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STUDIES ON SECONDARY METABOLITES OF *STREPTOMYCES SPECIES* CONTAMINATED BACTERIA

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ABSTRACT

Three marine soil strains of *actinomyces* were contaminated by *Bacillus* species designated as AIAS-5, AIAS-10 and AIAS-39 with spontaneous mutation during repeated transfer on slants media (storage at 4°C). Co-culture designated as AIAS-510, obtained from AIAS-5 & AIAS-10 and co-culture of AIAS-10 and AIAS-39 designated as AIAS-1039, results in the production of a new compound which were not found in their control culture. Antibacterial activities of AIAS-5, AIAS-10 and co-culture AIAS-510 were performed against a series of pathogenic bacteria. The results showed that co-culture AIAS-510 was highly active against a series of gram-positive and gram-negative bacteria compare to monoculture AIAS-5 and AIAS-10 alone. Crude extract of co-culture AIAS-1039 obtained from culture of AIAS-10 & AIAS-39 and crude extracts of AIAS-10 & AIAS-39 monoculture was also investigated for their biological activities. Antibacterial activities of AIAS-10, AIAS-39, and AIAS-1039 were performed against a series of pathogenic bacteria. The results showed that monoculture AIAS-10 was highly active against a series of gram-positive and gram-negative bacteria compare to monoculture AIAS-39 and co-culture AIAS-1039. Although antibiotic production was not induced in co-culture but antifungal activity was induced significantly. Co-culture AIAS-510 was more active against pathogenic fungi than monoculture AIAS-5 and AIAS-10. The Minimum Inhibitory Concentrations (MIC) of co-culture AIAS-510 was determined using broth-dilution method and found to be in the range of 16-32 µg/ml against a wide range of pathogenic bacteria.

KEYWORDS: *Actinomyces* strain; co-culture; inhibitory; antifungal & antibacterial.

INTRODUCTION

Microbial secondary metabolites have been considered one of the best resources for drug discovery (Pettit, 2011). Up to early 2013, more than 42, 000 natural compounds have been characterized from microorganisms and higher fungi. Given the fact that microorganisms naturally interact with each other, simulating microbial competition for nutrition and space is regarded as a major route for the induction of bioactive secondary metabolites (Oh, 2005). A previous study of a mixed cultivation of the marine-derived fungus *Pestalotia* sp. with a marine *R-proteobacterium*, closely related to *Thalassospira lucentensis*, yielded a new antimicrobial metabolite, pestalone (Cueto., 2001). More recently, a new antibiotic-antitumor metabolite, glionitrin-A was isolated during the co-culture of a mine drainage-derived *Aspergillus fumigatus* and a mine drainage-derived Sphingomonas isolate strain KMK-001 (Park, 2009), while co-culturing *A. fumigatus* with *Streptomyces peucetius* led to the production of the new formyl xanthocillin analogue fumiformamide (Zuck,2012). Bacteria may also produce antimicrobial compounds when they sense the presence of competing organisms. Co-culture of marine epibionts with human pathogens induced antibiotic production (Burgess., 1998). Co-culture of surface associated marine bacteria with live and heat-killed *Staphylococcus aureus* and live cultures of *Pseudomonas aeruginosa* and *E. coli* induced the antimicrobial activity (Mearns-Spragg, 1998). The co-cultivation-dependent formation of antibiotics by a strain of *Streptomyces* with different marine bacteria indicates the importance of co-culture fermentations for the discovery of new antibiotics (Slattery, 2001). Microbial competition is thought to be the selective force that promotes biosynthesis of bioactive natural products. Recently, the frequency of finding a new compound from *actinomycetes* is decreasing (Berdy J, 2005). Co-culture of *Streptomyces* with a bacterium *T. pulmonis* induced the production of antibiotics TPU-0043 (polyene macrolide) and alchivemycin which were not produced in a pure culture of *Streptomyces* (Igarashi Y, 2005). Co-culture of actinomycetes with *Bacillus subtilis* produced novel antibiotics Quinomycin A (Huang, 2009). Co-culture of marine bacteria with *Streptomyces tenjimariensis* induced istamycin A and B production (Slattery, 2001). Extracellular acidic polysaccharide production is induced by a two-membered bacterial co-culture (Kurata , 2003). In this current research work, we aim to investigate the effect of co-culture of two bioactive metabolite producing *actinomycetes* in a liquid fermentation medium maintaining standard culture conditions. We expect that due to competition of using culture media and cross-signaling between these bacteria will yield new secondary metabolites. Our results provide insights into the wide spectrum antimicrobial ability of the identified *Bacillus* species from the Bangladeshi coastal environment.

MATERIALS AND METHOD

Isolation of Microorganisms from soil sample that contaminate Streptomyces

Microorganisms exist in nature as mixed populations. The isolated Streptomyces/actinomycetes Species frequently contaminated by others bacteria during storage (Sarker, 2012, Haque, 2012,). Three strains were isolated and designated as AIAS-5, AIAS-10 and AIAS-39 which are respectively found in ANAM-5, AIAH-10 and ANAM-39. Morphological, biochemical and molecular characteristics of AIAS-5, AIAS-10 and AIAS-39 were studied by standard methods and these strain identified as a *Bacillus* sp.

Bioactive metabolite Production by Co-culturing of (AIAS-5, AIAS-10 and AIAS-39)

Transfer of organism from slant culture to yeast-extract glucose broth medium: A loop-full of the organisms of AIAS-5, AIAS-10 and AIAS-39 were transferred from preserved slant culture to a 100 ml flasks containing 60 ml yeast-extract glucose broth medium (Yeast extract 0.25g/100ml; Glucose 0.5g/100ml). The flasks were shaken in a rotary shaker at 220 rpm at 31^{o}C for 2 days.

Small-scale liquid fermentation of co-culture of AIAS-5 and AIAS-10

Inoculation with inoculums of AIAS-5 and AIAS-10: Inoculums of AIAS-5 and AIAS-10 were used as seed culture. **20 ml** 2 days inoculums of **AIAS-5** & **20 ml** 2 days inoculums of **AIAS-10** were mixed in a 500ml conical flask containing 200ml yeast-extract glucose broth medium. This seed culture was used to inoculate a number of 500 ml conical flasks containing 200 ml yeast extract glucose broth medium in the same process. **Control: 40** ml 2 days inoculums of **AIAS-5** were used to inoculate of 500 ml conical flasks containing 200 ml yeast extract glucose broth media. This process is followed for a number of 500 ml conical flasks containing 200 ml yeast extract glucose broth media. **40** ml 2 days inoculums of **AIAS-10** were used to inoculate of 500 ml conical flasks containing 200 ml yeast extract glucose broth media. This process is followed for a number of 500 ml conical flasks containing 200 ml yeast extract glucose broth media. The flasks were then shaken on a rotary shaker (220 rpm) at 31^{o}C for 7 days.

Small-scale liquid fermentation of co-culture of AIAS-10 and AIAS-39: (The procedure same as above).

Visual Observation: Microorganisms of co-culture of AIAS-5 & AIAS-10 and AIAS-10 & AIAS-39 were visually observed by means of magnifying glass and compare with monoculture of AIAS-5, AIAS-10 and AIAS--39.

Microscopic view: The morphology of microorganisms from co-culture of AIAS-5 & AIAS-10 and monoculture (control) of AIAS-5 & AIAS-10 microscopically were followed by Cover slip culture technique (Korzybsky, 1967). Similarly the morphology of microorganisms from co-culture of AIAS-10 & AIAS-39 and monoculture of AIAS-10 & AIAS-39 were observed.

Recovery of the product from the co-culture (AIAS-5& AIAS-10) medium

300 mg crude co-culture extract was obtained from 12 liter fermentation broth of **co-culture** (AIAS-5 & AIAS-10), **70 mg** from 5 liter fermentation broth of AIAS-5 **(monoculture)** and **55mg** from 3 liter fermentation broth of AIAS-10 **(monoculture).**

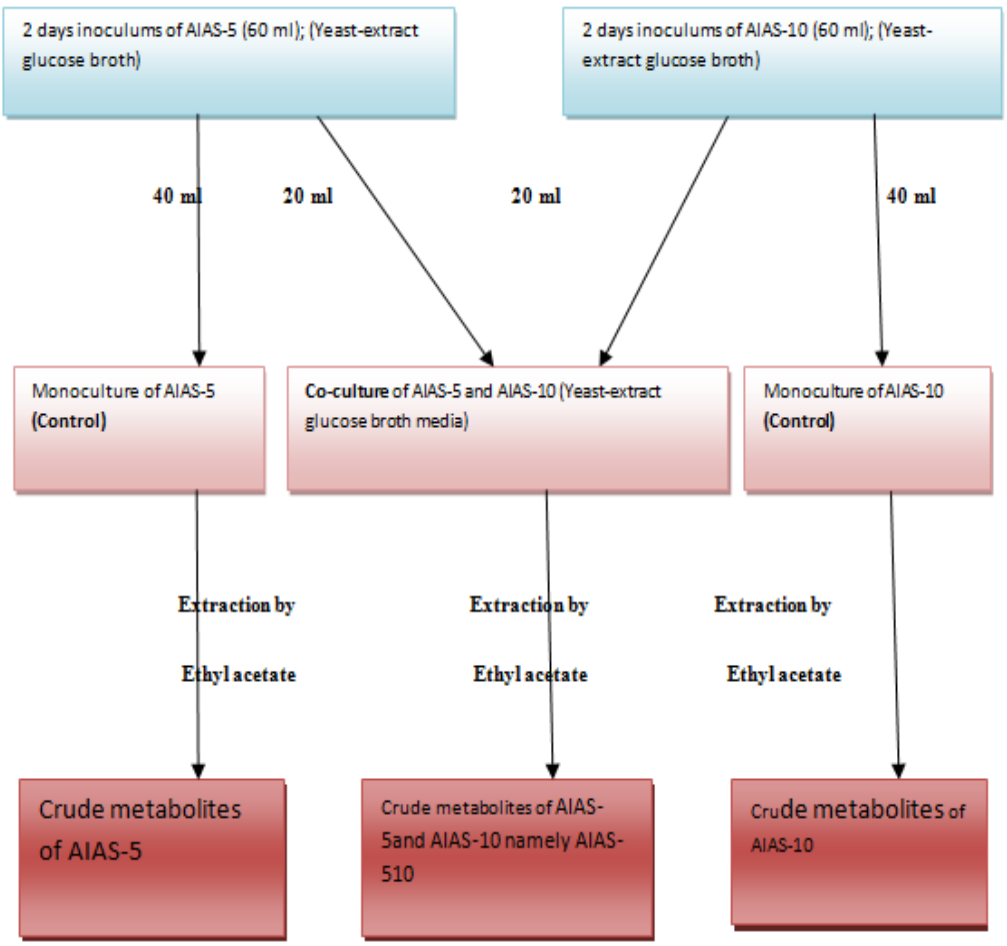


Figure: 1 Schematic representation of small-scale liquid fermentation of co-culture of AIAS-5 and AIAS-10.

Recovery of the product from the co-culture (AIAS-10 & AIAS-39) medium: 100 mg crude co-culture extract was obtained from 4 liter fermentation broth of **monoculture** (AIAS-10 & AIAS-39), **55 mg** from 3 liter fermentation broth of AIAS-10 **(monoculture)** and **40 mg** from 3 liter fermentation broth of AIAS-39 **(monoculture).**

Antibacterial activity of AIAS-510, AIAS-5 and AIAS-10: The disc diffusion method for susceptibility testing recommended by the FDR is a slight modification of the procedure developed by Bauer and his co worker in 1986 (Bauer, 1986). This is a highly standardized technique; the amount of antimicrobial agent contained in the disc is specified as well as the test medium, size of inoculums, conditions of the incubation and other details.

Test materials used for the study

Crude co-culture extract obtained from monoculture of AIAS-5 and AIAS-10 namely AIAS-510, crude extract of AIAS-5 and AIAS-10 were taken for investigation of antimicrobial activity. Solvent (methanol was used due to non-toxic) for dissolving the compounds. Solvent itself was used in blank disc to determine the effect. Kanamycin (K-5µg/disc) as standard.

Test organisms used for the study

The bacterial strains used in the sensitivity test are given bellow and pure cultures of the strains were collected from ICDDRB.

Gram positive: *Bacillus cereus***;** *Streptococcus agalactiae***;** *Staphyllococcus aureus.*
Gram negative: *Pseudomonas aeruginosa*; *Escherichia coli*; *Shigella dysenteriae.*

Preparation of discs containing test samples

Crude extracts of co-culture namely AIAS-510; crude extracts of monoculture AIAS-5 and AIAS-10 were dissolved in methanol in such a way to get 50µg/10µl. Filter paper discs were taken in a Petridis and sterilized by dry heat 10µl of the test solution was applied on a disc with the help of a micropipette. Thus each disc was contained 50µg of test compound. The discs were dried by air.

Standard disc

Kanamycin K-5 (5µg/disc) was used as standard disc for comparison. The test sample impregnated discs and standard Kanamycin disc were plated gently on solidified agar plates seeded with the organisms to ensure contact with the media, by means of a sterile forceps. The plates were then kept in a refrigerator at 4^{o}C for 12 hours thus the antimicrobial

compound(s) absorbed into discs get sufficient time to diffuse into the media. The plates were incubated at 37^{o}C for 24 hours.

Measurement of the zone of inhibition

After 12 hours incubation, the antibacterial activity of the compounds was determined by measuring the zone of inhibition in terms of mm by a transparent scale. Inhibitory zones obtained by the samples were compared to that of the standard disc.

Antibacterial activity of co-culture of AIAS-1039, crude extracts of monoculture of AIAS-10 and AIAS-39

Similarly we checked the antibacterial activity of AIAS-10, AIAS-39 and AIAS-1039 according to the procedure described above.

Thin Later Chromatography of the crude ethyl acetate extract from co-culture AIAS-510 and AIAS-1039

The dried crude extracts obtained from co-culture (AIAS-510) and monoculture (control) of AIAS-5 & AIAS-10 was dissolved in ethyl acetate in such a ratio that made 1% (w/v) solution. A fine small spot of the solutions were applied 4 mm above the lower edge on the activated TLC plate (coated with silica gel PF_{254}). The chamber was closed with the lid and kept in an undisturbed position. When the solvent front reached the target mark, the plate was taken out and dried with a hot air blower (Egon and Sthal 1969). Similarly TLC of co-culture (AIAS-1039) and monoculture of AIAS-10 & AIAS-39 were done by above method. Solvent systems applied are [Ethyl acetate: n-hexane (2:1); n-hexane: ethyl acetate (0:4) and Chloroform: methanol (9:1)].

Antifungal activity of the extracts AIAS-5, AIAS-10 and AIAS-510

The antifungal activity of the crude co-culture extract AIAS-510 obtained from monoculture of AIAS-5 and AIAS-10 and crude extracts of monoculture of AIAS-5 and AIAS-10 was investigated using disc sensitivity tests. The antifungal activity of AIAS-5, AIAS-10 and AIAS-510 was performed at a concentration of 50µg/disc against three pathogenic fungi comparing with standard ketoconazole 50µg/disc. The results of the antifungal activity are given in the Table 1. The result showed that the extract AIAS-510 obtained from co-culture of AIAS-5 & AIAS-10 is low active against various pathogenic fungi. The range of zone of inhibition is 10-11 mm. The antifungal activity of AIAS-510 (50µg/disc) is less than the standard antifungal drug ketoconazole at 50µg/disc.

Table 1: Antifungal activity of AIAS-5, AIAS-10 and AIAS-510 against *Candida albicans, Saccharomyces cerevaceae and Aspergillus niger*

Name of the fungi used	Zone of inhibition (in mm)			
	AIAS-5 (50µg/disc) Monoculture	AIAS-10 (50µg/disc) Monoculture	AIAS-510 (50µg/disc) Co-culture	Ketoconazole (50µg/disc)
Candida albicans	8	18	**11**	18
Saccharomyces cerevaceae	9	12	**10**	15
Aspergillus niger	9	13	**11**	16

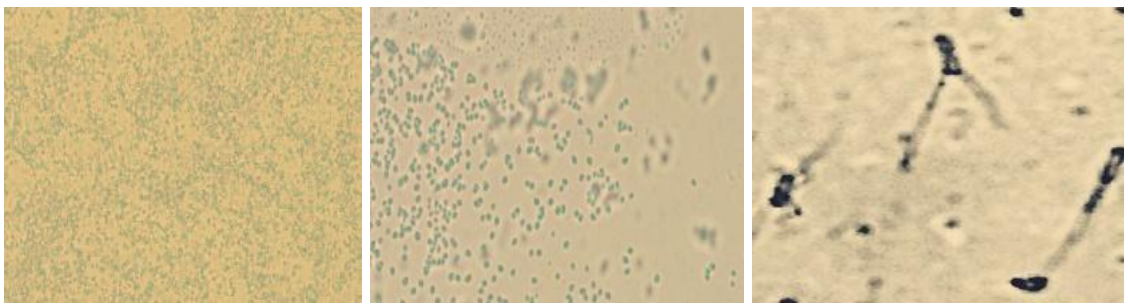
RESULTS AND DISCUSSION

Results

Observation of morphology

Co-culture of bacillus, AIAS-5 and AIAS-10 in yeast-extract glucose media the growth pattern of these strains was observed and compared with control. We observed that in the fermentation vessel both of the organisms appeared as aggregated round colonies 4 mm and 2 mm in diameter for AIAS-5 and AIAS-10 respectively. Fine needle-like outgrowth was also observed on the surface of the agglomerated colonies. But they grow separately and no attachment was found in between them when grown together. The media looked transparent and the colonies remained suspended. But, when they were grown separately, the pattern of growth was found to be somewhat different. The approximate size of the round-shaped agglomerates of AIAS-5 grown separately was about 5 mm, but a reduction of their size was observed in co-culture. Moreover, the needle-like out growth was not observed in monoculture system. Same result was observed in case of AIAS-10. The reason for this difference has not been studied, but we assume that the competition of growth and using nutrient is the reason for this.

Microscopic views of monoculture and co-culture of AIAS-5 and AIAS-10 (Figure 3).

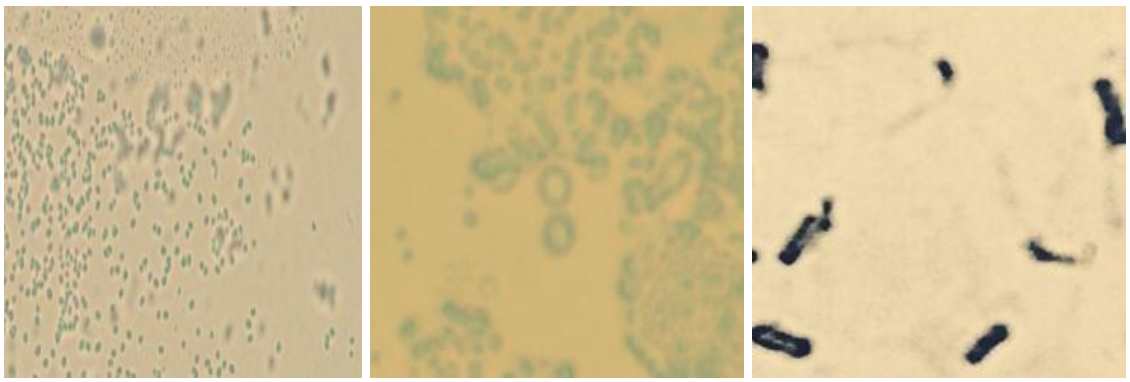


(i) (ii) (iii)

Figure: 3 Microscopic views of the monoculture of AIAS-5 (i), AIAS-10 (ii), microscopic view of AIAS-5 when co-cultured with AIAS-10 (iii); at magnification × 200.

Similarly, we cultured two species of bacillus, AIAS-10 and AIAS-39 in a conical flask in yeast-extract glucose media. During co-culture the growth pattern of these strains was observed and compared with control. Interestingly we observed that in the fermentation vessel both of the organisms appeared as aggregated round colonies 2mm and 3 mm in diameter for AIAS-10 and AIAS-39 respectively. Fine needle-like outgrowth was also observed on the surface of the agglomerated colonies. The media looked transparent and the colonies remained suspended. But, when they were grown separately maintaining the same culture conditions, the pattern of growth was found to be somewhat different. The approximate size of the needle-like outgrowth of AIAS-10 grown separately was about 2 mm, but a reduction of their size was observed in co-culture. We assume that the competition of growth and using nutrient is the reason for this.

Microscopic views of monoculture and co-culture of AIAS-10 and AIAS-39 (Figure-4).



(i) (ii) (iii)

Figure: 4 Microscopic views of the monoculture of AIAS-10 (i) AIAS-39 (ii) microscopic view of AIAS-10 when co-cultured with AIAS-39 (iii); at magnification × 200.

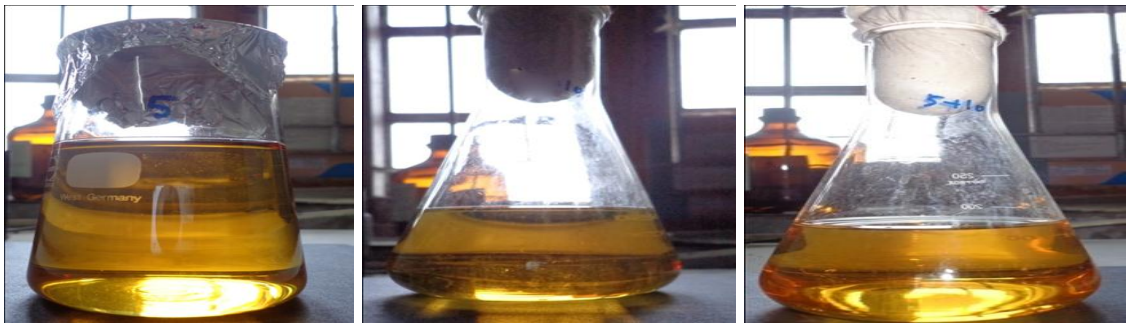
Small-scale liquid fermentation of monoculture and co-culture of AIAS-5, AIAS-10 and extraction of their secondary metabolites

About 300 mg crude extract was obtained with co-culture of AIAS-5 & AIAS-10 from 12 liter fermentation medium, 70 mg crude extract was obtained with AIAS from 5 liter fermentation medium (control) and 55 mg crude extract was obtained with AIAS-10 from 3 liter fermentation medium (control). The finding results are shown in Table-2.

Table: 2 Physical properties of the crude extracts of monoculture and co-culture of AIAS-5 and AIAS-10.

Physical characteristics	AIAS-5 (monoculture)	AIAS-10 (monoculture)	Co-culture of AIAS-5 & AIAS-10
Color	Reddish orange	Yellowish brown	Yellowish orange
Solubility	Soluble in ethyl acetate, DMSO, methanol and chloroform but insoluble in n-hexane.	Soluble in ethyl acetate, DMSO, methanol and chloroform but insoluble in n-hexane.	Soluble in ethyl acetate, DMSO, methanol and chloroform but insoluble in n-hexane.

Crude ethyl acetate extracts (before evaporation) have been shown in the Figure-5.



(A) (B) (C)

Figure: 5: Crude ethyl acetate extract (before evaporation) obtained from monoculture (control) and co-culture of AIAS-5 & AIAS-10; (A) Crude extract of AIAS-5 (control), (B) Crude extract of AIAS-10 (control), (C) Crude extract obtained from co-culture of AIAS-5 and AIAS-10.

Antibacterial activity of the crude extracts of AIAS-5, AIAS-10 and co-culture AIAS-510 using disc sensitivity test

The antibacterial activity of the crude extract obtained from co-culture of AIAS-5 and AIAS-10 (AIAS-510) and crude extracts of monoculture of AIAS-5 and AIAS-10 was investigated using disc sensitivity tests. The results of antibacterial activity are given in the Table-3.

Table: 3 Zone of inhibition of AIAS-5, AIAS-10 and AIAS-510 against a series of test bacteria.

Test organisms	Zone of inhibition (in mm)			
	AIAS-5 (50µg/disc) Monoculture	AIAS-10 (50µg/disc) Monoculture	AIAS-510 (50µg/disc) Co-culture	Kanamycin (5µg/disc)
Gram positive bacteria				
1.*Staphylococcus aureus*	9	11	20	28
2. *Bacillus cereus*	12	15	22	31
3. *Steptococcusagalactiae*	13	9	23	27

Gram negative bacteria:				
4. *Escherichia coli*	15	10	23	29
5. Shigelladysenteriae	10	12	23	33
6. *Pseudomonas aeruginosa*	9	11	19	30

Figure: 6 Antibacterial activity of AIAS 5, AIAS-10 and AIAS-510

The result showed that the extract AIAS-510 obtained from co-culture of AIAS-5 & AIAS-10 is strongly active against both Gram-negative and Gram-positive bacteria (Table-3). The zone of inhibition of this extract is quite comparable with kanamycin. The antibacterial activity of the crude co-culture obtained from mono-culture of AIAS-5 and AIAS-10 is more than that of crude extracts of individual monoculture. Zone of inhibition of AIAS-510 was 20 mm but AIAS-5 was 9 mm and AIAS-10 was 11 mm against *Staphylococcus aureus*,; against *E. coli*, zone of inhibition of AIAS-510 was 23 mm but AIAS-5 was 15 mm and AIAS-10 was 10 mm (Table-3). Such induction of antibacterial activity may be due to the following reasons. i) The compounds which are responsible for activity may be formed new compound with more activity when they are experiencing microbial competition in case of co-culture. ii) Production of active compounds may interfere with another compound resulting in better activity. iii) A new compound was formed in co-culture (figure: 5), this new compound may induced the antibacterial activity or may be due to the increased secretion of any active compound of monoculture.

MIC of the Crude Extracts

Table: 4. Minimum inhibitory concentrations (MIC) of extract AIAS-510 against pathogenic bacteria

Test tube no.	**Nutrient broth added (ml)**	**Concentration of AIAS-510 (µg/ml)**	**Inoculums added (µL)**	**Growth observation against**			
				Bacillus cereus	***Streptococcus agalactiae***	***Pseudomonas aeruginosa***	***Eshcherichia coli***
1	1ml	512	60	-	-	-	-
2	1ml	256	60	-	-	-	-
3	1ml	128	60	-	-	-	-
4	1ml	64	60	-	-	-	-

5	1ml	32	60	-	-	-	-
6	1ml	16	60	-	-	+	+
7	1ml	8	60	+	+	+	+
8	1ml	4	60	+	+	+	+
9	1ml	2	60	+	+	+	+
10	1ml	1	60	+	+	+	+
11	1ml	0.5	60	+	+	+	+
12	1ml	0.25	60	+	+	+	+
13	1ml	0.125	60	+	+	+	+
C_M	1ml	0	0	-	-	-	-
C_S	1ml	512	0	-	-	-	-
C_I	1ml	0	60	+	+	+	+
Results of MIC values (in µg/ml)				**16**	**16**	**32**	**32**

Legend, '+'= Growth and '-'= No growth

The results of MIC determination against bacteria are given in the Table 4. The result is very promising. The MIC value of AIAS-510 varies in between 16-32µg/ml. The lowest MIC value 16µg/ml was found against *Eshcherichia coli*. Whereas MIC value for *Bacillus cereus*, *Pseudomonas aeruginosa* and *Strepto coccusagalactiae* was found to be 32µg/ml.

Antifungal activity of the extracts AIAS-5, AIAS-10 and AIAS-510

The antifungal activity of AIAS-5, AIAS-10 and AIAS-510 was performed at a concentration of 50µg/disc against three pathogenic fungi comparing with standard nystatin 30µg/disc. The results of the antifungal activity are given in the Table-5 & Fig-7. The result showed that the extract AIAS-510 obtained from co-culture of AIAS-5 & AIAS-10 is strongly active against various pathogenic fungi.

Figure: 7 Antifungal Activity of the crude extracts AIAS-5, AIAS-10 and AIAS-510

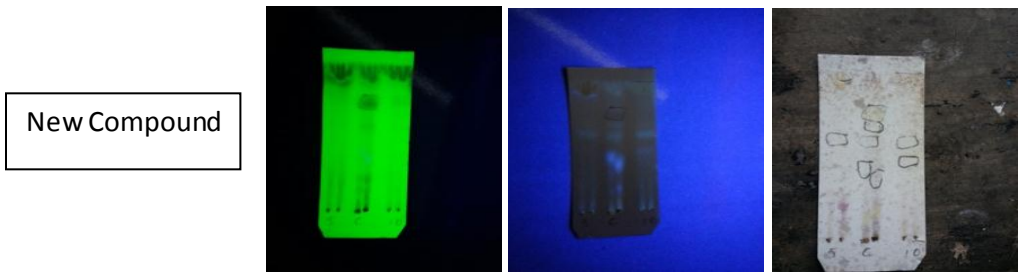
Table: 5 Antifungal activity of the crude extracts AIAS-5, AIAS-10 and AIAS-510

Test organisms	Diameter of the zone of inhibition (mm)			
	AIAS-5 (50µg/disc)	AIAS-10 (50µg/disc)	AIAS-510 (50µg/disc)	Nystatin (30µg/disc)
Candidaalbicans	16	16	19	23
Saccharomyces cerevaceae	12	13	17	22
Aspergillusniger	16	15	20	24

The result showed that the extracts AIAS-5 (ZOI: 12-16 mm) and AIAS-10 (ZOI: 13-16 mm) are moderately against various pathogenic fungi. From the above result, we observed that the antifungal activity of crude extract obtained from co-culture of AIAS-5 and AIAS-10 (AIAS-510:ZOI-15mm) is greater than the crude extracts obtained from monoculture of AIAS-5 and AIAS-10. The result indicates that co-culture of AIAS-5 and AIAS-10 induces antifungal activity against various pathogenic fungi (Table-5).

Thin layer chromatography (TLC) analysis of crude ethyl acetate extract obtained from co-culture of AIAS-5 & AIAS-10

As shown in the Figure 8, a new spot was observed for the crude extract obtained from culture. Interestingly this spot could be detected in case of the co-culture extract of AIAS-5 and AIAS-10. We also tried to separate the compounds using different solvent system but the 1: 2 combinations of n-hexane and ethyl acetate results in very good resolution**.**



A **B** **C**

Figure: 8 TLC analysis of crude ethyl acetate extract obtained from co-culture and monoculture of AIAS-5 & AIAS-10; (A) Short wavelength of UV, (B) Long wavelength of UV, , (C) After treatment of Vanillin-sulfuric acid reagent (Solvent, n-Hexane: Ethyl acetate= 1:2).

This finding is very interesting as the co-culture of different strains of *Bacillus* results in the production of a new compound (Carson, 2013). Production of new compound by co-culturing of different species of microorganism has been reported recently (Rateb, 2013). The result of our study is also similar to them, but the difference is that in this we have used two bioactive secondary metabolite producing *Bacillus*, which has not been reported before.

Small-scale liquid fermentation of monoculture and co-culture of AIAS-10 and AIAS-39 and extraction of their secondary metabolites

About 100 mg crude extract was obtained with co-culture of AIAS-10 & AIAS-39 from 4 liter fermentation medium, 55 mg crude extract was obtained with AIAS-10 from 3 liter

fermentation medium (control) and 40 mg crude extract was obtained with AIAS-39 from 3 liter fermentation medium (control) [Data not shown].

Table: 6 Physical properties of the crude extracts of monoculture of AIAS-10 & AIAS-39 and co-culture of AIAS-10 and AIAS-39 (AIAS-1039).

Physical characteristics	AIAS-10 (monoculture)	AIAS-39 (monoculture)	Co-culture of AIAS-10& AIAS-39 AIAS-1039
Color	Yellowish brown	Deep brown	Reddish brown
Solubility	Soluble in ethyl acetate, DMSO, methanol and chloroform but insoluble in n-hexane.	Soluble in ethyl acetate, DMSO, methanol and chloroform but insoluble in n-hexane.	Soluble in ethyl acetate, DMSO, methanol and chloroform but insoluble in n-hexane.

Crude ethyl acetate extracts (before evaporation) have been shown in the Figure -9.

(A) (B) (C)

Figure: 9 Crude ethyl acetate extract (before evaporation) obtained from (A) Crude extract of AIAS-10 (control), (B) Crude extract of AIAS-39 (control), (C) Crude extract obtained from co-culture of AIAS-10 and AIAS-39.

Antibacterial activity of the extracts AIAS-10, AIAS-39, AIAS-1039 using disc sensitivity test

The antibacterial activity of the crude extract obtained from co-culture of AIAS-10 and AIAS-39 namely AIAS-1039 and crude extracts of monoculture of AIAS-10 and AIAS-39 was investigated using disc sensitivity tests. The results of antibacterial activity are given in the Table-7.

Table: 7 Zone of inhibition of AIAS-10, AIAS-39, AIAS-1039 against a series of test bacteria.

Test organisms	Zone of inhibition (in mm)			
	AIAS-10 (50µg/disc) Monoculture	AIAS-39 (50µg/disc) Monoculture	AIAS-1039 (50µg/disc)	Kanamycin (5µg/disc)
Gram positive bacteria:				
1.*Staphylococcus aureus*	10	10	05	28

2. *Bacillus cereus*	17	12	09	31
3. *Steptococcusagalactiae*	12	10	10	33
Gram negative bacteria:				
4. *Escherichia coli*	11	09	08	35
5. Shigelladysenteriae	13	11	09	33
6. *Pseudomonas aeruginosa*	10	09	05	30

The result showed that the co-culture extract AIAS-1039 obtained from monoculture of AIAS-10 & AIAS-39 is less active against both Gram-negative and Gram-positive bacteria. The extract is more active against gram negative bacteria than gram positive bacteria. The zone of inhibition of this extract (50µg/disc) was significantly lower than standard kanamycin at 5µg/disc against both gram positive and gram negative bacteria.

DISCUSSION

For the past 50 years antibiotics have revolutionized medicine by providing cures for formerly life-threatening diseases. However many strains of pathogenic bacteria have recently emerged To develop new antibiotics from natural sources, the scientists in the field of drug discovery are working hard. Co-culture of different bacteria may be an alternative source to produce new bioactive metabolites. In our study, co-culture of AIAS-5 & AIAS-10 (*Baillus* Spp.) was carried out in yeast-extract glucose broth media. After small scale liquid fermentation, the fermentation broth of co-culture of AIAS-5& AIAS-10 was partitioned using ethyl acetate extract. TLC analysis of crude ethyl acetate extract obtained from co-culture of AIAS-5 and AIAS-10 reveals that one new compound was formed in co-culture (Fig-8), which was not observed in monoculture (control). The fermentation broth of co-culture of***AIAS-5 and AIAS-10*** were partitioned using ethyl acetate extract. About 300 mg of crude extract was obtained from the 8L liquid fermentation medium. TLC analysis of crude ethyl acetate extract obtained from co-culture of AIAS-5 and AIAS-10 reveals that one new compound (showes antibacterial activity) was formed in co-culture (Fig-8), which was not observed in monoculture (control). The compound was obtained as reddish brown crystals. R_f value of the compound was 0.74 with the solvent system ethyl-acetate and n-Hexane (2:1). The antifungal activity of AIAS-5, AIAS-10 and AIAS-510 was performed at a concentration of 50µg/disc against three pathogenic fungi comparing with standard ketoconazole 50µg/disc. The results of the antifungal activity are given in the Table 5 & 8. The result showed that the co-culture AIAS-510 obtained from monoculture of AIAS-5 & AIAS-10 is low active against various pathogenic fungi. The range of zone of inhibition is 10-11 mm. The antifungal

activity of AIAS-510 (50µg/disc) is less than the standard antifungal drug ketoconazole at 50µg/disc.

Table: 8 Antifungal activity of AIAS-10 AIAS-39, and AIAS-1039 against Candida albicans, Saccharomyces cerevaceae and Aspergillus niger.

Name of the fungi used	Zone of inhibition (in mm)			
	AIAS-10(50µg/disc) Monoculture	AIAS-39(50µg/disc) Monoculture	AIAS-1039 (50µg/disc) Co-culture	Ketoconazole (50µg/disc)
Candida albicans	8	18	**11**	18
Saccharomyces cerevaceae	9	12	**10**	15
Aspergillus niger	9	13	**11**	16

From the above result, we observed that the antifungal activity of crude co-culture extract (AIAS-1039, zone of inhibition 10-11mm) obtained from monoculture of AIAS-10 and AIAS-39 is less than the crude extracts obtained from individual monoculture of AIAS-10 (zone of inhibition 8-9mm) andAIAS-39 (zone of inhibition 13-18mm). The result indicates that co-culture ofAIAS-10 and AIAS-39induces lower antifungal activity against various pathogenic fungi. On the other hand the crude co-extract obtained from monoculture AIAS-10 and AIAS-39 is less active than standard kanamycin, AIAS-10 and AIAS-39. The antibacterial activity of the crude extract obtained from co-culture of AIAS-10 and AIAS-39 is less active than that of crude extracts of monoculture. Zone of inhibition against Staphylococcus aureus, of AIAS-1039 was 05 mm but AIAS-10 and AIAS-39 was 10 mm ; against E. coli, zone of inhibition of AIAS-1039 was 08mm but AIAS-10 was11 mm and AIAS-39 was 09 mm (Table-7). The antibacterial activity of crude extract of co-culture is less active than both monocultures. This may be due to the antibacterial activity may be due to the following reasons. i) The compounds responsible for activity may be formed new compound with less activity when they are experiencing microbial competition in case of co-culture. ii) Production of active compounds may interfere with another compound resulting in less activity. iii) A new compound was formed in co-culture, this new compound may reduced the antibacterial activity or may be due to the decreased secretion of any active compound of monoculture. Due to low production of metabolites obtained from co-culture of AIAS-5 & AIAS-10 and AIAS-10 & AIAS-39, we cannot characterize the new compound formed in co-culture. To characterize the new compound formed in co-culture, production in large scale is still going on in our laboratory.

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