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MINIATURIZED FERMENTATION IN EPPENDORF TUBES FOR THE DETECTION OF ANTAGONISTIC ACTINOMYCETES

Radhakrishnan M[1], J Priya[2], R Balagurunathan[3] and Vanaja Kumar[1]
[1]National Institute for Research in Tuberculosis, Department of Bacteriology, Chennai – 31, Tamil Nadu
[2]Department of Microbiology, Sri Sankara Arts & Science College, Kanchipuram – 631 561, Tamil Nadu
[3]Department of Microbiology, Periyar University, Salem – 600 011, Tamil Nadu

***Corresponding Author**: **Dr. R. Balagurunathan Ph.D.,** Associate Professor & Head, Department of Microbiology, Periyar University, Salem – 11, Tamil Nadu.

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Abstract: Actinomycetes are recognized as a prolific source for bioactive metabolites. Screening adequate number of strains by appropriate high quality screening protocol determines success of drug discovery programs. To accomplish screening for antagonistic actinomycetes at higher rate, the fermentation stage of the initial screening needs to be miniaturized. The present study is attempted for miniaturized production of bioactive compounds from 40 actinomycetes isolated from less explored ecosystems. Actinomycetes were cultivated in eppendorf tubes (2 ml) using 0.5 ml of yeast extract malt extract agar and modified nutrient glucose agar medium for 10 days and extracted using 1 ml of methanol. The crude extracts were tested against *S. aureus, B. subtilis* and *E. coli* by disc diffusion method. All the isolates were also tested for antagonistic activity by conventional cross streak method and disc diffusion method using extracts prepared by plate culture method. Crude bioactive compounds produced from five selective isolates by shake flask method showed good activity. Antagonistic activity, requirements, time duration and cost effect of all the three methods were compared. Miniaturized production process in eppendorf tubes was found to be more advantageous and suitable to develop as high throughput screening (HTS) method for the detection of antagonistic actinomycetes particularly in resource poor laboratories settings.

Keywords: Actinomycetes, Streptomyces, Miniaturized Culture, Antagonistic Activity, Eppendorf Tubes, High Throughput Screening (HTS)

INTRODUCTION

Actinomycetes have been the largest percentage of cultured members, which probably reflects both their relative ease of culturing and their importance in antibiotic discovery efforts[1]. Baltz[2] estimated that less than one part in 10^{12} of the earth's soil surface has been screened for actinomycetes. Screening applications such as search for bioactive molecules, medium optimization, or characterization of recombinant / mutagenized organisms require examination of large number of cultures. In this respect it is advantageous when representative and reproducible cultivation can be done in small scale preferably in standard format that is amenable to automation[3].

A variety of screening methods have been developed targeting different types of microorganisms. For antibiotic producing strains, the "agar-plug" method was widely used in 1980s and many modified versions were developed for preliminary screening with high efficiency[4,5]. However, extracts or culture filtrates must be needed for screening for antimicrobial activity against certain microorganisms like *Mycobacterium tuberculosis*, which employ liquid culture-based screening methods[6]. Mycelium-forming Streptomyces strains were grown in one milliliter liquid micro-cultures in square deep well micro titre plates, growth was evaluated with respect to biomass formation and production of secondary metabolites which were found to be very similar in the micro-cultures, bioreactors and shake-flask cultivation, respectively[3].

Certain issues are associated with when performing primary screening in solid medium and liquid culture based secondary screening. There can be loss of bioactive metabolite production in secondary screening while using a medium which is different from the one used for primary screening. Certain actinomycete strains produce antibiotic compounds only in solid medium but fail to produce the same in liquid medium[7,8,9].

To address all these issues, we made an attempted micro-scale production of bioactive metabolite production from *Streptomyces* species in eppendorf tubes using solid medium. The results were also compared with two solid culture and a submerged fermentation-based screening methods.

MATERIALS AND METHODS

Description of actinomycetes: Totally 40 actinomycete strains were obtained from Actinomycetes research laboratory at Department of Microbiology, Sri Sankara Arts & Science College, Kanchipuram. All the actinomycete isolates were previously isolated from different rare ecosystems such as Thar Desert, coffee plantation, rubber forest, and Western Ghats. Viability of all the strains were maintained on yeast extract malt extract (YEME) agar (ISP medium 2)[10].

Characterization of actinomycetes: Cultural characteristics were studied by inoculating the actinomycete cultures on YEME agar medium and incubated for 7-14 days. Recorded characteristics include growth, consistency, aerial mass color, reverse side and soluble pigment production. Micro morphological characteristics were studied by adopting slide culture method. During the incubation for 7-14 days at 28°C the presence or absence of aerial mycelium, substrate mycelium and spore chain structure[10,11].

Miniaturized production of bioactive compounds: In order to develop HTS method, miniaturized production of bioactive compounds was carried out in Eppendorf tubes (2 ml size). About 0.5 ml of YEME medium and modified nutrient glucose agar (MNGA) medium was added in to each set of eppendorf tubes. After autoclaving, all the tubes were kept in slanting position till it solidify. Then all the actinomycete isolates are inoculated in to YEME agar and MNGA medium and incubated at 28°C for 5 days.

Extraction of bioactive compounds: After fermentation, bioactive compounds from agar medium were extracted by solid liquid extraction method. About 1ml of methanol was added in to all the tubes and mixed with flame sterilized glass rods. All the tubes were kept at 4°C for 6 h for extraction. Then the agar portion was separated by centrifugation at 10,000 rpm for 10 min. The methanol portion was collected and concentrated by evaporation.

Antimicrobial activity testing: All the crude bioactive compounds produced from actinomycetes was dissolved by adding 100 µl of crude extract was impregnated on sterile filter paper disc (5mm in diameter) and allowed to dry. Crude compound impregnated discs were placed over nutrient agar medium inoculated with test bacterial strains such as *S. aureus, B. subtilis* and *E. coli*. All the plates were observed for zone of inhibition after 24 h of incubation at 37°C.

Screening of actinomycetes by cross streak method: All the actinomycetes strains were screened for antibacterial activity by cross streak method using MNGA medium[12]. Test bacterial pathogens used in this study include *S. aureus, B. subtilis* and *E. coli*. All the bacterial strains were obtained from actinomycetes Research laboratory, department of Microbiology, Sri Sankara Arts and Science College, Kanchipuram and maintained on nutrient agar slants. The actinomycete strains were streaked as parallel line on MNGA medium and incubated at 28°C for 5 days. After incubation 18 h old test bacterial strain were cross streaked between two actinomycetes strains and incubated at 37°C for 24 h. After incubation all the plates were observed for inhibition of bacterial growth.

Production of bioactive compounds from actinomycetes by plate culture method: Bioactive compound from all the actinomycete was produced by plate culture method using YEME medium. All the actinomycete strains are inoculated in to YEME medium and incubated at 28°C for 5 days. After incubation, the whole agar medium was cut in to pieces and extracted with each 10 ml of methanol in 50 ml beaker. All the extraction beakers were kept at 4°C for 24 h. Then the methanol portion was collected and concentrated by evaporation[13].

Antimicrobial activity of crude extracts was tested by disc diffusion method using *S. aureus, B. subtilis* and *E. coli* as test organism. Crude extract were measured and dissolved in 100 µl of methanol. About 10 µl of crude extract was impregnated in sterile filter paper disc with 5mm diameter. The test organisms were inoculated by making a lawn on nutrient agar by using sterile cotton swab. The crude compound impregnated discs were placed over the test organisms. The diameter of the inhibition zone was measured after 24 h of incubation at 37°C[12].

Production of bioactive compounds by submerged fermentation: Based on the results of different screening methods, 5 potential actinomycete isolates were selected for production of bioactive compounds by submerged fermentation. Submerged fermentation of potential actinomycete strains was carried out by shake flask fermentation method using soybean meal medium. Spores of potential actinomycete strains were scrapped from yeast extract malt extract agar (ISP2 medium) and inoculated in to 50 ml of soybean meal inoculation medium in 250 ml conical flask and kept in rotary shaker at 120 rpm for 48 h at 28°C. After 48 h of incubation, about 10% of inoculum from soybean meal inoculation medium was transferred in to 100 ml of soybean meal production medium and kept in rotary shaker at 120 rpm for 120 h at 28°C.

After fermentation, mycelium and supernatant was separated by centrifugation at 10, 000 rpm for 10 min at 4°C. The extracellular compounds from culture

supernatant were extracted by liquid-liquid extraction method using ethyl acetate and concentrated evaporation. Antimicrobial activity of crude extracts was tested by disc diffusion method as described earlier[12].

RESULTS

Characterization of actinomycetes strains: Out of 40 actinomycete strains studied most of the strains are powedery in consistency and white gray in color. Totally 31 isolates produced reverse side pigment on YEME medium and 9 isolates produced reverse side pigment on MNGA medium. Both on YEME and MNGA medium only 4 isolates produced soluble pigment. Cultural characteristics of certain strains differ from YEME agar to MNGA medium. Aerial and substrate mycelium was present in all the isolates. Spore chain morphology of most of the isolates is rectus flexibile which is followed by Retinaculum apertum and spirals. Based on their cultural and microscopic observation all the isolates are suspected as *Streptomyces* species.

Activity of crude extract produced by miniaturized production method: Crude extract from 12 actinomycetes inoculated on YEME medium showed activity against *S. aureus*, 15 strains against *B. subtilis* and 11 against *E. coli*. Crude extract from 18 actinomycete strains inoculated on MNGA medium showed activity against *S. aureus*, 22 strains against *B. subtilis* and 12 against *E. coli* (Table 1).

Screening by cross streak method: In cross streak method, 21 actinomycete strains inhibited *S. aureus* 7 inhibited *B. subtilis* and 8 strains inhibited *E. coli*, above five strains active against *S. aureus* alone (Table 1).

Activity of crude extract produced by plate culture method: In this study, crude extracts from 15 strains actinomycetes showed activity against S. aureus, 17 against *B. subtilis* and 12 against *E. coli*, crude extract from 8 strains inhibited all the three pathogens and none of the strains active against *S. aureus* alone (Table.1).

Table.1: Antagonistic activity exhibited by actinomycetes in different screening methods

S. No	Strain No	*S. aureus*				*B. subtilis*				*E. coli*			
		Cross streak	Agar plate	Miniaturized production		Cross streak	Agar plate	Miniaturized production		Cross streak	Agar plate	Miniaturized production	
				YEME	MNGA			YEME	MNGA			YEME	MNGA
1	HM10	+	8	6	-	+	13	10	-	+	15	11	8
2	D5	+	10	-	-	+	15	-	-	+	12	10	9
3	D6	+	11	-	-	+	15	-	-	-	12	8	-
4	D10	+	12	7	-	-	20	11	8	-	18	7	11
5	D13	+	-	-	-	+	-	-	9	+	-	-	-
6	D18	+	-	-	10	+	-	-	8	+	-	-	-
7	K5	-	-	-	10	-	-	-	-	-	-	-	-
8	K8	-	-	-	10	-	-	-	8	-	-	-	-
9	K10	-	-	-	10	-	-	12	7	-	-	16	-
10	K11	-	-	-	-	-	-	-	-	-	-	7	-
11	K22	-	-	-	-	-	-	10	8	-	-	7	-
12	K36	-	-	-	10	-	-	-	7	-	-	7	-
13	K38	-	-	-	-	-	-	9	8	-	-	7	-
14	NEK5	+	-	-	10	+	-	-	7	-	-	-	-
15	EE9	+	-	-	-	-	-	-	-	+	10	-	-
16	TA1	-	-	-	-	-	-	10	10	-	8	-	-
17	TA4	+	-	-	10	-	-	-	-	-	8	-	-
18	TA22	-	-	-	10	-	-	-	7	-	-	-	-
19	TA34	-	-	-	10	-	-	-	7	-	-	9	-
20	TA36	-	-	-	-	-	-	-	-	-	-	-	-
21	CSA3	-	-	-	10	-	-	8	-	-	-	-	-
22	CSA4	-	-	-	10	-	7	-	8	-	-	-	-
23	CSA11	-	-	-	-	-	-	10	7	-	-	-	-
24	CSA14	-	-	12	-	-	-	-	10	-	-	-	-
25	CSA24	-	-	-	-	-	-	-	-	-	13	-	-
26	S1A7	-	-	-	-	-	-	-	-	-	-	-	-
27	S1A25	-	-	-	10	-	-	-	7	-	-	-	-
28	S2A2	-	-	-	-	-	8	8	7	-	-	-	-
29	S2A5	+	7	13	12	-	8	8	7	-	15	-	-
30	S2A6	+	8	-	10	-	11	8	7	-	15	-	-
31	S2A7	+	9	-	10	-	9	14	8	-	15	-	8
32	S3A1	+	11	-	10	+	12	-	-	+	15	-	8
33	S3A2	+	-	-	10	-	-	-	-	-	-	-	9
34	S3A6	+	8	10	-	-	7	10	-	-	-	-	10
35	S3A7	+	9	-	-	-	7	-	-	+	-	-	12
36	S3A8	+	7	-	-	-	7	7	-	-	-	-	10
37	S3A16	+	12	-	-	-	8	-	-	-	-	-	9

38	S3A18	+	12	8	-	-	10	-	10	-	-	-	-
39	S3A20	+	10	-	-	-	9	-	-	+	-	-	13
40	S4A5	+	15	8	15	+	14	20	15	+	-	11	10

+ - presence of activity;
- - absence of activity

Antimicrobial activity of bioactive compound produced by submerged fermentation: All the five selected actinomycete strains showed good growth during submerged fermentation process. Antimicrobial activity of actinomycete extracts produced from selected actinomycete strains are given in table 2. Crude extract produced from all the five strains showed maximum activity against *S. aureus.*

Table.2: Antimicrobial activity of bioactive extracts produced by submerged fermentation

Actinomycetes strains	Test organisms (zone of inhibition in mm in dm)		
	S. aureus	*B. subtilis*	*E. coli*
HM10	14	9	7
D10	11	8	8
NEK5	20	9	9
CSA11	12	7	8
S4A5	13	20	8

Comparative analysis of different screening methods evaluated for the detection of antagonistic actinomycetes: Comparative analysis of different screening methods evaluated for the detection of antagonistic actinomycetes was given in Table 3.

Table.3: Comparative analysis of screening methods (data from 40 actinomycete strains and 3 test organisms / trial)

Parameters	Cross streak method	Agar plate culture method	Miniaturized production method
Number of steps	Two steps Streaking of test organisms Cross streaking of test pathogens	Three steps Cultivation of actinomycetes Extraction of crude compounds Testing by disc diffusion method	Three steps Cultivation of actinomycetes Extraction of crude compounds Testing by disc diffusion method
Requirements	Petriplates	Petriplates	Eppendorf tubes Petriplates
Media used	MNGA	YEME agar Nutrient agar (for activity testing)	YEME agar/MNGA Nutrient agar (for activity testing)
Quantity of media needed	400 ml MNGA	200 ml MNGA 200 ml Nutrient agar	20 ml YEME medium/20 ml MNGA medium 200 ml nutrient agar
Other chemicals needed	-	Methanol	Methanol
Cost of chemicals (in Rs)	App. 30 Rs	App. 30 Rs	App. 15 Rs
Duration of study Incubation Extract preparation Activity testing	 5 days - 24 hours	 5 days 2-3 days 24 hours	 5 days 2-3 days 24 hours
Contamination chance	High	Moderate	Nil
Further use of different media	Increase use of chemicals and glassware usage	Increase use of chemicals and glass ware usage	Less when compared to other methods
Growth of actinomycetes	+/-	+	+ in YEME agar +/- in MNGA
Others			
Number of strains to be screened	Less	Less	It allows to screen large number of strains
Possibilities to study culture conditions and other parameters	Increase medium cost	Increase medium, other chemicals and glassware's cost	High possibilities to study the effect of different media and culture conditions on bioactive compound production using less amount of chemicals
Extraction of crude compound from the medium	-	+	+
Further testing of crude compounds	Further testing of crude extract is needed before the selection of potential strain	Not needed	Not needed
	It did not indicate the activity of intra cellular compounds	It did not indicate the activity of intracellular compounds	It also indicate the activity of intracellular metabolites due to the whole medium extraction along with the biomass

DISCUSSION

In general, research towards any natural products, always starts with screening for desired activity. The suitability of screening protocol and culture condition are the key factors in novel drug discovery[14]. There are so many preliminary screening methods for the detection of antibiotic producing actinomycetes are in practice. This includes crowded plate method; agar overlay method, cross streak method, agar plug method. But the success of drug discovery program is depends on two things such as (i) having appropriate high quality screening protocols and (ii) having adequate numbers of high quality numbers to screen. With this view the present study was attempted to develop miniaturized production method as High Throughput

Screening (HTS) for the detection of antagonistic actinomycetes from less explored ecosystems.

Cross streak method is in routine practice for the detection of antagonistic actinomycetes from routine and less explored ecosystem[11,12, 15, 16,17]. But this method is allowed to detect the activity of extracellular compounds. In the present study also considerable number of actinomycetes showed activity against one or more of the pathogens tested.

The crude extract prepared from agar plate culture also showed good activity against the test pathogens. This method allowed to detection of activity of extracellular compounds secreted in to the medium and intracellular compounds present within the mycelium. Bredholt et al[13] screened around 3200 marine actinomycetes isolates for antimicrobial activity by culturing them in three different agar media. Crude compounds were extracted using DMSO and tested for antimicrobial activity. Lu *et al*[18] produced ansamitosins from *Actinosynnema pretiosum* using yeast extract malt extract glucose (YMG) agar medium.

One way to increase the probability of finding new antibiotics is simply to screen at a higher rate. To accomplish this, the fermentation stage of the initial screen needs to be miniaturized to accommodate millions of actinomycetes per year rather than tens of thousands screened per year as in the past[2]. In the present study, miniaturized production of bioactive compound from actinomycetes was carried out in eppedorf tube containing YEME and MNGA medium. The whole medium was extracted with methanol and tested for activity by disc diffusion method. Both the medium supported antibiotic production form actinomycetes tested. The antimicrobial activity of crude extract produced by eppendorf method was comparable with other two methods used (Table 3). Based on the results the present study reported that the following are the advantages of miniaturized production process.

- Miniaturized production of bioactive compound in eppendorf needs simple requirements when compared to other methods.

- Chemical needed for miniaturized production is very less (20 ml medium/40 strains) when compared to cross streak method (400 ml medium/40 strains).

- Crude extract produced in miniaturized process (100 µl) is easy to screen against at least 10 test organisms at 10 µl/disc concentration.

- Amount of solvent needed for extraction of crude compound from miniaturized process (1ml/strain).

- Miniaturized process allowed detecting intracellular bioactive compounds also. But it is not possible in cross streak method.

- Overall the cost effect of miniaturized process is also good which needs just 3 rupees for chemical for the production of bioactive compounds from 40 actinomycetes strains but the agar plate culture and cross streak methods need approximately 30 rupees for the same 40 strains.

- Further utility of this miniaturized production process includes Optimization of different culture conditions for antibiotic production.

- Quantity and type of antibiotic production are greatly influenced by various culture conditions[14].

- Due to the advances in analytical techniques, small quantity (even 10 mg) of crude extract is enough to detect the nature of compounds present in it. Fiedler[19] reported the screening of secondary metabolite by HPLC and UV visible absorbance spectral libraries. In this study, secondary metabolites were produced from Streptomyces species in 100 ml of medium. Mycelium was extracted with 50 ml of methanol and culture filtrate was extracted twice with 50 ml of ethyl acetate at pH 4. In HPLC analysis only 10 µl of culture filtrate or extract was injected. The production of this small quantity of extract is achievable simply by using the miniaturized production process in this study rather than the method described by Fiedler[19].

- More over it is possible to preserve actinomycetes in eppendorf tubes as stock cultures

- Further, based on the results of miniaturized production process bioactive compounds were produced from five selected actinomycetes isolates and tested for antimicrobial activity. All the isolates showed good antimicrobial activity. This result showed that scale up of fermentation process is possible, based on the miniaturized production process.

CONCLUSION

The present study concluded that miniaturized production process developed in this study will leads to the further development of good High throughput screening (HTS) method to screen large number of actinomycetes for the detection of antagonistic activity. This may be useful for developing country like India to exploit its natural microbial resource with simple investment cost. Further standardization of this method may strengthen its potential.

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