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Influence of cultural parameters on antimicrobial activity of endophytic streptomyces sp. Cr 12 isolated from *catharanthes roseus* leaves

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Abstract

Streptomyces sp. (Cr 12) was isolated from Catharanthes roseus leaves during the screening of endophytic actinomycetes for bioactive metabolites. Cell growth as well as antimicrobial metabolite production was studied in different culture media. Secondary metabolites from the strain were active against bacteria - Bacillus subtilis, Staphylococcus aureus and Proteus vulgaris as well as fungi - Botrytis cinerea, Curvularia lunata and Rhizoctonia solani. Production of bioactive metabolites by the strain was high in ISP2 broth as compared to other tested media in still conditions. The influence of different cultural parameters on antibiotic biosynthesis by Streptomyces Cr 12 was studied which showed highest antimicrobial activity at pH 7 in 28^{o}C after 16 days of growth.

Keywords: Antimicrobial metabolites, Catharanthes roseus, optimization, Streptomyces sp.

Introduction

Actinomycetes, the gram-positive, free living, saprophytic bacteria widely distributed in soil, water, marine sediments and colonizing plants show marked chemical and morphological diversity and form a distinct evolutionary line of organisms [1,2]. Majority of the world's antibiotics have been obtained from soil-born Streptomyces spp. [3]. Among the microorganisms, actinomycetes are significant being the most potent source for bioactive secondary metabolites especially for antibiotics with diverse clinical effects and agricultural profile and have received special attention for resolving the problem of antibiotic resistance to conventional drugs and important applications in human medicine [4-6]. Streptomyces and Micromonospora are reported as most prolific producers of pharmacologically and agriculturally active agents [7,8]. Ecological role of the endophyte to the host plant is yet to be fully ascertained, though there are reports of certain endophytes confering heat tolerance, salt tolerance, enhanced vegetative growth [9-11] and protection to the plant from the invading fungal pathogens [12]. The internal biologically complex plant tissue is a distinct microhabitat within the terrestrial ecosystem due to its varying content of alkaloids, terpenoids, steroids besides aromatic compounds. Healthy plant tissues thus represent an untapped reservoir of novel endophytic microorganisms producing bioactive metabolites [13]. In the search for plants hosting such endophytic streptomycetes it is suggested that the bioactive compounds within the plant might not be the product of the plant, but products of the endophytes that live therein. Antimicrobial agents of some enodophytes may be in symbiotic association with a host plant [14]. The finding that some Streptomyces spp. have

taken up residence in plants opens the possibility that this may be an entirely untapped source for novel pharmaceuticals and agents for agriculture etc. The main objective of the present study is to screen an endophytic Streptomyces sp. for growth pattern and antimicrobial metabolites and to optimize cultural conditions for enhancing production of the bioactive metabolites.

Materials and Methods

Antimicrobial screening

The selected strain, Streptomyces sp. Cr 12 was isolated from Catharanthes roseus (family Apocynaceae) leaves on starch casein agar medium (SCA) [15]. The antibiotic potential of the isolate in broth media was biologically determined by agar well diffusion method [16]. The isolate was grown in submerged culture in 250 ml flasks containing 50 ml of ISP2 liquid broth, Starch-casein broth, Starch-nitrate broth, Starch-soybean yeast extract broth, Potato dextrose broth, Glycerol asparagine broth, Oatmeal broth, Production medium broth, Benett's broth and Nutrient broth separately. The flasks were inoculated with 1 ml of active Streptomyces seed culture and incubated at 28 ±2ºC for 15days under both static and shaken (150 rpm) conditions. Seed culture was prepared by inoculating 50 ml medium with 5 ml spore suspension and incubated on a rotary shaker at 180 rpm/min at 28^{o}C for 48 hr. [17]. After growth, 50 µl aliquots of the cell-free filtrate were transferred to wells bored in Mueller Hinton agar (MHA) plates that were previously inoculated with the test bacteria- Bacillus subtilis (MTCC 441), Staphylococcus aureus (MTCC 96), Proteus vulgaris (MTCC 744), Pseudomonas aeruginosa (MTCC 424) and Candida albicans and the plates were incubated at 37ºC for 24-48 hr. At the end, the zone of inhibition was measured and recorded. Botrytis cinerea, Curvularia lunata and Rhizoctonia solani were used for antifungal screening. Fungal mycelial discs (6 mm) of each of the three fungi were aseptically transferred and positioned in the centre of PDA (pH 6.5) plates. Agar wells were made in each plate using sterile 6 mm diameter cork borer, 3 cm away from the centre of the plate. 50 µl aliquots was added individually into each well and incubated at room temperature for 3-5 days. The zone of inhibition between the edge of the fungal colony and the well was measured. The medium in which the strain exhibits high levels of inhibitory activity was selected for further studies.

Influence of cultural parameters on the biosynthesis of antibiotics by streptomyces sp. (Cr 12)

Bioactive metabolite production by the strain was optimized by using different cultural parameters. viz., pH, temperature and incubation period. To test the effect of pH on biosynthesis of antibiotics, 50 ml aliquots of ISP2 medium were adjusted to initial pH values (4-12), using 1N NaOH or 1N HCl and sterilized at 121^{o}C for 15 min. The flasks were inoculated with 1 ml of seed culture and incubated at 28 ±2ºC for 15 days under static conditions. The effect of incubation period was studied in ISP2 broth at pH 7, 50 ml aliquots of sterile media were inoculated and incubated at 28 ± 2ºC under static conditions for 30 days. At every 2 days interval, the culture was harvested and tested for activity by agar well diffusion method. The effect of different temperatures was tested by incubating the media at pH 7, 50 ml aliquots of sterile media were inoculated and incubated at 20ºC-40ºC under static conditions for 16 days. At the end of each incubation period, the dry weight of the biomass was recorded and antibiotic activity determined. 50 µl of the broth was transferred aseptically to wells of agar plates inoculated with the test organism S. aureus and C. lunata. The diameter of the clear inhibition zone for bacteria was measured after 24 hrs of incubation and the zone of inhibition between the edge of the fungal colony and the well was measured after 3-5 days. All the tests were performed in triplicate.

Results and discussion

Antimicrobial metabolite production by Cr 12 isolate, when tested in static and shake conditions

showed higher activity against the test microbes under static condition and the isolate was more active towards bacteria than fungi. Out of four bacteria tested the two gram positive bacteria were highly sensitive and had maximum inhibition in ISP2 but gram negative bacteria were less sensitive and P. vulgaris was mildly inhibited and no activity was seen for P. aeruginosa. Out of the four fungi tested, 3 filamentous fungi were inhibited by the metabolites of Cr 12. There was no activity seen in C. albicans. Inhibition was much less for B. cinerea in all the other media when compared to ISP2 medium. This was to a lesser extent seen with C. lunata in most of the media. Inhibition was consistently good in S. aureus and B. subtilis in all the tested media except for B. subtilis in Oatmeal medium. The ability of the Streptomyces isolate to produce antibiotics could be increased or decreased under different cultural conditions. Inhibition was noticed in most of the media tested, but highest inhibition was recorded in ISP2 medium (Table 1). Inhibition activity was less in shake condition compared to static condition and the highest inhibition zone was recorded for S. aureus (Table 2). Streptomyces species are heterotrophic feeders and can utilize simple molecules as nutrients [18]. However it depends on the isolate of Streptomyces and the type of medium. A clear elucidation of the antagonistic properties is largely impacted by the quality of the medium or type of organisms [19].

Influence of different cultural parameters on biomass yield and antimicrobial activity

There was an increase in growth on the second day of cultivation and maximum growth was achieved on the sixteenth day of incubation. The antibacterial activity was detected starting from the second day and reaching its maximum on the sixteenth day of incubation (Fig. 1)

The initial pH value of the fermentation (Fig. 2) indicated that the production of biomass and antibacterial agents by Streptomyces Cr 12 were strongly dependent on the pH of culture broth with 7 pH as optimum for both biomass production and antimicrobial activity. Glazebrook et al. 1993 [20] observed high productivity of 5-Hydroxy-4-oxonorvaline by S. akiyoshiensis at the initial pH ranging between 6.3 to 6.6 while Gupte and Naik 1998 [21] optimized the pH as 6.5 for the production of a new tetraene polyene antibiotic, HA-2-91 from S. arenae var ukrainiana. However Sujatha et al 2005 [22] reported that initial pH of 7.2 with an incubation time of 96 hours was optimal. The effect of temperature on biomass and bioactive metabolite production of the strain was recorded (Fig. 3), where cell growth and the yield of bioactive metabolites were found to be optimum when the strain was cultured at 28^{o}C. Actinomycetes such as S. galbus, [23] showed optimum levels of antibiotic production at 30^{o}C. Our results also revealed that the condition of incubation period, pH and temperature influenced the quantity of biosynthesis of antibiotics. Nutritional and cultural conditions for biosynthesis of antibiotics by Streptomyces psammoticus under shake-flask conditions have been optimized and resulted in 1.82-fold increase in antibiotic yield [22]. Maximum production of antimicrobial activity by Streptomyces Cr 12 was observed in ISP2 medium at pH 7, after sixteenth day of incubation. Species of the genus Streptomyces produce a wide variety of antibiotics and form a major source of useful secondary metabolites [5,24] suggested that the ability of microbes to form antibiotics is not a fixed property but can either be increased or completely lost depending on the conditions in which they are grown. A perusal of literature clearly indicates Streptomyces sp. as a potential agent for screening novel bioactive compounds. In the present study, optimal conditions for the production of bioactive metabolites by Streptomyces sp. Cr 12 were determined and the metabolites showed good antimicrobial activity

Table 1. Antagonistic properties of 15-day old cultures of *Streptomyces* Cr 12 in different liquid media, under static conditions.

	Bacterial species (Inhibition zone in mm)				**Fungal species (Inhibition zone in mm)**			
Media	*B. subtilis*	*S. aureus*	*P. vulgaris*	*P. aeruginosa*	*C. albicans*	*B. cinerea*	*C. lunata*	*R. solani*
ISP2	17(± 0.57)	18 (± 0.57)	13.66(± 0.33)	-	-	11.33(± 0.33)	12(± 0.57)	11.66(± 0.33)
Starch-casein	16(± 0.57)	12.33 (± 1.20)	12(± 0.0)	-	-	5 (± 1.15)	9.33 (± 0.33)	8 (± 1.15)
Starch-nitrate	16.33(± 0.33)	17 (± 0.57)	12.66(± 0.33)	-	-	8.33(± 0.33)	10 (± 1.15)	9 (± 0.0)
Starch-soybean yeast extract	14 (± 2.08)	15.66 (± 2.02)	11.33(± 0.66)	-	-	8 (± 0.57)	9.33 (± 0.88)	9.33(± 0.33)
Potato dextrose	12 (± 1.15)	12.33 (± 1.85)	11 (± 0.57)	-	-	6(± 1.15)	9 (± 1.15)	8.33 (± 0.88)
Glycerol asparagines	15(± 0.57)	15(± 0.0)	-	-	-	5.33(± 0.33)	7.33 (± 0.33)	6.66(± 0.33)
Oatmeal	-	11(± 0.0)	-	-	-	-	10 (± 0.0)	4.66 (± 0.33)
Production medium	16 (± 0.0)	13(± 1.15)	-	-	-	5.66 (± 0.33)	10.33(± 0.33)	9.33 (± 0.33)
Benett's	12 (± 0.57)	10(± 0.0)	-	-	-	5.33(± 0.33)	6 (± 0.0)	7.66 (± 0.33)
Nutrient	10.66(± 0.33)	10.66 (± 0.66)	-	-	-	6 (± 0.57)	7.66(± 0.33)	6(± 0.0)

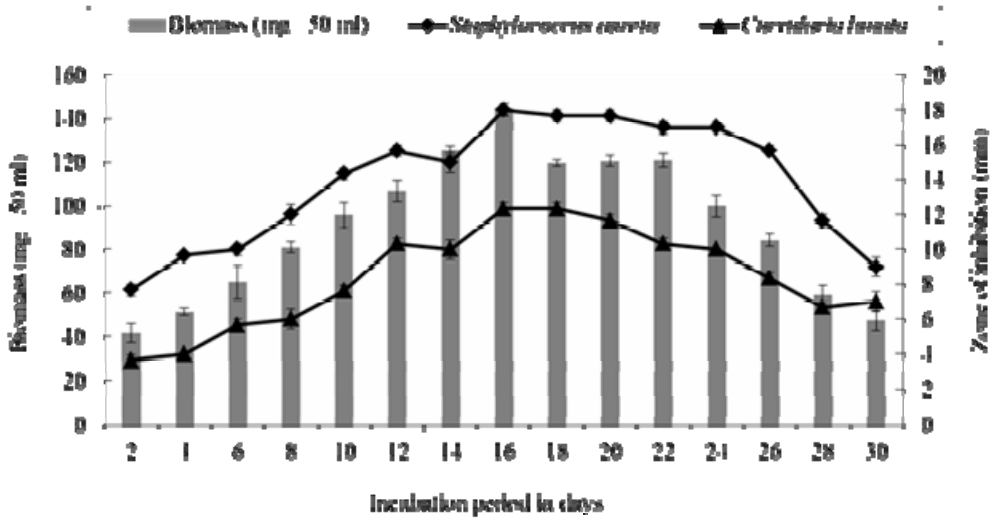

Fig. 1 Effect of incubation period on growth and antibiotic production by *Streptomyces* isolate Cr 12.

Table 2. Antagonistic properties of 15-day old cultures of *Streptomyces* Cr 12 in different liquid media, under shaking conditions.

	Bacterial species (Inhibition zone in mm)				**Fungal species (Inhibition zone in mm)**			
Media	*B. subtilis*	*S. aureus*	*P. vulgaris*	*P. aeruginosa*	*C. albicans*	*B. cinerea*	*C. lunata*	*R. solani*
ISP2	11.66(± 0.33)	12.33(± 0.33)	11 (± 0.0)	-	-	5.66 (± 0.33)	4 (± 0.0)	7.66(± 0.33)
Starch-casein	10.33(± 0.33)	10.33(± 0.33)	10 (± 0.57)	-	-	-	4.33 (± 0.33)	5 (± 0.57)
Starch-nitrate	13(± 0.57)	12 (± 0.57)	-	-	-	5.66 (± 0.33)	7.33 (± 0.33)	6 (± 1.15)
Starch-soybean yeast extract	6.33 (± 0.33)	10.33(± 0.33)	-	-	-	6 (± 0.57)	5.66 (± 0.33)	-
Potato dextrose	9.33 (± 0.33)	11.33(± 0.33)	10.66 (± 0.66)	-	-	2.33 (± 0.33)	4.33 (± 0.33)	6 (± 0.0)
Glycerol asparagines	8 (± 0.57)	10.33(± 0.33)	-	-	-	3 (± 0.0)	-	-
Oatmeal	-	-	-	-	-	-	-	-
Production medium	9.33 (± 0.33)	8.33 (± 0.33)	-	-	-	4.33(± 0.33)	3.33 (± 0.33)	5.33 (± 0.88)
Benett's	12.66(± 0.66)	15 (± 0.0)	12.33(± 0.33)	-	-	-	3 (± 0.0)	-
Nutrient	6.66 (± 0.33)	7.66(± 0.33)	-	-	-	-	3.33 (± 0.33)	-

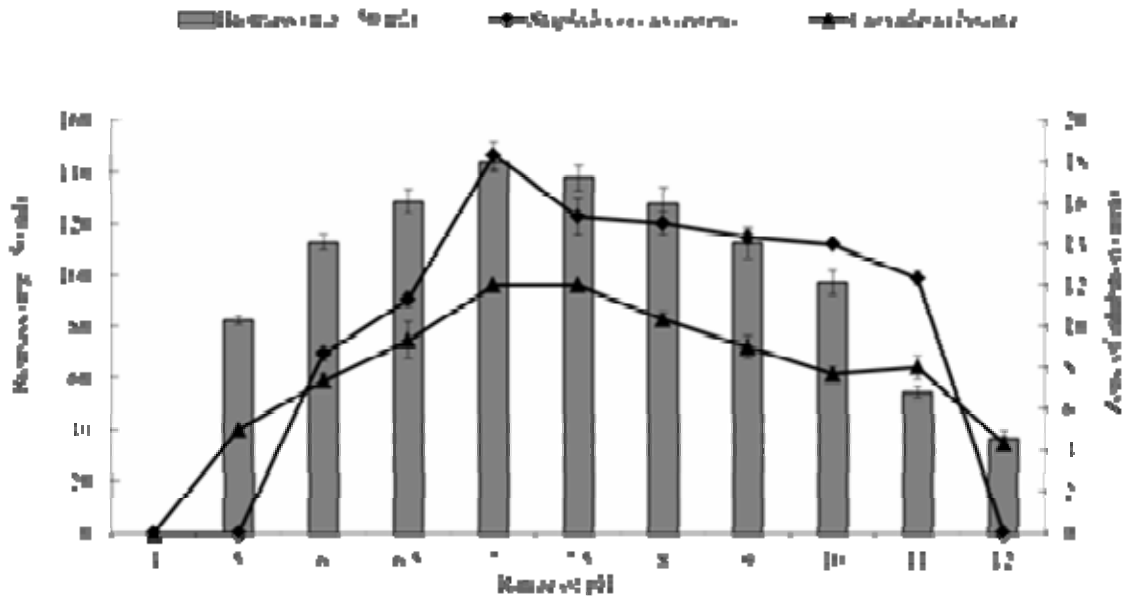

Fig. 2 Effect of different pH on growth and antibiotic production by *Streptomyces* isolate Cr 12

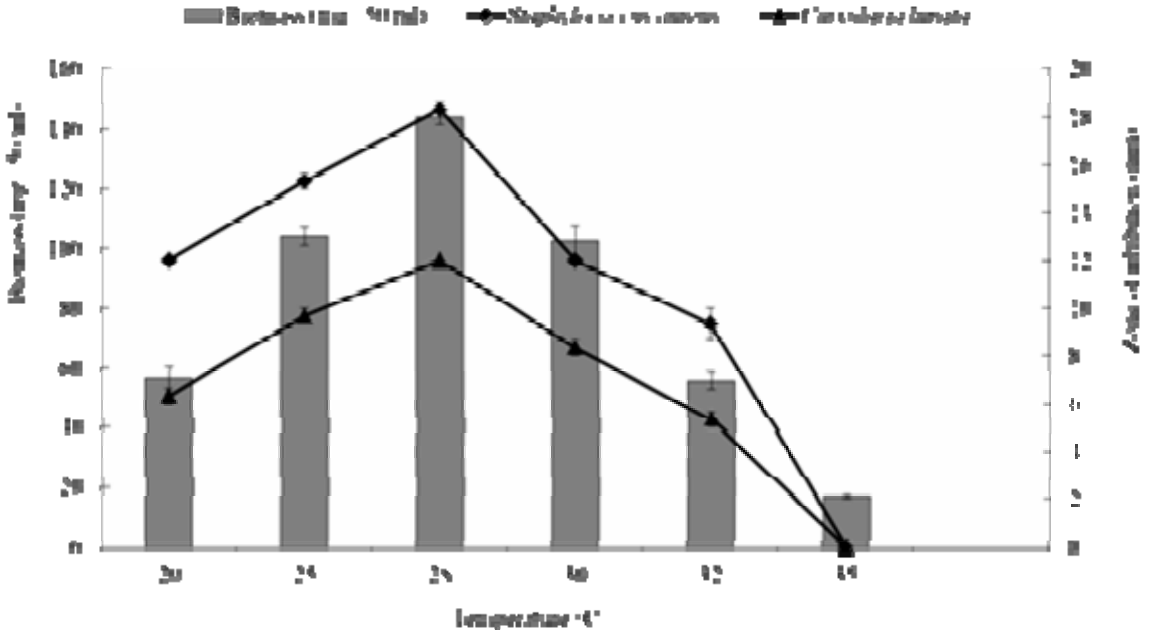

Fig. 3 Effect of temperature on growth and antibiotic production by *Streptomyces* isolate Cr 12

against gram positive bacteria and fungi. Further studies on the purification and characterization of potent bioactive metabolites produced by the strain are in progress.

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References

1. Goodfellow M, O'Donnell AG. Search and discovery of industrially-significant actinomycetes. Proceedings of the 44th Symposium on Society for General Microbiology, (SCGM'89), Cambridge University Press: Cambridge 1989;343-83.
2. Mincer TJ, Jensen PR, Kauffnan CA, Fenical W. Wide spread and persistent populations of a major new marine actinomycete taxon in ocean sediments. Applied Environ Microbiol 2002;68:5005-5011.
3. Arai T. Actinomycetes: The Boundary Microorganisms. Toppan Co. Ltd., Singapore; 1976.
4. Watve MG, Tichoo R, Jog MM, Bhole BD. How many antibiotics are produced by the genus Streptomyces. Arch Microbiol 2001;176:386-90.
5. Berdy J. Bioactive microbial metabolites. J. Antibiot 2005;58:1-26.
6. Wise R. The worldwide threat of antimicrobial resistance. Curr Sci 2008;95:181-187.
7. Okamoto-Hosaya Y, Okamoto S, Ochi K. Development of antibiotic-overproducing

strains by site-directed mutagenesis of the rps1. gene in Streptomyces lividans. J Appl Environ Microbiol 2003;69:4256-4259.
8. Magarney NA, Keller JM, Bernan V, Dworkin M, Sherman DH. Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. Applied Env Microbiol 2004;70:7520-7529.
9. Porter JK, Bacon CW, Robbins JD. Lysergic acid amide derivatives from Balansia epichloe and Balansia claviceps (Clavicipitaceae). J Nat Prod 1979;42:309–314.
10. Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM. Thermotolerance generated by plant/fungal symbiosis. Science 2002;298:1581.
11. Rodriguez R, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim Y, Redman R. Stress tolerance in plants via habitat-adapted symbiosis. International Society for Microbial Ecology Journal 2008;4:404–416.
12. Arnold A, Mejia L, Kyllo D, Rojas E, Maynard Z, Robbins N, Herre E. Fungal endophytes limit pathogen damage in a tropical tree. Proc Natl Acad Sci USA. 2003;100:15649–15654.
13. Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol 2003;67:491-502.
14. Yang X, Strobel G, Stierle A, Hess WM, Lee J, Clardy J. A fungal endophyte-tree relationship; Phoma sp. In Taxus wallichana. Plant Sci 1994;102:1-9.
15. Kuster E, Williams ST. Selection of media for isolation of streptomycetes. Nature (London) 1964;202:928-929.
16. Gramer A. Antibiotic Sensitivity and Assay Test. In: Collins CH, Lyne PM (ed) Microbiological Methods. Butterworth and Co, London 1976. p: 235.
17. Balagurunathan R, Subramanian. Studies on marine Streptomyces nigrifaciens. I-taxonomy and standardization of antibiotic production. Cienc Mar 1993;19:435-443.
18. Yang SS, Wang JY. Protease and Amylase Biosynthesis of Streptomyces rimosus in Submerged and Solid State Cultivations. Bot Bull Acad Sin 1999;40:259-265.
19. Salama AM, Mansour FA, Kamel Z. Studies on Blue-Pigmented Streptomyces Isolated from Egyptian Soil. Egypt J Bot 1980;23:1.
20. Glazebrook MA, Vining LC, White RL, Smith KC, Chedrawy EG. Nutrient effects on growth and production of 5-hydroxy-4-oxonorvaline by Streptomyces akiyoshiensis. Can J Microbiol 1993;39:536-542.
21. Gupte TE, Naik SR. Optimization of nutritional requirements and process control parameters for the production of HA-2-91, a new tetraene polyene antibiotic. Hindustan Antibiot Bull 1998;40:5-13.
22. Sujatha P, Bapi Raju KV, Ramana T. Studies on a new marine streptomycete BT-408 producing polyketide antibiotic SBR-22 effective against methicillin resistant Staphylococcus aureus. Microbiol Res 2005;160(2):119-26.
23. Paul AK, Banerjee AK. Determination of optimum conditions for antibiotic production by Streptomyces galbus. Folia Microbiol (Praha) 1983;28:397-405.
24. Krassilnikov NA. Intra-Strain and Intra-sp. Antagonism among Microorganisms. Doklady Akad Nauk SSSR 1960;77:117-119:725-728.