hospital waste management etc. ("Progress on antibiotic resistance," 2018; Rahman & Huda, 2014; van Belkum et al., 2018). The current situation in Bangladesh is very alarming since the resistance by major Gram negative bacteria such as *Escherichia coli*, *Klebsiella* sp., *Salmonella* sp., *Pseudomonas* sp., *Acinetobacter* sp. and major Gram positive bacteria such as *Staphylococcus aureus*, *Enterococcus* sp was reported against imipenem (3- 84 %), third generation cephalosporin (61.6- 94.9 %), aminoglycosides (10.8- 88.6%), ciprofloxacin (56- 90.1%), cotrimoxazole (58- 80.3%), nitrofurantoin (14.3- 91.7%), tazobactum+piperacillin (20.8- 81.4%) and colistin (2.2- 16.4%) (Barai et al., 2017).

Although research and development for new generation of antibiotics initially helped in solving the resistance problems, the situation deteriorated with time. To combat the multidrug resistance, a sustainable holistic approach including production of new potent antibiotics from novel sources is urgently needed (A & M, 2012; Ahmad et al., 2017). Since, numerous essential bioactive compounds e.g. antibiotics, enzymes, hormones, vitamins, anticancer and antiviral drugs, herbicides, fungicides, insecticides, immunomodifiers, therapeutic agents, were obtained from different microorganisms, systematic screening for microbes with potential antimicrobial agents is always a focus of interest to the researchers (Ferdous, Shishir, Khan, & Hoq, 2018; Khatun, Haque, & Islam, 2018; Lee, Chan, Stach, Wellington, & Goh, 2018; Mustafa, A., & Cem, 2004). Discovery and development of new antimicrobial agents is therefore beside other approaches to combat antibiotic resistance, an important area (Ahmad et al., 2017; Bérdy, 2005; V & S, 2018).

Among 22,500 bioactive compounds, almost half were reported to be produced by actinomycetes (mainly *Streptomyces*) followed by fungi and other bacteria (Bérdy, 2005; Lee et al., 2018; Ser et al., 2017). A large number of antibiotics (nearly 80% of all) currently in use including Erythromycin, Streptomycin, Rifamycin and Gentamycin are obtained from soil actinomycetes which necessitates the novel biotopes, niche, ecosystems and extreme environments to be explored on regular basis for more potential and novel actinomycetes (Ahmad et al., 2017; Crits-Christoph, Diamond, Butterfield, Thomas, & Banfield, 2018; Lee et al., 2018; S. Ningthoujam & Sanasam, 2011). Among the soil actinomycetes, two major groups i.e. *Streptomyces* and *Micromonospora* are predominant in obtaining different antibiotics.

Streptomyces is renowned for producing secondary metabolites with different biological activities, such as antibacterial, antifungal, antiparasitic, herbicidal, antitumor, anticancer and anti-immunosuppressant activities (Anderson & Wellington, 2001; Jiang et al., 2018; Nishat & Alam, 2017). Like other members of actinobacteria, *Streptomyces* are filamentous Gram-positive bacteria with high GC content in the genome, mostly spore-forming and noted for their distinct "earthy" odor (Ahmad et al., 2017; Martinko & Madigan, 2005). In fact, thousands of antibiotics obtained to date represent a small portion of the repertoire of bioactive compounds and hence, it is very likely to discover novel *Streptomyces* with potent bioactive compounds (Bérdy, 2005; Crits-Christoph et al., 2018; Ser et al., 2017).

Bangladesh is a tropical country with vast ecological diversity which increases the chance of obtaining diverse *Streptomyces* with novel antimicrobials. But this area remained mostly untouched in this country except few recent studies (Abony, Alam, Banik, Jannat, & Datta, 2017; Khatun et al., 2018; Nishat & Alam, 2017; Sharmin, Rahman, Sayeed, Anisuzzaman, & Islam, 2017). *Streptomyces bangladeshensis*, a new species of *Streptomyces*, from the soil of Natore, Bangladesh was reported to produce bis-(2-ethylhexyl)-phthalate, an antibacterial and antifungal agent (Al-Bari, 2005) and *Streptomyces banglaensis* strain ANTS-1^T obtained from the soil of Rajshahi, Bangladesh was reported to produce Actinomycin D, an antitumor protein (Sharmin et al., 2017). The present study was therefore undertaken with the objectives of characterizing 8 potential indigenous *Streptomyces* isolates based on certain physico-chemical parameters, biotyping based on their carbohydrate utilization ability and to determine the influence of pH levels on their antimicrobial production.

Methods and materials

Bacterial strains

Four pathogenic bacterial strains, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 14579, *Salmonella typhimurium* ATCC 14028 and *Escherichia coli* ATCC 25922, used in this study were kindly provided by Dr. Md. Mahfuzul Hoque, Department of Microbiology, University of Dhaka, Bangladesh. *Streptomyces* strains A-9, A-12, B-5, B-7, C-1, D-5, E-6 and E-8, isolated previously from different locations of Dhaka and Comilla (Abony et al., 2017) with antagonistic activity against pathogens, were considered for further characterization.

Phenotypic characterization

The culture of the *Streptomyces* isolates were maintained on ISP-1 (g/L: pancreatic digest of casein- 5.0, yeast extract- 3.0), ISP-2 (g/L: yeast extract- 4.0, malt extract- 10.0, dextrose- 4.0, agar- 20.0), ISP-4 (g/L: soluble starch- 10.0, K_2HPO_4- 1.0, $MgSO_4$- 1.0, NaCl- 1.0, $(NH_4)_2SO_4$- 2.0, $CaCO_3$- 2.0, $FeSO_4$- 0.001, $MnCl_2.4H_2O$- 0.001, $ZnSO_4$- 0.001, agar- 20.0), ISP-6 (g/L: bacto-peptone- 15.0, proteose peptone- 5.0, ferric ammonium citrate- 0.5, K_2HPO_4- 1.0, $Na_2S_2O_3$- 0.08, agar- 15.0, yeast extract- 1.0; pH: 7.0±0.2), Soybean Casein Digest Agar (HiMedia, India) and Starch Casein Agar (g/L: soluble starch- 10.0, casein (vitamin free)- 0.3, KNO_3- 2.0, $MgSO_4.7H_2O$- 2.0, K_2HPO_4- 2.0, $CaCO_3$- 0.02, $FeSO_4.7H_2O$- 0.01, Agar- 20.0, Nystatin- 0.05, Benzyl Penicillin - 0.0008) media. The isolates were incubated at 20 °C for 7-14 days and the growth morphologies were recorded (Supp.

Table 1) following the International *Streptomyces* Project (ISP) guidelines (S. Ningthoujam & Sanasam, 2011).

Determination of tolerance to growth conditions

a. Temperature

ISP-4 agar medium (pH 7.2 ± 0.2) was inoculated with isolates and incubated at different temperatures such as 4, 10, 20, 37, 40, 50 and 60 °C to determine the temperature tolerance ranges (Priyanka, 2011; S. Ningthoujam & Sanasam, 2011).

b. pH

The [H^+] ion concentration of the ISP-1 broth was adjusted to the pHs 3, 5, 7, 9 and 11 by using pH- Buffer tablets (Merck, India) earlier in the water. Growth of the isolates at varied pHs were checked by inoculating them into 10 ml of ISP-1 broth of different pHs in Erlenmeyer flask and incubated at 37 °C for 7- 14 days. One loopful of each culture was then streaked onto ISP-2 agar plates of similar pHs and incubated at 20 °C for 10-12 days with regular monitoring for growth (Priyanka, 2011; S. Ningthoujam & Sanasam, 2011).

c. Salinity

Different concentrations of NaCl i.e. 1, 3, 5, 7, 10, 12 and 15 % were maintained in the ISP-4 agar media (pH 7.2 ± 0.2) and the isolates were inoculated into those media to check the salinity tolerance. The media were then incubated at 20 °C for 7- 15 days and the presence or absence of growth was recorded from 7th day onwards (Priyanka, 2011; S. Ningthoujam & Sanasam, 2011).

Biochemical typing

The ability of *Streptomyces* isolates to utilize various sugars (Mannose, Xylose, Raffinose, Rhamnose, Glucose, Sucrose, Lactose, Galactose, Mannitol, Glycerol and Inositol) as the source of carbon and energy was assessed by growing the isolates on sole carbon utilization medium. The carbohydrate stock solutions were prepared in sterile distilled water and further sterilized by filtering through Millipore membrane filters (0.22 µm). The growth of isolates was checked by adding 1% of each mentioned sugar instead of Dextrose in ISP-2 agar media (pH 7.2 ± 0.2) separately. Plates inoculated with the isolates were incubated at 37 °C for 7 to 10 days. The growth of the isolates was considered as sugar utilization ability whereas lack of growth implied inability of utilization. Binary matrices of the isolates based on the sugar utilization abilities were prepared assuming ability as 1 and inability as 0. These sugar profiles were used to biotype the isolates by calculating the similarity and dissimilarity matrices. Dendrogram was constructed which exhibited the relatedness as well as the distances among the isolates following the method described by Shishir et al. (Shishir, Pervin, Sultana, Khan, & Hoq, 2015).

Antimicrobial activity of isolates

Antimicrobial activity of the isolates was determined by following Kirby Bauer method (Barai et al., 2017; Hoque et al., 2011). In brief, to prepare bacterial lawns of interests, test pathogens, were grown overnight in Muller Hinton broth (Oxoid, UK) and the desired turbidity was adjusted using 0.5 M McFarland standard (Hoque et al., 2011). Muller Hinton Agar (MHA) plates were then streaked with sterilized cotton swab, soaked with liquid culture of test pathogens, evenly in three directions and keeping at an angle of C. 60 °C onto the surface. Surplus suspension was removed before the plate was seeded and after the moisture of the inocula was dried up, sterile borer was used to make well (8 mm diameter) (Shishir et al., 2018). The culture supernatant of the *Streptomyces* isolates was added into the prepared wells and allowed to diffuse around through the media. The plates were then incubated at 37 °C for 24 hours and the zone of inhibition was measured and recorded for crude culture supernatant obtained at different pHs and also for the ethanol extracted as well as heat treated supernatants (Bizuye, Moges, & Andualem, 2013; S. Ningthoujam & Sanasam, 2011).

Statistical analysis

The objective was to compare the antimicrobial effects of 8 *Streptomyces* strains against the test pathogens. On the other hand, the influence of pH at 5 levels on the antimicrobial effects was also checked. In these connections, a two-factor experiment was developed with one observation per cell (Montgomery, 2006). The following additive model was set for each pathogen:

$$y_{ij} = \mu + \tau_i + \beta_j + \epsilon_{ij}, \qquad i = 1,2,\ldots,8; \quad j = 1,2,\ldots,5,$$

Where μ is an overall mean, τ_i is the effect of the i - th antibiotic, β_j is the effect of the j- th pH level, ϵ_{ij} is the random error and ϵ_{ij} follows normal distribution with mean 0 and constant variance, σ^2, y_{ij} is the observed response for the i - th antibiotic and j- th pH level. Both antibiotics and pH are fixed factors. Additional assumptions are, $\sum_{i=1}^{8} \tau_i = 0$ and $\sum_{j=1}^{5} \beta_j = 0$. The analyses were performed using the software R version 3.4.1.

Results

Cultural characteristics

Most of the indigenous *Streptomyces* isolates demonstrated affluent growth on ISP media supplemented with antibacterial and antifungal drug and soluble pigments were produced by the isolates in ISP-6 media. Colony morphologies of the isolates were recorded which followed the typical growth pattern of *Streptomyces*. In ISP-1 medium, all isolates produced whitish colony with slight variations like milky white or creamy appearance. In ISP-2, creamy or grayish appearance were observed. In ISP-4, it was white or grayish or greenish. In ISP-6, all isolates exhibited whitish appearance except isolate E-6 which failed to grow. In SCDA medium, whitish or grayish appearance were mostly observed with waxy texture by E-6. In starch casein agar, all except isolate B-7 (yellowish) were mostly whitish and D-5 did not grow (Supp. Table 1).

Tolerance of isolates at different growth conditions

Table 1 | **Tolerance of indigenous Streptomyces isolates to different growth conditions.**

Parameters		Isolates							
		A-9	A-12	B-5	B-7	C-1	D-5	E-6	E-8
Temperature	4 °C	+	+	+	-	-	-	-	+
	10 °C	+	+	+	+	+	+	+	+
	20 °C	+	+	+	+	+	+	+	+
	37 °C	+	+	+	+	+	+	+	+
	40 °C	+	+	+	+	+	+	+	+
	50 °C	+	+	-	-	-	-	-	+
	60 °C	+	+	-	-	-	-	-	+
Salinity	1%	+	+	+	+	+	+	+	+
	3%	+	+	-	-	-	+	-	+
	5%	+	+	-	-	-	+	-	+
	7%	+	+	-	-	-	+	-	+
	10%	+	+	-	-	-	+	-	+
	12%	-	-	-	-	-	+	-	-
	15%	-	-	-	-	-	-	-	-
pH	3	-	-	-	-	-	-	-	-
	5	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+
	11	-	-	-	-	-	-	-	-

Table 2 | **Summary of ANOVA performed for the test pathogens.**

Test pathogens	Source of Variation	Sum of Squares	Mean Square	F-value	Pr (>F)
S. typhimurium	Isolates	234.0	33.43	1.493	0.210
	pH	217.6	54.41	2.431	0.071
E. coli	Isolates	99.97	14.282	1.567	0.1863
	pH	125.65	31.412	3.447	0.0207
S. aureus	Isolates	99.6	14.23	1.241	0.315
	pH	955.7	238.91	20.830	0.0000000466
B. cereus	Isolates	79.9	11.41	0.619	0.736
	pH	1036.6	259.15	14.046	0.0000021

The *Streptomyces* isolates tolerated varied ranges of temperature, pH and salinity specified in this study (Table 1). Interestingly, isolates A-9, A-12 and E-8 exhibited identical tolerance pattern for all three parameters. They were able to grow at temperatures from 4 °C to 60 °C, pH from 5 to 9 and salinity up to 10% of NaCl concentration. Isolates B-5, B-7, C-1 and E-6 could not survive at salinity more than 1% whereas D-5 tolerated up to 12% of salinity. In case of H^+ ion concentration, no isolates could manage to grow beyond the pH range 5 to 9.

Types and their relatedness

The biochemical ability of the *Streptomyces* isolates in utilizing various sugars as carbon and energy source was determined. The *Streptomyces* isolates were able to utilize all the sugars tested in this study with variations among the isolates.

Based on the sugar profiles, the similarity and dissimilarity among the isolates were estimated and a dendrogram was constructed which revealed the biochemical distance among the isolates. Two major clusters, C_a and C_b with substantial distance were observed from the tree, i.e. the dendrogram (Fig. 1). Cluster two, C_b was branched with two more clusters, C_{b1} and C_{b2} with substantial distance. Isolates B-5 and B-7 were closely related as positioned in the same cluster, C_a which based on the sugar profile was similar to *Streptomyces mutabilis*. Similarly, isolates C-1 and E-6 from the same cluster C_{b1} were closely related which resembled *Streptomyces subrutilis*. The relatedness of comparatively larger cluster C_{b2} was toward *Streptomyces mirabilis* which contained isolates A-9, A-12, D-5 and E-8. The sugar profiles of A-9 and A-12 were identical whereas the similitude of isolates D-5 and E-8 from Comilla was found to the isolates collected from Dhaka (A-9 and A-12). Again the isolate C-1 was much similar to isolate E-6 and both of them were collected from Comilla.

Antimicrobial activity of *Streptomyces* isolates

The *Streptomyces* isolates were observed to exert antagonistic activity against the test pathogens and the crude supernatant i.e. antimicrobial compounds at their native state were functional. Rest of the forms of the supernatants i.e. ethanol extract or the heat treated portion, were not antagonistic at all.

The inhibition of the test organisms by crude supernatant was occurred at varied intensity levels. While considering the antimicrobial activity of *Streptomyces* isolates against test pathogens irrespective of pH variation, *S. typhimurium*, was found to be inhibited maximum by isolate C- 1 (13.6± 1.14) followed by A-12 (13.4± 2.8) and B- 7 (13± 1.0). Against *E. coli*, isolate B- 5 (15.2± 1.92) was found to be most potent followed by A- 9 (14.6± 0.89) and E- 6 (14.4± 1.14). Again, *S. aureus* was more susceptible to isolate B-7 (11.2± 6.69) followed by D- 5 (11± 6.67) and A- 9 (10.8± 6.22). And *B. cereus* was inhibited maximum by isolate B- 5 (12.05± 4.64) followed by B- 7 (11.95± 4.75) and A- 12 (11.85± 4.42) (data not shown).

While considering the efficacy of antimicrobial compounds against the test pathogens irrespective of variation of the isolates (Fig. 2), it was observed that *S. typhimurium* was highly susceptible to the antimicrobial compounds produced at pH 9 followed by pH 7 and pH 6. The susceptibility of *E. coli* was more or less similar to the antimicrobial compounds produced at pH 5 to 8 but at pH 9, it was reduced. *S. aureus*, was more susceptible to the antimicrobial compounds produced at pH 6 than those at other pHs and interestingly, antimicrobial compounds of all the *Streptomyces* isolates produced at pH 9 inhibited all the test pathogens at varied degrees except *S. aureus* for which the zone of inhibition was nil. A similar instance of inactivity was observed for *B. cereus* with the antimicrobial compounds produced at pH 5 although the other test pathogens were inhibited with the very same agent. *B. cereus* was mostly inhibited with the antimicrobial compounds

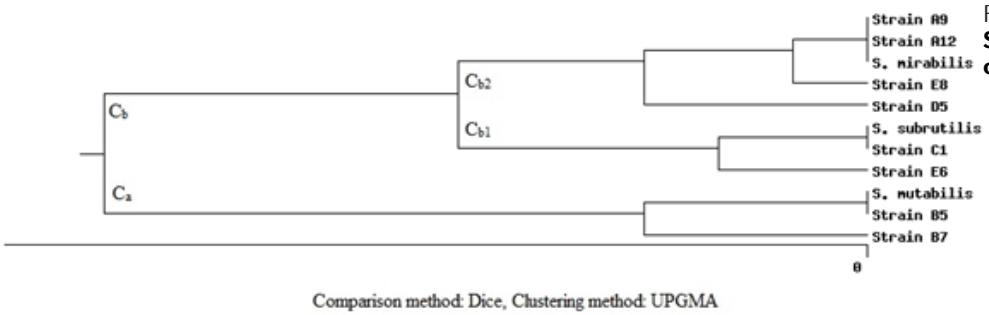

Figure 1 | **Relatedness of indigenous Streptomyces isolates based on their carbohydrate utilization capability.**

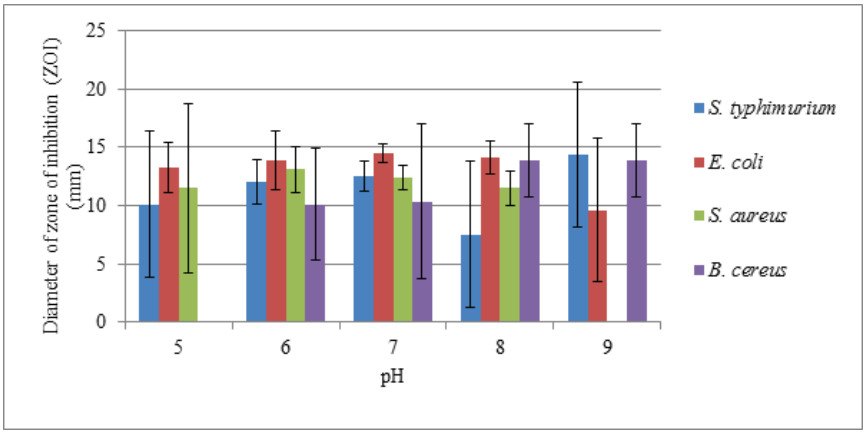

Figure 2 | **Degree of antagonism of Streptomyces isolates against the test pathogens with antimicrobials produced at different pH.**

produced at pH 8 and 9.

pH influenced variation in antimicrobial activity

Against *S. typhimurium*, it was determined that neither the different *Streptomyces* isolates nor the different pH levels affected the mean zone of inhibition (ZOI) at 5% levels of significance (Table 2). Similarly, against *E. coli*, it could be concluded that variation in the mean ZOI was not affected by the different *Streptomyces* isolates. But the effect of pH was significant at α =0.05 (Table 2). To identify exactly which pH affected the antagonism, a post-hoc test called "Tukey HSD" was performed.

It was revealed from Tukey HSD test that there was significant difference in mean ZOI caused by the antimicrobials produced at pH 9 and pH 7 (p-value= 0.0241855) at α=0.05. Significant difference was observed as well for pH 9 and pH 8 (p-value= 0.0429481) (Supp. Table 4). Thus differences in antimicrobial compounds production, especially against *E. coli* was observed due to pH variations.

Against *S. aureus*, significant variation in antagonism was not caused by different *Streptomyces* isolates rather different levels of pH affected the antimicrobial production [α=0.05] (Table 2). So, further test was performed to check which means differed significantly.

It was revealed that there were significant differences in mean ZOI caused by the antimicrobials produced at different pHs, especially of pH 9 which, even though exerted antagonism against other test pathogens, was completely inactive against *S. aureus*. These were also observed through Tukey HSD test (Supp. Table 6), i.e. against *S. aureus*, the efficacy of pH 9 derived antimicrobial compounds was significantly different from those of other pHs (5, 6, 7 and 8) derived compounds whereas such differences among pH 5 to pH 8 were not significant.

A similar scenario was observed in case of antagonism against B. cereus (at α=0.05) which was affected significantly by different pH levels rather than the *Streptomyces* isolates (Table 2). Hence, "Tukey HSD" test for multiple comparisons was performed and it was revealed that the antimicrobials produced at pHs (6 to 9) caused significantly different antagonisms than produced at pH 5. Antimicrobial compound derived at pH 5 was completely inactive against *B. cereus* although exerted antagonism against other test pathogens (Supp. Table 8). Contrarily, such differences were not significant for the antimicrobial compounds derived at pH 6 to pH 9..

Discussion

Treating bacterial infections by antibiotics became difficult nowadays since the antimicrobial resistance continues to increase leaving the pipeline for new antibiotics depleted (MacGowan & Macnaughton, 2017). The situation is even more deteriorated due to the emergence of multi-drug resistant pathogens. Although there are plenty of factors involved in development of antibiotic resistance, the ultimate fate is the cumulative resistance among the pathogens which is getting unmanageable day by day in Bangladesh (Barai et al., 2017; Faiz & Ariful, 2011; Rahman & Huda, 2014). Besides appropriate administration and management of existing antibiotics, additional antimicrobial resources must be prepared in advance to be useful in need. Unfortunately, here in Bangladesh, quite a very few initiatives were taken to explore the

indigenous *Streptomyces* which covered Natore, Rajshahi, Cox's Bazar, Dhaka and Comilla to identify and develop potential strains with their bioactive compounds (Abony et al., 2017; Al-Bari, 2005; Nishat & Alam, 2017; Sharmin et al., 2017). The prospects of those preliminary findings were very inspiring since a wide range of tolerance of different growth conditions was also evidenced by the isolates besides the demonstration of antimicrobial and antitumor activities. In this study, 8 potential *Streptomyces* isolates were therefore characterized based on the tolerance of different growth conditions and differentiated based on their carbohydrate utilization ability. At the same time, the influence of pH on their antimicrobial compound production and efficacy was also investigated.

Since wide ranges of tolerances to different growth conditions are always advantageous and preferential traits of microorganisms, it is an essential task to characterize these properties. Hence, the growth of *Streptomyces* isolates at different physical conditions i.e. temperature, salinity and pH was checked and the ranges were determined. Temperature range for growth was determined on inorganic salt starch agar medium (ISP-4) incubating the isolates from 4 °C to 60 °C. Isolates typically showed optimum growth in the range of 20- 40 °C with scant growth at 10 °C. Growth of most of the isolates decreased beyond the range 20 °C- 40 °C which is very similar to the properties of Nambul river isolates of Manipur, India as reported by Ningthoujam (S. Ningthoujam & Sanasam, 2011). However, A-9, A-12 and E-8 demonstrated tolerance towards lower as well as higher temperature i.e. 4 °C- 60 °C which could facilitate the addition of special feature to the relevant bioactive molecules like functionality at wider temperature range.

In terms of growth at different salinity, tolerance level was variable among the isolates though all isolates showed maximum growth at 1% of NaCl concentration. Isolates A-8, A-9 and E-6 tolerated up to 10% of NaCl concentration and in case of D-5, tolerance up to 12% of NaCl concentration was evidenced which is similar to the salt tolerance of isolates reported from mangrove origin (Priyanka, 2011). Interestingly, reports of isolation of certain *Streptomyces* sp. from high saline environment i.e. Cox's Bazar marine ecosystem with a salinity of 32% to 34.5%, were also made (Nishat & Alam, 2017). On the other hand, the *Streptomyces* isolates were found to grow around a neutral pH range i.e. pH 5 to 9. The growth of the isolates A-8, A-9 and E-6 were abundant in comparison to other isolates in these pHs.

Assimilation of different carbon sources by actinomycetes is considered as the basis of their differentiation (Pandey, Shukla, & Majumdar, 2005). Utilization of carbon sources such as Mannose, Xylose, Raffinose, Rhamnose, Glucose, Sucrose, Lactose, Galactose, Mannitol, Glycerol and Inositol were considered in this study. Isolates exhibited varied capability in utilizing these carbon sources. Almost all the isolates have shown abundant growth in rhamnose and galactose followed by mannitol, xylose and glycerol. Isolate A-9, A-12, C-1, E-6 and E-8 were able to utilize most of the sugars. For optimum production of antibiotics, the influence of certain carbon sources was also reported beside different pH (Pandey et al., 2005). Hence the varied capability of utilizing different sugars could be indicative of the potentials of diverse antibiotic production.

The sugar profile of the isolates i.e. the ability of utilizing different carbon sources was used here as the tool for biochemical typing of the *Streptomyces* isolates. In this connection, dendrogram was constructed based on the binary matrices of the isolates which were prepared by converting the sugar profiles. From the dendrogram, two major biotypes C_a and C_b were observed and biotype C_b was further divided into C_{b1} and C_{b2} biotypes (Fig. 1). Biotype C_a was found to have similar sugar profiles of *S. mutabilis* and biotype C_b with further branching was similar to *S. subrutilis* (C_{b1}) and *S. mirabilis* (C_{b2}). Thus dendrogram analysis assisted in classification of the isolates based on their sugar profiles and this relatedness could further be assessed using molecular techniques like RAPD-PCR, 16S rRNA gene sequence analysis etc.

While tested against the test pathogens, the crude culture supernatant of *Streptomyces* isolates exhibited varied antagonistic activities in its native form. Heat treated supernatant or ethanol extraction of the supernatant was not at all inhibitory to the pathogens. This indicates the nature of the antimicrobial molecules produced by the *Streptomyces* isolates i.e. they could be of protein in nature. Since both heat and ethanol inactivated them, it is clear that the active components are not heat stable and readily denatured by ethanol.

In native form, the maximum antagonism i.e. the zone of inhibition was recorded against *E. coli* by isolate B-5 at pH 6 (18 mm), against *S. typhimurium* by isolate A- 9 at pH 9 (20 mm), against *S. aureus* by isolate B-7 at pH 5 (18 mm) and against *B. cereus* by isolate B- 7 at pH 8 and 9 (18 mm) as well as by isolate D-5 at pH 7 (18 mm). It was reported that the *Streptomyces* strain EFAI-1, isolated from Rajshahi of Bangladesh, demonstrated antibacterial activity against *B. cereus* (19 mm), *Listeria monocytogenes* (15 mm), *S. aureus* (13 mm), *E. coli* (14 mm), *Shigella sonnei* (18 mm) and *S. typhi* (21 mm) (Khatun et al., 2018). Again, the Nambul river isolates from Manipur of India were reported with moderate antagonism against bacteria and fungi (S. Ningthoujam & Sanasam, 2011). In another study, Singh L.S. reported the antibacterial activities of *Streptomyces tanashiensis* strain A2D against *B. subtilis* (15 mm), *S. aureus* (25 mm), *E. coli* (21 mm) and *K. pneumonia* (23 mm) (Singh, Mazumder, & Bora, 2009). The antibacterial activities of the studied isolates were thus found to be highly comparable to other *Streptomyces* strains isolated from nearby places and reported so far.

The yields of antimicrobial compounds at different pH were variable for different isolates as observed by the degree of antagonism against the test pathogens. Isolate A-9, A-12, B-5, C-1, E-6 and E-8 demonstrated maximum antagonistic activity against *S. typhimurium* at pH 9 whereas isolate B-7 scored maximum at pH 7 and 9. Interestingly, isolate D-5 was most active at pH 5 and loss of activity was observed at pH more than 6. Antagonism of both isolate E-6 and E-8 was nullified at pH 5 and 8 but retained activities at pH 6 and 7. Antimicrobial activity of *Streptomyces* isolates against *E. coli* fluctuated slightly along with the pH. Isolates A-9 and A-12 exhibited highest activity at pH 8. Isolate B-5 was most active at pH 6. Isolates B-7 and C-1 inhibited *E. coli* maximum at neutral pH i.e. at pH 7 and D-5 showed maximum activities at pH 6. Isolate E-6 and E-8 were most active at pH 5. Again, the activities of isolates C-1 and D-5 were nullified at pH 9. Against *S. aureus*, *Streptomyces* isolates A-9, A-12, B-5, B-7 and C-1 exerted their maximum activities at pH 5 whereas D-5 did maximum at pH 6. Isolate E-6 and E-8 exhibited maximum antagonism at pH 8 and pH 7 respectively and they were also inactive at pH 5. At pH 9, antimicrobial compounds of all of the isolates were inactive against *S. aureus*. On the other hand, *Streptomyces* isolates exerted highest antagonism against *B. cereus* at neutral to slightly alkaline pH. Isolate A-9 was most active at pH 7, A-12 at pH 6, B-5, B-7, C-1 and E-6 at pH 9 and E-8 was most active at pH 6. Antimicrobial compounds of all of the isolates were inactive against *B. cereus* at pH 5. Moreover, isolate A-9 was inactive at pH 6 and E-6 as well as E-8 was inactive at pH 7.

Since variation in antagonism against the test pathogens at different pH level was observed, it was analyzed statistically to reveal whether the isolates or the pH levels somehow influence the intensity of antimicrobial activity or not (Supp. Table 2-8). The variation in antagonism due to the variation of *Streptomyces* isolates was deduced as non-significant against all four test pathogens. But it was found to be significant due to the different pH levels against the test pathogens except *S. typhimurium* (Table 2). Although the antagonism against *S. typhimurium* was observed to be maximum for the antimicrobials produced at pH 9 (Fig. 2), it was not significant at α=0.05 (Table 2). The maximum production of organism specific antimicrobial compounds, as indicated by higher zone of inhibition, occurred at pH 9 and the production was slightly higher than of other pH. Against *E. coli,* the antimicrobial activity of the *Streptomyces* isolates decreased significantly from pH 7 to pH 9 which indicates that the production of *E. coli* specific antimicrobial compounds were maximum at neutral pH and reduced gradually at alkaline pH. Significant influence of pH levels in production of antimicrobial compounds specific against *S. aureus* and *B. cereus* was also observed. Although the antimicrobials produced at pH 5 and pH 9 were effective against other test pathogens, *B. cereus* and *S. aureus* specific antimicrobials might not be produced at pH 5 and 9 respectively. The variation in antagonism caused by the antimicrobials produced at pH levels other than 5 and 9, even though observed, were not significant against *B. cereus* and *S. aureus* respectively. It was thus very meticulously deduced that the pH as a growth condition significantly influenced the production of pathogen specific antimicrobial compounds.

Conclusion

The ability of the *Streptomyces* isolates in tolerating wide range of growth conditions as revealed in this study would be special advantage in developing sustainable bioprocess for large scale production of antimicrobial compounds with them. At the same time, the influence of pH in pathogen specific antimicrobial production could enhance the chances of obtaining diverse antimicrobials and in pursuance of this, the *Streptomyces* isolates should be tested against more pathogens to reveal the spectrum of their antagonism as well. Further research in characterizing the antimicrobial compounds should be carried out involving purification, structure analysis of the active compounds and optimization of upstream as well as downstream processes to be able to develop them as potential antibiotics.

Author contributions

MA and AB conducted the research work, MA, AB and MEU prepared the Manuscript. NJA performed the statistical analyses. MAS did data analysis and meticulous revision. SD supervised the research and approved the final manuscript.

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Competing financial interests

Authors declared that there was no competing interest.

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