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Effect of critical medium components and culture conditions on antitubercular pigment production from novel Streptomyces sp D25 isolated from Thar desert, Rajasthan

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ABSTRACT

Effect of culture conditions and critical medium components on antitubercular pigment production from novel *Streptomyces* sp D25 isolated from Thar desert, Rajasthan was investigated. Antitubercular pigment from *Streptomyces* sp. D25 was produced by Agar Surface Fermentation (ASF) using yeast extract malt extract agar (YEME) as a basal medium. Effect of incubation period, temperature, carbon source, nitrogen source, minerals and sodium chloride concentration was studied by adopting one-variable-at-a-time method. Crude pigment produced under different conditions was extracted solid-liquid extraction method using ethyl acetate. Responses measured include the growth rate, quantity of crude extract and activity against *S. aureus* MTCC96 and *M. tuberculosis* H37Rv at 100 µg concentrations. Growth and pigment production was correlated with the bioactivity. Of the various conditions tested, maximum growth, pigmentation and bioactivity was observed on 6th day of incubation. Of the various medium components tested, 1% glucose, fructose and malt extract, pH 7 and 9, temperature 30^{0}C and 40^{0}C, 0.1% KNo3 and 0 – 5% NaCl was found to influence the growth, bioactive pigment production and antimicrobial bioactivity. Further statistical based optimization is in progress to prove the effect of interaction of the above variables on antitubercular pigment production from *Streptomyces* sp D25.

INTRODUCTION

Actinomycetes are the economically valuable bacteria which are ubiquitous in nature with the ability to produce novel secondary metabolites including antibiotics (Berdy, 2012). Secondary metabolites from actinobacteria have a long history in the treatment of TB. Many clinically used anti-TB compounds have been reported from actinomycetes with new structure and novel mechanism of action (Butler and Buss, 2006). Since the discovery of streptomycin, the first antibiotic used for anti-TB therapy obtained from *Streptomyces griseus*, during 1944, numerous anti-TB antibiotics have been reported from various actinobacterial genera. Importantly, promising candidates such as rifamycin, erythromycin, pacidamycin, caprazamycin, capuramycin and thiolactomycin in clinical trials against MDR-TB are also of actinomycete origin (Souza, 2009). Inspired by this excellent track record, a significant effort was made that resulted in the successful isolation of actinobacteria from terrestrial sources for anti-TB screening programs in the past 50 years.

However, the rate of discovery of new compounds from normal terrestrial actinobacteria has decreased whereas the rate of re-isolation of known compounds has increased (Lam, 2006). Undiscovered species inhabiting unique environments with differing environmental constraints have been thought to be resources of novel compounds (Kurtboke, 2010; George *et al.*, 2012; Radhakrishnan *et al.*, 2014a). Actinomycete strain D25 used in this study was isolated from Thar Desert soil, Rajasthan, India using Starch casein nitrate (SCN) agar medium. Strain D25 produced powdery growth with diffusible yellow-orange pigment production on yeast extract malt extract (YEME) agar medium. The crude pigment showed promising activity against multi drug resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis* and methicillin resistant *aureus* by disc diffusion method and luciferase reporter phage (LRP) assay, respectively (Radhakrishnan *et al.*, 2014a).

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Moreover, strain D25 produced bioactive yellow pigment when grown only on solid medium but not in liquid medium (Radhakrishnan *et al*., 2014b). Production of secondary metabolites by microorganisms highly depends on the strains and species of microorganisms and their nutritional and cultural conditions (Wang *et al*., 2010; Jose *et al*., 2011). Minor changes in media composition exert a huge impact on quantity and quality of secondary metabolites and general metabolic profile of microorganisms (Greasham, 1983; Wang *et al*., 2011).

Hence, optimization of culture medium is essential to ensure enhanced production of desired metabolites. Optimization of culture medium is conventionally done by one factor at a time (OFAT) method which is workable as long as the production process is influenced by a less number of variables (Kanmani *et al*., 2013). The present study reports the effect of culture conditions and critical medium components on antitubercular pigment production from novel *Streptomyces* sp D25 isolated from Thar desert, Rajasthan.

MATERIALS AND METHODS

Effect of incubation period

Hundred microliter spore suspension of *Streptomyces* sp D25 was transferred into ten yeast extract malt extract (YEME) agar plates and spreaded using sterile L- rods. All the plates were incubated at 28^0C for 10 days. For every 24 hours of fermentation, after scrapping the mycelial growth, crude pigment secreted into the agar medium was extracted using 50 ml of methanol for 24 hours. The solvent portion was collected and concentrated using eppendorf concentrator at 40^0C and quantified (Eccleston *et al*., 2008).

Antibacterial activity was tested against *Staphylococcus aureus* MTCC96 by disc diffusion method at 100µg/disc concentration (Radhakrishnan *et al*., 2007). Activity was expressed as zone of inhibition in millimeter. Antitubercular activity was tested against *Mycobacterium tuberculosis* H37Rv by adopting luciferase reporter phage (LRP) assay at 100 µg/ml concentration. Activity was expressed as percentage reduction in relative light unit (RLU). Extracts showing more than 50 % reduction in RLU, when compared with control, was considered as showing antitubercular activity (Radhakrishnan *et al*., 2010).

Effect of solvents on extraction of antitubercular pigment

Streptomyces sp D25 was grown on YEME agar plates for 10 days at 28^0C. At the end of fermentation, crude pigment secreted onto the agar medium was extracted by solid liquid extraction using different organic solvents such as n-hexane, dichloromethane, diethyl ether, chloroform, ethyl acetate and methanol at 1:2 ratio. All the solvent extracts were concentrated using rotary evaporator and quantified. Antibacterial and antitubercular activity was tested against *S. aureus* MTCC96 and *M. tuberculosis* H37Rv at 100µg concentration by adopting disc diffusion method and LRP assay, respectively.

Effect of culture conditions and medium components

Effect of various critical medium components on antitubercular pigment production was studied by adopting classical one-variable-at-a-time method (Shekar *et al*., 2011). Factors and variables used in this study are given in table 3. Effect of carbons sources were studied by replacing glucose from YEME agar with 1% of different sugars. Each five plates were prepared for each sugars.

Crude pigment was extracted from different sugar supplemented medium using ethyl acetate after 10 days of incubation at 28^0C. Effect of nitrogen sources were studied by replacing the malt extract with 1% of different organic and inorganic nitrogen sources. Effect of temperature was studied by incubating twenty five YEME agar plates seeded with 100 µL of strain D25 spore suspension. Each five plates were incubated at 10^0C, 20^0C, 30^0C, 40^0C and 50^0C. After 10 days of incubation, all the plates were taken and extracted using ethyl acetate. Effect of medium pH on antitubercular pigment production was also tested using YEME agar.

Effect of minerals was studied by supplementing 100 mg of different minerals such as MgSo4, CaCo3, KCl. Effect of sodium chloride was studied by supplementing different concentration of NaCl viz., 1%, 2.5%, 5%, 7.5% and 10%.

Response measurement

Growth

Effect of different variables on the growth of *Streptomyces* sp D25 was compared with the growth of same strain in YEME agar. Growth pattern was expressed as good (3+), moderate (2+), poor (1+) and no growth (-).

Pigment production

Quantity of the pigment produced using different variables was estimated using pre-weighed eppendorf tubes and the quantity of crude pigment was expressed in milligrams /50 ml of medium.

Activity

Antibacterial activity was tested against *S. aureus* MTCC96 by disc diffusion method at 100µg/disc concentration (Radhakrishnan *et al*., 2007). Activity was expressed as zone of inhibition in millimeter. Antitubercular activity was tested against *M. tuberculosis* H37Rv by adopting LRP assay 100 µg/ml concentration. Activity was expressed in the percentage reduction in relative light unit (RLU). Extracts showing more than 50 % reduction in RLU, when compared with control, was considered as showing antitubercular activity (Radhakrishnan *et al*., 2010).

RESULTS AND DISCUSSION

Streptomyces sp D25 produced powdery growth with gray aerial mycelium and soluble yellow pigment on the YEME agar medium. The growth, colour intensity and quantity of

pigment were increased with incubation period. While testing for bioactivity, crude pigment obtained from 6th day of incubation was showed maximum activity against *S. aureus* MTCC96 and *M. tuberculosis* H37Rv (Table 1).

Table 1: Antituberculous pigment production by strain D25 at different incubation period in agar surface fermentation.

Incubation period (Days)	Growth	Pigment production (mg/40 ml)	Bioactivity against *S. aureus* MTCC96 (Zone of inhibition)	Bioactivity against *M. tuberculosis* H37Rv (% red in RLU)
1	-	0	NT	NT
2	1+	7	8	42.52
3	1+	10	10	51.24
4	2+	14	12	57.49
5	2+	30	12	65.31
6	3+	37	14	80.78
7	3+	45	14	87.78
8	3+	48	14	89.91
9	3+	48	14	89.23
10	3+	49	13	85.24

Secondary metabolites are usually produced only at the end of the stationary phase (Sanchez *et al.*, 2010) and secondary metabolism occurs best at sub-maximal growth rates. In many cases, the distinction between the growth phase and production phase is clear (Demain and Fang, 1995). In the present study maximum quantity of pigment production and activity was noticed between the 6th day and 10th day of fermentation. The timing between the two phases can be manipulated by medium optimization in order to improve the product yields.

Among the six different solvents tested for the extraction, yellow pigment got extracted well in all five solvents other than n-hexane. In antibacterial activity testing, all the five solvent extracts showed 19-25mm zone of inhibition against *S. aureus* MTCC96. In LRP assay, except n-hexane extract, all other extracts showed more than 80% reduction in RLU against *M. tuberculosis* H37Rv (Table 2).

Table 2: Quantity and activity of crude pigment extracted from strain D25 using different solvents.

Extracts	Quantity of crude extract (mg/40 ml of medium)	*S. aureus* MTCC 96 (zone of inhibition)	*M. tuberculosis* H37Rv (% reduction in RLU)
ME	41	21.3±0.57	88.58±2.31
ChE	30	23±1.00	84.74±3.60
EAE	42	25±0.00	90.65±2.09
DCME	35	21.3±0.82	91.59±4.02
DEEE	39	19.6±0.59	91.15±2.94
n-HE	-	0.00	0.00

ME – methanol extract; DCME – dichloromethane extract; DEEE – diethyl ether extract; ChE – chloroform extract; EAE – ethyl acetate extract; n-HE – n-hexane extract; 0 – No zone of inhibition

Most of the secondary metabolites including antibiotics produced by actinomycetes are extracellular in nature (Radhakrishnan *et al.*, 2011). In addition, the results evidenced that the bioactive metabolite present in the crude extract is polar or medium polar in nature. Of the various carbon sources tested, glucose, arabinose, xylose, mannitol, fructose, Inositol and rhamnose were found to influence the growth of strain D25. However, the pigment production and its activity were influenced only by glucose and fructose. Secondary metabolites such as antibiotics are frequently inhibited by a rapidly utilized carbon source such as glucose (Lounes *et al.*, 1996). The basic mechanism(s) of the phenomenon is not understood completely. Glucose is an excellent carbon source for growth, but a high concentration of glucose usually inhibits the biosynthesis of antibiotics (Chu and Li, 2002; Cao, 2003).

All nitrogen sources can be divided into two groups; inorganic nitrogen sources are regarded as quick metabolized nitrogen sources, which are beneficial for fast growth of microorganisms relieving the need of long-time accumulation of product. Moreover, the biosynthesis of antibiotics is also inhibited by rapidly utilized nitrogen sources such as ammonium and regulated by inorganic phosphate (Shikha *et al.*, 2005; Hulya and Tarhan, 2006). Whereas the organic nitrogen sources are sustainable nitrogen sources, which are beneficial for steady product accumulation (Baoxin *et al.*, 2011). In the present study, among the selected organic nitrogen sources tested, growth, pigment production and activity was influenced by the malt extract. Other nitrogen sources like asparagine and glutamine were influenced only the growth of *Streptomyces* sp D25.

Actinomycetes have the ability to tolerate wide range of pH and temperature. Alkaliphilic and halophilic actinomycetes and their antimicrobial and enzymatic potential were reported from desert ecosystems around the world (Hozzein *et al.*, 2004; Norovsuren *et al.*, 2007). Except the pH 5, all other pH ranges such as 7, 9 and 11 were found to enhance the growth, pigment production and activity. Except 7.5% and 10% of NaCl, all other concentrations of NaCl influenced the growth, pigment production and its bioactivity. Of the various temperature ranges tested, fermentation at 30^0C and 40^0C was found to influence the growth, pigment production and activity.

Although many studies of the mineral requirements of microorganisms for growth have been reported, there have been only a few detailed investigations on the requirements of actinomycetes for metals in the biosynthesis of antibiotics (Majumdar and Majumdar, 1965). El-Tayeb *et al.*, (2004) reported that the addition of KNo3 in the production medium was found to influence the anti TB antibiotic rifamycin production by *Amycolatopsis mediterranei* Among the different minerals tested, only the KNo3 was found to influence the growth, pigment production and activity.

Good correlation was noted between the growth, pigment production and its activity against *S. aureus* MTCC96 and *M. tuberculosis* H37Rv. Correlation between the quantity of crude pigment and bioactivity was also observed. But there is no correlation between growth and antimicrobial activity and growth and pigment production.

Table 3: Effect of agar surface fermentation conditions on the antibiotic production by the *Streptomyces* sp. D25.

			Bioactivity against		
Parameters	**Variables**	**Growth**	**Quantity of crude pigment (mg)**	***S. aureus MTCC96* (Zone of inhibition)**	***M. tuberculosis H37Rv* (% Reduction in RLU)**
Carbon source	Glucose	3+	50	10	80.12
	Arabinose	3+	0	0	0.00
	Sucrose	1+	15	7	52.45
	Xylose	3+	15	8	48.23
	Inositol	3+	0	0	0.00
	Mannitol	3+	0	0	0.00
	Fructose	3+	30	9	83.12
	Rhamnose	3+	0	0	0.00
	Raffinose	1+	0	0	0.00
	Cellulose	No Growth	0	0	0.00
Nitrogen source	Asparagine	3+	12	7	51.00
	Glutamine	3+	9	7	49.21
	Tyrosine	1+	9	9	45.20
	Malt extract	3+	47	13	87.21
	Peptone	2+	14	8	60.21
	Beef extract	2+	11	8	53.12
pH	5	No Growth	0	-	0.00
	7	3+	47	12	81.22
	9	3+	47	10	87.24
	11	3+	46	10	88.24
Temperature	10	No growth	0	0	0.00
	20	2+	39	11	75.24
	30	3+	45	12	83.12
	40	2+	43	9	85.44
	50	No Growth	0	-	0.00
Minerals	MgSo4	1+	7	7	32.21
	KNo3	3+	45	13	83.22
	KCl	2+	39	11	79.24
	MgCl2	2+	36	10	75.21
	FeSo4	1+	8	7	44.21
NaCl %	0	3+	48	13	78.22
	1	3+	51	11	84.12
	2.5	2+	47	13	85.39
	5	2+	47	14	86.24
	7.5	1+	25	9	66.21
	10	No Growth	0	-	0.00

3+ good; 2+ moderate; 1+ poor

CONCLUSION

Findings of the present study as well as our previous studies (Radhakrishnan *et al*., 2014b) revealed that solid state fermentation is the only choice for the production of antitubercular pigment from the desert soil *Streptomyces* sp D25. Because the synthetic and purified carbon and nitrogen sources are economically costly, utilizing cheaper substrates like agricultural wastes may be the economically viable alternative for the production of antitubercular pigment from *Streptomyces* sp D25.

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