CIBTech Journal of Microbiology ISSN: 2319-3867 (Online)
An Online International Journal Available at http://www.cibtech.org/cjm.htm
2013 Vol. 2 (3) July-September, pp.8-17/Bharat Prasad
Research Article

INVESTIGATION OF ANTIMICROBIAL SECONDARY METABOLITES OF SOIL ACTINOMYCETES OF SARAN DISTRICT, BIHAR

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ABSTRACT
Fifty two different actinomycetes stains have been isolated from difference soil samples collected from Saran, Bihar. Well diffusion method has been used to detected secondary metabolites producing strains Results indicated that 35% of all isolated are active against at least one of the test organisms. Six isolates showed strongly actve against test organism. Formation of antimicrobial substances is affected by differents physiological and biochemical factors. The result of present investigation reveals that fomation of inhibition zone have been started after 14^{th} days and maximum after 55^{th} day in alkline condition at **37** ^{o}C.tenperature. It has been also observed that maximum inhibition zone are formed in starch and $(NH_4)H_2PO_4$ containing media.

Key Words: *Actinomycetes, Saran District, Secondary Metabolites, Test Organisms, Inhibition Zone*

INTRODUCTION
Soil microorganisms continue to provide pharmacologically important secondary metabolites which are unique and novel chemical compounds. Soil actinomycetes have been commercially exploited as they are known to produce important biologically active metabolites. Although extensively studied over the past four decades, actinomycetes continue to prove themselves as reliable source of novel bioactive compounds. Among the well-characterized pharmaceutically relevant microorganism, actinomycetes remain major source of novel and therapeutically relevant natural product (Jensen *et al.,* 2007). Searching for novel actinomycetes constitutes an essential component in natural product based drug discovery. Analytical methods continue to improve to allow the rapid elucidation of structures of these natural products valuable components of modern drug discovery actinomycete groups are mainly considered as antibiotic producer (Awa *et al.,* 2005). These antibiotics are used in curement of various diseases and show a wide range of chemical structure (Ouhdouch *et al.,* 2001). Over 500 distinct antibiotic substances have been shown to be produced by actinomycete and a large number of them have been studied chemically. Actionmycetes sps produce some pharmaceutically important antibiotics like Tetracycline, Streptomycin, Macroeides, Ivermectin, Rifamycin and other clinically useful antibiotics. Earlier workers have reported some antifungal agents Nkkomycin and oloxins produce by actinomycetes which inhibit chitin synthesis (Nalan and Croses, 1998). However many compounds polyenes in particular, cannot be used because of their toxicity, in animal therapy, agriculture and industry (Olddauch *et al.,* 2001) Antimicrobial substances synthesis takes place in the older part of the substrate mycelium, whereas sporulation takes place in specialized aerial hyphe (Thompson *et al.,* 2002) The formation of aerial mycelium coincides with the phase in which secondary metabolites such as pigments and antibiotics are produced. The possibility of common regulatory elements for both morphological and physiological differentiation has been widely investigated (Demain, 1989; Kuster *et al.,* 1964). The isolation and characterization of gene involved in differentiation and the study of pleiotropic mutants effective in both sporulation and antibiotic production have strongly supported interdependence of the regulatory pathways of these two aspects of differentiation in *Streptomyces coelicolor.* There are evidences to assert that the secondary metabolites is initiated by nutrient limitation and reduction in growth rate (Demain, 1989). Secondary metabolites synthesized by long pathways which are often control and influenced by primary metabolites (Demain *et al.,* 1979). In most of the cases an intermediate metabolite produced during

primary metabolites serves as precursor for the biosynthesis of antibiotics. The composition of the culture medium, closely connected with the metabolite capacity of the antibiotic producing organism greatly influences

The biosynthesis of secondary metabolites changes the nature and concentration of carbon, nitrogen, phosphorus and trace elements have been reported to affect antibiotic biosynthesis in different organism (Demain, 1979). Early reports showed the actinomycetes species could utilize sugars, alcohols and some organic acids. On the basis of the utilization of different carbon sources, *Pridham and Gottieb* (1948) characterized different actinomycetes Grove *et al.,* (1955) studied the carbon utilization of *Streptomyces kanamyceticus* for kanamycin production in complex media and reported that glucose, maltose, dextrose, starch, lactose and sucrose are better carbon sources than glycerol. Other authors have also studied the effect of different sugar and nitrogen sources on antibiotic production (Bharat, 2012).

pH has profound effect on both primary and secondary metabolisms (Foster, 1949). Maximum and minimum yield of secondary metabolites and mycelial yield of organisms are affected with variation in pH. Optimal temperature and aeration rates also play an important role in secondary metabolites production (Pandey *et al.,* 2005).

The need of search for new and efficient antibiotic producing strains keeps rising due to the emergence of drug resistant pathogens (Wise, 2008). Many antifungal compounds have been identified, but safe and effective antifungal drugs have not yet been developed because of the high degree of similarity between fungi and mammalian cells (Berdy, 2005). As there is lack of effective and safe antifungal antibiotic, there is a need of nontoxic and effective antifungal antibiotics (Gupta *et al.,* 2002). The pioneering work of Waksman (1967) showed that the actinomycetes are capable of producing medically useful antibiotic (Nolan and Cross, 1998).

Antibiotic biosynthetic genes have been found on giant linear plasmid that could be transferred not only among actinomycetes strains but also between different species. Similarly many actinomycete passes resistance to multiple antibiotics to different strain. Antibiotic resistance may spread readily among Streptmyces strains by horizontal transfer of plasmids (Goodfellow *et al.,* 1988) Antibiotic producing Streptomyces can inhibit a broad range of soil borne microbes, including gram +Ve and Gram – ve bacteria, fungi and nematodes (Samac and Kinel, 2001; Saadoun *et al.,* 2003; Bharat, 2012).

As a result the potential for antibiotic producing streptomyces to control soil borne plant pathogen on diverse crop species have been widely investigated (Saadoun *et al.,* 2003) with the increasing missuse of antibiotics, a serious problem of antibiotic resistance is coming up very fast (Saadoun *et al.,* 2003) Intensive search for new antibiotic is going on world wide (Haque *et al.,* 1996). The great diversity amongst actinomycete has been observed and a new species are continually being discovered for their capabilities to produce unique array of secondary metabolites of commercial values. However these require thorough genetically, biochemical and toxicological investigations before the strain are explanted commercially. Therefore my present study has been carried to know the biochemical parameters of secondary metabolites production by selected strains of actinomycetes through stationery batch culture experiments.

MATERIALS AND METHODS

Isolation of microbes from the soil sample was carried out by soil dilution plate technique using starch casein agar medium (Kuster *et al.,* 1964). Purification of actinomycetes strain has been done by streak plate technique (Williams and Cross, 1971; MTCC, Chandigarh, 2002). Well diffusion method was used to detected secondary metabolites producing strains. Flasks containing 30ml in 250 ml flask of starch casein broth were inoculated separately with spores of selected strains. These were incubated at 37ºC in stationary condition. Secondary metabolites production potentiality was detected in 7^{th}, 14^{th}, 21^{st}, 28^{th}, 35^{th} and 60^{th} day's old culture filterates. The culture filtrates were extracted twice with ethyl acetate and the pooled solvent extracts were evaporated to dryness under vacuum to yield a crude residue. Similar

protocol was followed for all the 5 strains. The extracts were used for antimicrobial activity against the test organism. Photographs of isolated strains were taken with the help of Sony Digital camera.

Determination of the Nature of Secondary Metabolites

Biurete test:- 5 ml. of culture filtrate of the all selected strain were taken in tube separately 10 drops of copper sulphate solution (1%) was added to each tube and 5 ml of sodium hydroxide (40%) were further added to each tube. These were thoroughly mixed and change in colour was observed. The presence of violet colour complex show the presence of amino acid in secondary metabolites.

Iodine test:-Iodine solution (0.005N in 3% potassium iodine) has been prepared 5 drops of each filtrate of different strains have been taken in test tubes separately. These solution acidi fied by adding five drops of NHCl and 2 drops of iodineSolution Red Bricks has been observed.

Test of carbohydrate: Fehling's solution has been prepared just before use by mixing equal volume of copper sulphate (A) and alkaline sodium potassium tartars (B) 10 ml of the test solution mixed in equal volumes of Fehling's solutions A and B have been taken in tube separately.

1 ml of culture filtrate after growing each strain has been added to each of the test tube and boiled precipitates have been observed indicating the absence of carbohydrate in the test solution. The same experiment was done with Benedict's reagent (by boiling a mixture of 10 drops of culture filtrates and 5ml of Benedict's regent in a water bath.

TEST ORGANISMS: Following test organism has been purchesed from IMTECH Chandhigarh.

A. *Candida albicans* MTCC 183,B. *Microporum gypseum* MTCC 2819, C. *Aspergillus nidulans* MTCC 404, D. *Escherichia coli* MTCC 118, E. *Staphylococus aureus* MTCC 737

RESULTS AND DISCUSSION

Results

Result has been noted in table 1-8 and figure 1-3

Table 1: Antimicrobial activity of selected strains after 14th 35th and 60th day's incubation

Days	**Diameter of inhibition zone (in mm)**		
	14th	**35th**	**60th**
Test organisms	**A B C D E**	**A B C D E**	**A B C D E**
Strains			
BP7	0 0 0 0 0	5 7 13 10 8	9 10 14 15 10
BP16	0 0 0 0 0	8 6 17 19 15	12 10 12 15 10
BP19	0 0 0 0 0	5 10 10 17 10	9 1 12 9 2
BP22	0 0 0 0 0	5 4 10 3 4	8 8 12 5 6
BP30	0 0 2 0 0	3 6 12 4 6	5 10 16 10 12
BP49	1 2 0 0 0	8 7 9 6 1	8 12 12 10 1

Table 2: Production of antimicrobial secondary metabolites in starch glucose, dextrose Sucrose and Maltose carbon sources

	Zone of diameter inhibition (in mm)																			
Carbon Sources	**Starch**					**Glucose**					**Dextrose**					**Sucrose**					**Maltose**				
Test organism	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**
Isolated Strains																									
BP7	16	19	16	18	14	11	11	10	12	6	13	21	12	16	17	10	16	12	9	18	4	6	9	8	4
BP16	16	19	20	12	8	10	9	20	17	6	11	15	16	16	15	10	6	12	6	12	6	8	10	8	7
BP19	17	11	9	6	12	18	6	12	16	14	16	12	20	21	22	14	15	6	12	18	6	4	2	5	7
BP22	16	17	20	16	19	14	13	11	12	9	16	14	13	13	14	10	16	14	8	13	9	12	16	4	8
BP30	18	16	12	16	12	15	16	15	12	8	18	17	16	17	9	6	12	9	4	8	2	6	8	8	9
BP49	9	12	14	10	16	13	10	10	14	14	13	12	11	19	15	6	12	9	4	8	6	8	4	12	6

Table 2: Effect of dextrose concentration on production of the antimicrobial metabolites by selected strains BP7, BP16, BP19BP22 &BP30

Dextrose concentration (%)	**BP7**					**BP16**					**BP19**					**BP22**					**BP30**				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
0.5	14	16	13	12	10	8	15	11	10	7	14	9	19	17	18	13	10	18	17	17	15	16	13	14	7
1.0	11	5	14	14	15	9	19	12	15	9	15	10	19	17	18	15	12	19	16	18	15	17	15	6	8
1.5	11	10	16	16	16	10	19	15	18	9	10	9	20	17	6	15	12	19	16	19	16	17	15	16	9
2.0	15	13	15	16	17	11	20	12	12	10	16	12	20	21	22	16	14	19	18	20	18	17	16	17	9
2.5	10	12	15	11	17	10	12	13	15	9	12	10	17	19	21	12	12	17	18	16	17	15	14	17	7

Table 3a: Secondary metabolites production in Yest extract,$NaNo_3$ $(NH_4)H_2PO_4$ and KNO_3 nitrogen sources

	Zone of diameter inhibition (in mm)			
Nitrogen	**Yeast extract**	**$NaNO_3$**	**$(NH_4)\ H_2PO_4$**	**KNO_3**
Test organisms	A B C D E	A B C D E	A B C D E	A B C D E
Strains				
BP7	19 20 16 12 6	20 20 18 12 4	22 20 17 18 16	16 19 18 12 10
BP16	11 5 14 14 15	9 19 12 15 9	16 2 15 12 15	3 10 15 14 20
BP19	16 18 14 6 9	12 16 14 16 6	18 18 20 19 8	16 10 15 16 6
BP22	16 8 19 20 4	14 14 18 20 19	16 14 20 21 23	14 16 20 19 22
BP30	18 16 16 8 4	18 16 11 11 9	19 19 17 17 10	16 14 13 12 7
BP49	13 16 6 14 9	12 10 8 16 8	12 13 15 21 10	16 14 20 21 23

Table 3b: Effect of (NH_4) H_2PO_4 Concentration of production of antimicrobial metabolites by selected strains BP7,BP16,BP19,BP22, BP30 & BP49

(NH_4) H_2PO_4 concentration(%)	**BP7**	**BP16**	**BP19**	**BP22**	**BP30**	**BP49**
Test organisms	A B C D E	A B C D E	A B C D E	A B C D E	A B C D E	A B C D E
0.5	19 18 14 16 15	9 19 20 22 16	17 16 18 17 6	14 12 18 16 20	18 16 15 15 9	10 10 18 14 13
0.7	22 20 17 18 16	10 10 12 1118	18 18 20 19 8	16 14 20 21 23	19 19 17 17 10	12 12 10 20 16
1.0	20 18 16 17 15	8 18 19 20 15	17 17 17 14 7	15 10 17 20 19	17 17 14 16 9	9 10 16 15 15
1.5	18 15 14 16 14	6 12 18 19 14	12 15 17 13 4	13 9 14 17 18	15 14 13 12 6	8 10 16 12 14

Table 4: Production of secondary metabolites by selected strain on different pH 5 ,6 ,7,8,9&10

pH	**5**					**6**					**7**					**8**					**9**					**10**				
Test Organism	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**
strains																														
BP7	0	0	0	5	2	11	5	14	14	15	9	19	12	15	9	12 19	20	20	20	10 9	15	15	3	9 6	14	10	2			
BP16	6	5	9	2	0	10 16	12	16	18		20 16	20	22	18		16 18	16	12	15	15 7	15	13	8	10 8	12	13	7			
BP19	6	8	7	2	1	9	12	10	16	9	8 13	13	15	2		9 10	13	14	20	9 9	12	13	14	9 16	16	6	12			
BP22	8	12	0	8	9	16 14	18	16	15		15 14	16	16	15		16 15	11	11	12	16 13	10	10	11	6 4	12	9	6			
BP30	5	4	5	4	6	16	12	10	9	6	18 16	10	20	12		14 8	16	10	9	13 9	15	10	9	12 8	9	12	4			
BP49	0	0	5	7	6	22 15	8	9	12		16 14	10	21	12		16 13	9	10	11	13 9	12	10	4	12 9	10	9	8			

Table 5: Difference of pH of culture filtrate in starch casein broth

Time in	**Strains**					
Hours	**BP7**	**BP16**	**BP19**	**BP22**	**BP30**	**BP49**
0	7	7	7	7	7	7
24	6.8	6.5	6.6	7.0	6.7	7.1
48	6.7	6.6	6.8	6.9	6.6	7.1
12	7.1	6.7	6.8	6.9	7.2	7.1
96	7.5	71	7	7.1	7.2	7.1
120	72	7.5	7.5	7.2	7.2	7.1
144	7.3	7.2	7.6	7.2	7.3	7.1
168	7.2	7.3	7.6	7.2	73	7.1
192	7.2	7.3	7.6	7.2	73	7.1

Table 6: Production of antimicrobial secondary metabolites in dextrose casein broth at different temperature and pH7

Temperature	**Zone of diameter inhibition (in mm)**				
	25ºC	**30ºC**	**37ºC**	**42ºC**	**50ºC**
Test organism	**A B C D E**	**A B C D E**	**A B C D E**	**A B C D E**	**A B C D E**
Strain					
BP7	2 5 7 9 6	18 16 6 12 9	16 18 14 15 19	12 14 13 9	0 0 0 0 0
BP16	5 7 3 2 11	12 20 15 16 4	22 23 14 16 8	8 7 6 5 4	0 0 0 0 0
BP19	6 8 7 2 0	3 12 6 12 6	9 18 6 13 10	4 10 12 6 9	0 0 0 0 0
BP22	3 6 0 0 0	19 9 18 6 4	16 4 18 12 6	12 11 2 8 7	0 0 0 0 0
BP30	5 7 8 0 1	8 8 9 2 7	12 11 2 8 7	16 10 6 4 12	0 0 0 0 0
BP49	3 5 7 001	16 18 16 4 6	16 18 19 10 15	12 11 2 8 7	0 0 0 0 0

Table 7: Production of secondary metabolites by selected strain in synthetic media (pH 7and temp37 ºC

Media	**SCB**	**DCB**
Test organism	**A B C D E**	**A B C D E**
Strains		
BP 7	8 15 16 4 5	22 23 18 8 12
BP16	2 4 10 8 13	8 6 18 8 15
BP19	0 0 1 3 2	8 6 5 10 11
BP12	10 5 15 0 0	24 20 20 10 6
BP30	0 2 14 10 5	6 13 14 15 18
BP49	2 3 4 6 10	9 10 12 16 20

Table 8: Determination of the nature of secondary metabolites

	Biochemcal test		
Isolates	**Biurete test**	**Iodine test**	**Carbohydrate test**
BP 7	+	-	-
BP16	+	-	-
BP19	+	-	-
BP12	+	-	-
BP30	+	-	-
BP49	+	-	-

+= Positive - = Negative

Discussion

Actinomycets is a distinct group of important soil microorganisms which are clinically and economically valuable. These bacteria produce about 75% of the commercially and medically useful antibiotic (Mustafa, 2004). Approximately 60% of antibiotic developed for agricultural uses were also isolated from actinomycetes species as well (Krssilninkov, 1981). During the present investigation all six selected isolated strains produce result against test organisms.

Antibiotic production usually occurs in stationary phase (Reichenbach *et al.*, 1988) and primary metabolism is the precursor for the biosynthesis of antibiotic (Demain, 1979). During present investigation, all strains showed increased in the mycelial growth up to 21 days of incubation, which represented the late log phase as well as maximum atibiotic production The antibiotic production remain constant after three weeks of incubation.

It has been observed that the slower the growth rate of mycelial the greater the amount of antibiotic synthesis takes place. The present findings are in agreement with the earlier workers of James and Edward (1991) in case of S. Thermoilaceus strain NCIB 10076. During present investigation, all strains showed maximum production of metabolite in late log phase, which remained constant during stationary phase, indicating that the metabolite production was directly proportional to the growth.

Regulation of antibiotic production is influenced by a number of factors like carbon and nitrogen sources (Pridham *et al.,* 1958). Changes in the nature and concentration of carbon, nitrogen, phosphorus and trace elements have been reported to affect antibiotic biosynthesis in different organism (Demain, 1979).

All together five types of carbon sources were tested during present investigation to determine the most suitable carbon source for antibiotic production. Amongst all, dextrose showed its superiority for the growth and anitibiotics production Besides dextrose other sugars viz. starch, glucose, sucrose also showed good for the growth but not as dextrose. Our present findings for all the strains also suported by the earlier workers. In case of (NH_4), H_2PO_4 nitrogen containing media it was found that most of selected strains have shown maximum inhibition zone but this result is not constant to all test organism. This finding is also supported the result provide by Jeffrey (2007).

Temperature is very important for biosythesis of Streptomycin and the growth of the Streptomycete (Krassilnikov, 1981). Many investigations have shown that the secondary metabolism in microorganism is influenced by environmental factors (Jenesen, 2007). James and Edward (1989) have also observed optimum antibiotic synthesis between 30º - 55ºC in *Streptomyces thermoviolaceus*. Although nutrient availability controls activity of soil actinomycetes, other factor such as temperature also plays an important role (Goodfellow and Williams, 1983). The present investigation showed optimum secondary metabolites production at 37ºC in all selected strains. It was observed that at 37ºC maximum inhibition zone was formed. Apart from this 28ºC – 37ºC was also favorable for the secondary metabolites production (Shriling *et al.,* 2005). The pH of the medium is very important for the growth of microorganism's active metabolism as well as biosynthesis of antibiotic (James, 2002). Changes in the acidity of the medium have significant effect on the yield of the end products of actinomycets metabolism (James *et al.,* 1998). Earlier studies have found a pH ranges between 6.5 to 7 as must suitable for production of antibiotic It has been found that the pH affect both cell growth and secondary metabolites production rate. The result of present investigation reveals that the optimum pH was found to be 7.0 for all the selected strain and alkaline condition for production of secondary metabolites is favorable.

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