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RESEARCH ARTICLE

Optimization of Cultural Conditions for the Antimetabolites Production by *Streptomyces aureus* strain BG03

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ABSTRACT

The growth of *Streptomyces aureus* strain BG03 was the best on Ken Knight`s agar medium. Among the different media tested, Crawford`s media was the best for the production of bioactive compounds by S. aureus strain BG03. The culture filtrate of *S. aureus* strain BG03 extracted from Crawford`s broth amended with starch as a carbon source, peptone as a nitrogen source, temperature of 35°C and pH 7.5 recorded the highest growth inhibition and inhibition zone against *Rhizoctonia bataticola* causing dry root rot in urdbean. Therefore the above mentioned parameters were optimum for the maximum production of bioactive compounds by *S. aureus* strain BG03. The crude metabolites extracted from S. aureus strain BG03 was found to be promising in reducing the mycelial growth of *R. bataticola.* Gas Chromatography-Mass Spectrophotometry (GC-MS), analysis of crude metabolites of *S. aureus* strain BG03 revealed the presence of seven compounds having antifungal activity.

Keywords: *Streptomyces aureus, Optimization, Cultural conditions, Antimetabolite production,*

Streptomyces is the largest genus of the actinomycetes which are Gram-positive, aerobic, non-motile with a filamentous form that resembles fungi. *Streptomyces* have relatively large genomes of approximately 8–9 Mbp in size with a high GC content of more than 70 per cent (Singh *et al.*, 2012). *Streptomyces* are prolific producers of wide variety of bioactive compounds which are found to have antifungal (Lim *et al.,* 2015), antibacterial, antiviral (Abd and Hatem, 2013), immunosuppressive, and antioxidant properties (Wang *et al.*, 2008). They account for nearly 60 per cent of the production of agriculturally important antibiotics (Liu *et al.*, 2013).

The production of secondary metabolites from the genus *Streptomyces* can be influenced by optimization of the nutritional requirements and cultural conditions. These conditions play an important role in the production of these secondary metabolites (Khattab *et al*., 2016). Antibiotic biosynthesis is a specific property of microorganisms which depends greatly on culture conditions. Improvement in the growth and antibiotic production can be carried out by manipulating the nutritional and physical parameters of the culturing conditions (Gao *et al.,* 2009).The present work was carried out with a view to investigate the effect of different media, carbon and nitrogen sources, on bioactive compound production by the *S. aureus* strain BG03. The influence of pH and temperature on bioactive compound production was also studied.

MATERIAL AND METHODS

Cultural characteristics of S. aureus strain BG03

Cultural characteristics *viz.,*growth pattern, color of aerial mycelium, color of substrate mycelium, and surface characteristics of the *S. aureus* strain BG03 were recorded on different media *viz*.,Ken Knight`s, Crawford`s, Potato dextrose, Yeast extract malt extract, Nutrient, Starch casein hydrolysate, starch agar, AF/MS, and Tryptone soya agar medium.

Optimization of cultural and nutritional conditions for the production of bioactive compounds

Streptomyces aureus strain BG03 was inoculated in different broth and incubated for 10 d at 30°C on a rotary shaker (150 rpm) to find out the broth which produces maximum bioactive compound. The media used were Knight`s, Crawford`s, Potato dextrose, Yeast extract malt extract, Nutrient, Starch casein hydrolysate, AF/MS, and Tryptone. After incubation actively grown culture of 100 ml quantity from each broth was centrifuged at 10,000 rpm at 4ºC for 10 min and the supernatant containing, culture filtrate of respective broth was

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obtained. From that culture filtrate 400µl of each broth were taken aseptically and the antifungal activity was measured using agar well diffusion assay.

Nitrogen source of Crawford`s broth (Yeast extract) was substituted with potassium nitrate, peptone, beef extract and malt extract inoculated with *S. aureus* strain BG03 and incubated for 10 d at 30°C on a rotary shaker (150 rpm). Culture filtrate of each nitrogen source broth was used to identify antifungal activity against *R. bataicola* by agar well diffusion assay.

The *S. aureus* strain BG03 was inoculated on Crawford`s broth (pH 7.0) at various levels of pH 6, 7.5, 8 and 9 using a phosphate buffer and incubated for 10 d at 30°C on a rotary shaker (150 rpm) to optimize the pH for maximum bioactive compound production in broth medium. Culture filtrate of each pH source broth was used to identify antifungal activity against *R. bataicola* by agar well diffusion assay.

The *S. aureus* strain BG03 was inoculated in Crawford`s broth and incubated at different temperatures 20,25, 30, 35 and 40°C for determining the optimum temperature for maximum bioactive production. After the incubation period the culture filtrate of broth at above mentioned temperature was prepared and tested for antifungal activity against *R. bataicola* by agar well diffusion assay.

Efficacy of crude metabolites against Rhizoctonia bataticola

The *S. aureus* strain BG03 was inoculated into Crawford's broth in a 250 ml conical flask, and the culture was incubated at 28°C for 48 h on a shaker at 120 rpm. At the end of incubation period the culture filtrate was collected by centrifugation at 10,000 rpm for 15 min and the supernatants were collected. Antimicrobial compound was partially purified from the culture filtrate by solvent extraction method. Ethyl acetate was added to the culture filtrate in the ratio of 1:1 (V/V) and shaken vigorously for 1 h for complete extraction. The ethyl acetate phase that contains bioactive metabolites was separated from the aqueous phase by using separating funnel. The ethyl acetate fraction was evaporated to dryness in a water bath and residue obtained was used to determine antifungal activities against *R. bataticola* by agar well diffusion method and effectiveness was measured by zone of inhibition.

Characterization of antibiotics

The crude metabolites from the *S. aureus* strain BG03 was analyzed through Gas Chromatography-Mass spectrometry (GCMS) at Agricultural College and Research Institute, Madurai. GC-MS analysis was carried out on a Shimadzu GCMS-QP2010 Ultra system. The injector temperature was 280°C. The samples were injected in the split mode with split ratio 1/25. Injection volume was 1 µL. A capillary column Rtx-5MS (5% Diphenyl-95% Dimethyl Polysiloxane), 30 m x 0.25 mm x 0.25µm, was used. Carrier gas was helium with constant flow of 1.00 ml min-1. The oven temperature was as follows: initial temperature of 60°C, held for 2 min, increased to 10° C min-1 up to 260°C and held for 10 min. The MS ionization potential was 70 eV, and the temperatures were as follows: interface 260° C, Ion source 280°C. Mass scan range 40-550.

RESULTS AND DISCUSSION

The actinobacterial isolate *S. aureus* strain BG03 was grown on different solid media *viz.,*Crawford`s agar, Ken Knight`s agar, Potato dextrose agar, Yeast extract malt extract agar, Nutrient agar, Starch casein hydrolysate agar, Starch agar, AF/MS agar, Oat meal agar, Tryptone soya agar to determine the best media for its growth.

Table 1. Cultural characteristics of *S. aureus* strain BG03 on different agar media

Medium	Cultural characterization			
	Growth	Aerial mycelium	Substrate mycelium	Surface
Crawford`s agar	Good	White	Pale yellow	Slight powdery
Ken Knight`s agar	Excellent	Whitish yellow	Pale yellow	Powdery
Potato dextrose agar	Moderate	White	Pale yellow	Slight powdery
Yeast extract malt extract agar	Moderate	Pure white	Pale yellow	Slimy
Nutrient agar	No growth	-	-	-
Starch casein hydrolysate agar	Excellent	White	Pale yellow	Slimy
Starch agar	Excellent	White	Pale yellow	Slight powdery with Slimy
AF/ MS agar	No growth	-	-	-
Tryptone soya agar	Good	Creamy white	Pale yellow	Slimy
Oat meal agar	No growth	-	-	-

The result indicated that the growth of *S. aureus* strain BG03 was excellent in Ken knight`s agar, starch casein hydrolysate and starch agar medium. The colour of the aerial mycelium was white to creamy white and the substrate mycelium was pale yellow in colour and the colony showed powdery to slimy texture. The growth of *S. aureus* strain BG03 was found to be good in Tryptone soya agar, Crawford`s agar and it was moderate in Potato dextrose agar, Yeast extract malt extract agar. On oat meal agar and nutrient agar medium no growth of *S. aureus* BG03 was observed (Table 1). Yasmeen *et al.* (2016) found that *Streptomyces* sp. strain VJSY-2 exhibited excellent growth on YMD agar medium and *S.carpaticus* strain VJSY-3 exhibited good growth on tryptone yeast extract agar (ISP-1), and tyrosine agar.

Optimization of cultural condition for production of bioactive compounds by S. aureus strain BG03

The actinobacteria *S. aureus* strain BG03 was grown in different medium for 10 d to determine the best medium for the maximum production of bioactive compounds. The culture filtrate of *S. aureus* train BG03 from each culture broth was extracted and it was used for assessing the antifungal activity against the growth of *R. bataticola*. The result showed that *S. aureus* strain BG03 had the highest antifungal activity in Crawford`s broth which recorded 49.26 percent growth inhibition and 40.33 mm inhibition zone. It was followed by starch casein broth with 38.88 per cent and 30.00 mm growth inhibition of *R. bataticola* and inhibition zone respectively. The lowest inhibition of 2.96 per cent and inhibition zone of 0.0 mm was recorded by nutrient broth. Hence, the Crawford`s broth which showed highest growth inhibition of *R. bataticola* was suitable media for bioactive metabolite production (Table 2)

Table 2. Effect of different media on the antifungal activity of *S. aureus* strain BG03 culture filtrate against *R. bataticola*.

Medium	Mycelial growth of pathogen (mm)	Per cent reduction over control	Inhibition zone (mm)*
Ken Knight`s	61.33	31.85^{c} (34.35)	10.00^{d}
Crawford`s	45.66	49.26^{a} (44.57)	40.33^{a}
Potato dextrose	78.33	12.96^{f} (21.10)	00.20^{f}
Yeast extract malt extract	69.00	23.33^{e} (28.88)	10.46^{c}
Nutrient	87.33	2.96^{g} (09.90)	0.00^{h}
Starch casein	55.00	38.88^{b} (38.57)	30.00^{b}
AF/ MS	68.66	23.71^{e} (29.13)	00.76^{e}
Tryptone	65.00	27.77^{d} (31.80)	00.04^{g}
Control	90.00	0.00^{h} (0.64)	00.00^{h}
CD(p=0.05)		0.10	

*Values are mean of three replications

Values in parentheses are arcsine transformed values

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

In this investigation *S. aureus* strain BG03 grown in Crawford`s broth exerted maximum inhibition of *R. bataticola* with highest inhibition zone. This indicated that Crawford`s broth favour the highest production antibiotics of *S.aureus* when compared to other media. Starch casein broth ranked next in enhancing the production of bioactive compounds. Singh *et al.* (2017) found that starch casein broth was the best medium for the antibiotic production by *S. chilikensis* strain ACITM-1. *Streptomyces* sp. grown in yeast malt medium showed the maximum growth inhibition of *M. phaseolina* (Verma *et al.*, 2018).

The Crawford`s broth was amended with various carbon sources *viz.,* fructose, sucrose, starch and maltose to determine the best carbon source for bioactive compound production. Among these, culture filtrate of *S. aureus* strain BG03 grown in media amended with starch was found to be significantly superior in inhibiting the growth of *R. bataticola* with 46.30 per cent growth inhibition and 20.10 mm inhibition zone. It was followed by sucrose which exhibited growth inhibition of 33.17 per cent. The metabolites production by *S. aureus* strain BG03 was low in fructose amended medium as it inhibited the mycelial growth of *R. bataticola* only to the extent of 27.91 per cent (Table 3).

Carbohydrate *viz.,* glycerol, maltose, mannose, sucrose and xylose are reported to interfere with production of metabolites (Shobha *et al.*, 2014). In this study among the different carbon source, starch was found to be the best for the production of bioactive compound by *S. aureus* strain BG03 this findings was in concordance

with Osman *et al.* (2011) who found that starch was the best carbon source for antibiotic production. Similar results were also reported by El-Nasser *et al.* (2010) and Singh *et al.* (2017).

The growth and antimetabolite production is greatly influenced by the nature and type of nitrogen source supplemented in the Crawford`s broth. The antifungal activity of *S. aureus* strain BG03 was found to be high 45.93 per cent growth inhibition and 20.10 mm inhibition zone in medium amended with peptone. The culture filtrate of potassium nitrate incorporated medium recorded the lowest growth inhibition (10.34 %) against *R. bataticola* (Table 3).

Table 3. Effect of nutritional conditions on the antifungal activity of *S. aureus* strain BG03 culture filtrate against *Rhizoctonia bataticola.*

Nutritional							
Carbon				Nitrogen			
Source	Mycelial growth of pathogen (mm)	Per cent reduction over control	Inhibition zone (mm)*	Source	Mycelial growth of pathogen (mm)	Per cent reduction over control	Inhibition zone (mm)*
Fructose	65.33	27.41^{d} (33.96)	0.23^{d}	**Potassium nitrate**	79.66	10.34^{d} (18.75)	0.26^{d}
Maltose	63.83	29.07^{c} (28.77)	0.33^{c}	**Peptone**	48.66	45.93^{a} (42.66)	20.10^{a}
Starch	48.33	46.30^{a} (25.23)	20.10^{a}	**Beef extract**	78.66	12.60^{b} (20.79	10.80^{b}
Sucrose	59.66	33.71^{c} (31.62)	0.60^{b}	**Malt extract**	87.33	02.90^{c} (09.80)	0.90^{c}
Control	90.00	00.00^{d} (09.97)	0.00^{d}	Control	90.00	00.00^{e} (00.44)	0.00^{e}
CD (p=0.05)		0.12		CD (p=0.05)		0.05	

*Values are mean of three replications

Values in parentheses are arcsine transformed values

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

It is evident from the present study *S. aureus* strain BG03 showed the highest antifungal action against *R. bataticola* when it was grown in medium amended with peptone as a nitrogen source. This was attributed to the addition of peptone in the medium enhanced that production of bioactive compound, therefore peptone was found to be good nitrogen source for antibiotic production. This was in agreement with findings of Bundale *et al.* (2015) who found that medium amended with peptone resulted in maximum bioactive metabolite production in *S. spectabilis*. Singh *et al*. (2017) reported that peptone and potassium nitrate were best the nitrogen source for the highest antifungal activity and biomass production for *S. chilikensis* strain ACITM-1.

pH of the culture medium affects not only growth of the *Streptomyces* but also production of the antibiotics. The result on pH of the medium revealed that *S. aureus* strain BG03 showed the maximum antifungal activity against *R. bataticola* at pH 7.5. At this pH, *S. aureus* strain BG03 recorded the highest growth inhibition 48.74 per cent with the inhibition zone of 20.00 mm. Growing of *S. aureus* at pH 6.0 and 9.0 was not suitable for antimetabolite production as indicated by the lowest growth inhibition of *R. bataticola* at these pH (Table 4).

Table 4. Effect of physical conditions on the antifungal activity of *S. aureus* strain BG03 culture filtrate against *Rhizoctonia bataticola*.

Physical						
PH				Temperature (^{o}c)		
Level	Inhibition zone (mm)*	Mycelial growth of pathogen (mm)	Per cent reduction over control	Level	Inhibition zone (mm)*	Per cent reduction over control
6.0	0.36^{b}	87.66	12.60^{b} (20.79)	**20**	10.40^{d}	35.68^{c} (36.67)
7.5	20.06^{a}	80.33	48.74^{a} (44.27)	**25**	10.76^{c}	14.45^{e} (22.34)
8.0	0.03^{c}	87.83	22.90^{c} (28.59)	**30**	20.10^{b}	40.74^{b} (39.66)
9.0	0.00^{d}	88.5	11.60^{d} (19.91)	**35**	20.60^{a}	46.90^{a} (43.22)
Control	0.00^{d}	90.00	0.00^{d} (0.44)	**40**	0.23^{e}	15.93^{d} (23.52)
CD (p=0.05)			0.05	Control	0.00^{f}	00.00^{f} (00.44)
				CD (p=0.05)		0.10

*Values are mean of three replications

Values in parentheses are arcsine transformed values

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Changes in the initial external pH affect many cellular processes *viz.,*regulation and biosynthesis of secondary metabolites (Reddy *et al.*, 2011). Optimum pH for antibiotic production in *Streptomyces* cultures has been reported to be near neutral (Oskay *et al.*, 2004). In this investigation the optimum pH for the maximum production of antibiotics was found to be pH 7.5. The actinobacteria *S. aureus* strain BG03 cultivated at this pH showed the highest antifungal activity against *R. bataticola* compared to other pH level. Similar findings were earlier reported by Singh *et al.* (2017) who observed maximum antibiotic production in *S. chilikensis* ACITM-1 at pH 7.5.

Table 5. Antimicrobial compounds identified in the ethyl acetate extract of *S. aureus* strain BG03 by GC-MS analysis

R. Time	Compounds	Molecular formula	Compound nature	Area %	Activity	References
18.79	9,12- Octadecadienoic acid (Z,Z)- methyl ester	$C_{19}H_{34}O_2$	Linoleic acid	40.47	Antifungal, Nematicide	Yu *et al*., 2005
18.98	6- Octadecenoic acid, methy ester,(Z)	$C_{19}H_{36}O_2$	Stearic acid	1.41	Antifungal	Rahuman *et al*., 2000
21.06	1h-1, 2, 4- triazole – 1-ethanol, alpha-butyl	$C_{14}H_{17}Cl_2N_3O$	Alcohol	4.32	Antifungal, antimicrobial.	Chandrasekaran *et al*., 2011
14.10	Pyrrolo{1,2-a}pyrazine-1,4-dione, hexahydro-3 (2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	Alkaloid	1.34	Antimicrobial, anti-inflammatory	Yassa *et al*., 2009
11.52	1- Nonadecene	$C_{19}H_{38}$	Alkene compound	0.85	Anti-fungal activity	Dalli *et al*., 2007
5.88	Undecane	$C_{11}H_{24}$	Alkene compound	0.11	Anti-fungal activity	Dalli *et al*., 2007
11.96	Pentadecanoic acid	$C_{15}H_{30}O_2$	Fatty acid	0.33	Antibacterial, Antifungal	Aparna *et al.,* 2012
19.18	9-Octadecenoic acid (z)-, methyl ester	$C_{19}H_{36}O_2$	Oleic acid methyl ester	1.00	Antimicrobial Nematicidal	Yu *et al*., 2005
9.18	Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	Alkane compound	0.21	Antifungal, Nematicide	Balogun *et al*., 2013
22.57	Docosane	$C_{22}H_{46}$	Acyclic alkanes	0.46	Antimicrobial	Ghosh *et al*., 2011

Praveen *et al.,* (2008) reported that extreme pH was unfavorable for production of antibiotics. The optimum pH for growth and bioactive production in *S. purpurascens* (R3) was reported to be pH 7.0 (Bundale *et al.*, 2015). Ahmed *et al*., (2016) found that *Streptomyces* strain (SP 1 and SP 28) showed high antifungal activity at the pH ranged from 7-7.5.

The incubation temperature also was found to have an effect on growth as well as bioactive metabolite production. The culture filtrate of *S. aureus* strain BG03 extracted from the medium incubated at 35° C showed the highest growth inhibition of *R. bataticola* (46.90 % inhibition and 20.66 mm inhibition zone). The medium incubated at 30° C reduced the mycelial growth of *R. bataticola* by 5.68 per cent. The highest temperature of 40° C showed the lowest growth inhibition of 15.93 mm with inhibition zone of 0.23 mm (Table 4).

It was evident from the present study *S. aureus* strain BG03 showed antifungal action against *R. baticola* at 35° C. The media incubated at 30° C also inhibited the mycelial growth of *R. bataticola* to an extend of 35.68 per cent. Therefore the optimum temperature for the maximum production of bioactive metabolites in *S. aureus* strain BG03 found to be 35° C. The maximum antibiotics production by *S. violates* was observed at 30° C (Hassan *et al.*, 2018). *Streptomyces* sp. KGG32 produced maximum biomass and antimicrobial production at 30 ˚C (Oskay *et al.*, 2004). The optimum growth and antimicrobial compound production of *S. chilikensis* ACITM-1 was reported to be 30° C (Singh *et al*., 2016).

Characterization of antibiotics produced by S. aureus strain BG03

The crude metabolites from the *S. aureus* strain BG03 was analyzed through Gas Chromatography-Mass Spectrometry (GCMS) at Agricultural College and Research Institute, Madurai. A total of seven compounds with antifungal activity were identified from *S. aureus* strain BG03, among these four compound were identified as major compounds *viz*., 6- Octadecenoic acid, methy ester,(Z), 9,12-Octadecadienoic acid (Z,Z)- methyl ester, 1h-1, 2, 4- triazole–1- ethanol, alpha-butyl and undecane were having maximum probability in the crude metabolite extracts (Table 5)

This results were in agreement with findings of Awla *et al*., (2016) and Taswar *et al*., (2017). twenty seven different organic compounds were detected from the crude extract of *Streptomyces* EF37141 through GC– MS analysis. These compounds were antimicrobial and antifungal (Taswar *et al*., 2017).

CONCLUSION

From this study, it was concluded that Ken Knight`s agar medium is the best medium for growth *S. aureus* strain BG03. The Crawford`s media amended with starch as the carbon source and peptone as the nitrogen source with the pH of 7.5 and incubated at the temperature of 35° C were found to be optimum for the maximum production of bioactive compound by *S. aureus* strain BG03.

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