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Bioautography guided Screening of Antimicrobial Compounds Produced by *Microbispora* V2

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Abstract

The aim of this study was to investigate the antimicrobial activity of MicrobisporaV2. The genus Microbispora is known to produce antimicrobial compounds like phenazines and others. The isolate Microbispora V2 can be exploited to mine out its capabilities of producing antimicrobial compounds utilizing cost effective production media. The screening is defined as the first step, which is applied to a sample, in order to establish the presence or absence of antimicrobial compounds. Bioautography belongs to microbiological screening methods commonly used for the detection of antimicrobial activity of a compound under study. Bioautography is a technique that combines thin Layer chromatography with bioassay in situ. It is one of the simplest and cheapest methods for detecting antimicrobial compounds in partially purified extracts because the method is easy to run, reproducible and requires less equipments. The fermentation process can be made cost effective by utilizing renewable energy sources like oil cake as sources of macronutrients of fermentation media. Therefore various oil cakes were chosen as nutrient source of fermentation media. Hence bioautography was used as a tool to screen out the production of antimicrobial compounds by Microbispora V2 employing oil cake fermentation media.

Keywords: Bioautography, screening, antimicrobial compounds, *microbispora*V2, *sclerotium rolfsii*, *Bacillus cereus.*

Introduction

The genus *Microbispora* is known to produce antimicrobial compounds like phenazines, phenoxaziones and C2-symmetrical phenazines, nebularine gluosylquestiomycin, antifungal triacetylene dioxolone, tyramine and indole alkaloids, microbiaeratin, bispolides and microbisporicin a lantibiotic[1-10]. The isolate *Microbispora* V2 can be exploited to mine out its capabilities of producing the antimicrobial compounds utilizing cost effective production media.

The antimicrobial activity of an isolate can be detected by various methods like dilution methods (agar diffusion and MIC) and bioautography methods. Bioautography is sensitive method for detection of antimicrobial compounds even in small amounts[11]. Hence for detection of antimicrobial compounds, bioautography is suitable method in initial stages as compared to agar dilution methods[12,13].

Bioautography can be employed in the target directed isolation of active constituents[14]. Paper chromatography followed by bioautography was used for the first time in 1946 by Goodal and Levi to estimate the purity of penicillin[15]. Thin layer chromatography - bioautography was introduced by R. Fisher and H. Lautner in 1961[16]. There are three different approaches for bioautography to localize antimicrobial activity on a TLC chromatogram - agar diffusion or contact bioautography, direct bioautography and immersion or agar overlay bioautography[17,18]. Those assays supply a quick screen for new antimicrobial compounds through bioassay-guided isolation[19].

The fermentation process can be made cost effective by utilizing renewable energy sources like oil cake as sources of macronutrients of fermentation media. Oil cakes/oil meals are by-products obtained after oil extraction from the seeds. Oil cakes are of two types, edible and non-edible. Edible oil cakes have a high nutritional value; especially have protein content ranging from 15% to 50% (www.seaofindia.com). Their composition varies depending on their variety, growing condition and extraction methods. Various types of edible oil cakes are available in and around Pune, Maharashtra, India. The oil cakes chosen as nutrient source of fermentation media are almond (*Prunus dulcis),*coconut (*Cocos nucifera*), cotton seed (*Gossypium arboreum*), flax (Linseed) (*Linum usitatissimum*), groundnut (*Arachis hypogaea*), mustard [black mustard (*Brassica nigra*)], safflower (*Carthamus tinctorius*), sesame [white (*Sesamum indicum*)], soybean (*Glycine max*) and sunflower (*Helianthus annuus*).

Hence bioautography was used as a tool to screen out the production of antimicrobial compounds by *Microbispora* V2 employing oil cake fermentation media.

Material and Methods

The strain of *Microbispora* V_2 was kindly provided by Dr Neelu Nawani isolated from hot water spring at Vajrashwari near Mumbai, Maharashtra, India.

Preparation of inoculum of *Microbispora*V2: The oil cakes were used as carbon and nitrogen source in growth media.[20] The

oil cakes employed for making fermentation media are: i. Almond (*Prunus dulcis),* ii. Coconut (*Cocos nucifera*), iii. Cotton seed (*Gossypium arboreum*), iv. Flax (Linseed) (*Linum usitatissimum*), v. Groundnut (*Arachis hypogaea*), vi. Mustard [black mustard (*Brassica nigra*)], vii. Safflower (*Carthamus tinctorius*) viii. Sesame [white (*Sesamum indicum*)], ix. Soyabean (*Glycine max*), x. Sunflower (*Helianthus annuus*).

The oil cake of each type was added as 0.75 gm% to minimal agar. The minimal agar composition is (gm/100ml): $MgSO_4$ -0.05, K2HPO4 -0.02, KH2PO4 -0.03, agar -2.0 and pH7.0. After autoclaving, sterile 1 ml trace salt solution is added. The composition of trace salt solution (gm/100ml): $FeSO_4.7H_2O$-0.1, $ZnSO_4.7H_2O$-0.1and $MnCl_2.7H_2O$-0.1.

*Microbispora*V2 was inoculated on sterile oil cake agar medium and incubated at 40°C for 7 days. After incubation, growth was harvested in sterile teepol saline(0.02%). 1% inoculum of *Microbispora*V2 ($0.2A_{540}=10^7$spores/ ml) was added into sterile respective oil cake fermentation broth (100ml in 500ml Erlenmeyer flask) and incubated at 40˚C with agitation 180 rpm for 10 days on rotary shaker. The fermented broth was centrifuged at 5000 rpm for 15 minutes. The cell free supernatant was used for extraction of antimicrobial compounds and the pellet was preserved as innoculum.

Extraction of antimicrobial compounds and down streaming: Chloroform was added to cell free supernatant in 1:1 proportion. Organic phase was taken and kept at 40 ˚C for evaporation of chloroform. Residue was redissolved in 2ml chloroform and used for bioautography.

Detection of antimicrobial activity of *Microbispora* V2 by bioautography: Test microorganisms selected for antimicrobial activity: i. *Bacillus cereus*: It is the causative agent of food-borne illnesses. The majority of *B. cereus* strains appear to be capable of producing either diarrhoeal or emetic toxin[21-23]. ii. *Sclerotium rolfsii*: It is a versatile soil born pathogen attacking several crop plants. It is found to cause variety of diseases namely damping of seedlings, collar or stem rot, foot rot, crown rot, Sclerotium wilt and blight[24, 25].

The bioautography: The agar overlay method (immersion bioautography) was used for detection of antimicrobial compounds. TLC plate (Merck Silica Gel 60 $F_{254)}$ was loaded with 10µl of solvent extract of fermented oil cake medium in duplicate. The solvent system used was Butanol: acetic acid (90:10). The chromatogram was kept for evaporation of the solvent. Developed chromatogram was placed on sterile potato dextrose agar plate (15 ml) for detection of antifungal activity and on sterile Muller Hinton agar plate (15 ml) for detection of antibacterial activity. For detection of antifungal activity, molten potato dextrose agar 5 ml seeded with 1ml of spore suspension of *Sclerotium rolfsii* ($0.2A_{540}$ $=10^7$spore/ml) was poured on chromatogram. For detection of antibacterial activity, Molten Muller Hinton agar 5 ml seeded with 1ml of spore suspension of *Bacillus cereus* ($0.1A_{540}=10^7$spores/ ml) was poured on chromatogram. After agar got solidified the petri plates were kept at 4˚C for diffusion for 3 hours. Plates were then incubated at room temperature for 24 hours for bacterial culture and for 4 days for fungal culture. The antimicrobial activity was checked along with standard 2 hydroxyphenazine and phenazine-1-carboxylic acid (Colour your enzyme, Bath, Ontario, Canada). After incubation plates were checked for zone of inhibition using 2.0mg/ml phenyl tetrazolium chloride.[26]

Results and Discussion

The chloroform extract of cell free supernatants of oil cake broths fermented by *Microbispora* V2 were assessed for antimicrobial activity against targeted pathogens *Bacillus cereus* and *Sclerotium rolfsii* by immersion bioautography method. The R_f of the spots detected by bioautography were identical with the standards i.e. R_f of phenazine-1-carboxylic acid was found out to be 0.17 and of 2 hydroxyphenazine was 0.04. The maximum zones of inhibition of the pathogens were seen in case of sesame oil cake medium, 30mm and 20mm against *Bacillus cereus* and *Sclerotium rolfsii* respectively (figure 1). The chloroform extracts of cell free supernatants of ground nut and soybean oil cake fermented media exhibited only antifungal activity.

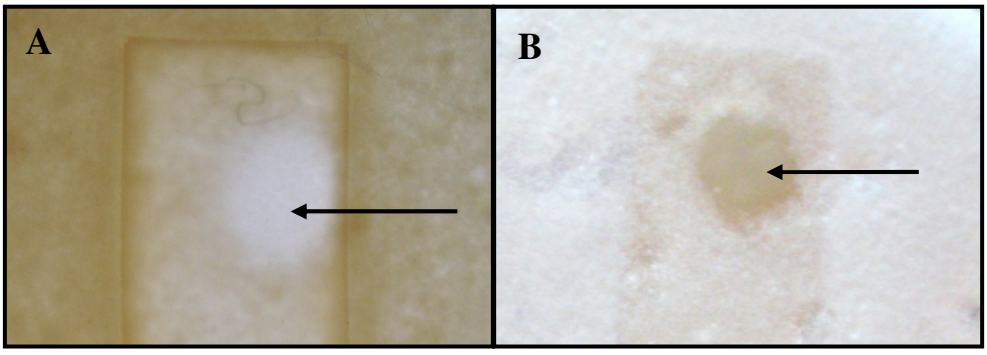

Figure-1
Antimicrobial activity solvent extract of cell free supernatant of sesame oil cake broth fermented by M*icrobispora* V2 (Growth inhibition zones are indicated with arrows. X.15) (A) Zone of inhibition of *Bacillus cereus* after incubation for 24 hours at 37ºC (Medium-Muller-Hinton Agar); (B) Zone of inhibition of *Sclerotium rolfsii* after incubation for 4 days at 30ºC (Medium-Potato dextrose agar), (Sony Camera, Model-DSC-450:15X optical zoom; 9.1mega pixels

Antimicrobial activity was not detected in solvent extracts of cell free supernatants of other oil cake broth fermented by *Microbispora* V2 (table 1). Solvent extract of cell free supernatant of pablum medium fermented by *Microbispora* V2 also showed antimicrobial activity but was less than in case of sesame oil cake medium.

Table-1
Antimicrobial activity* of crude extract of *Micrbispora*V2

Sr. No.	Oil cake medium	Inhibition zone (mm) against *Bacillus cereus Sclerotium rolfsii*	
1	Almond	-	-
2	Coconut	-	-
3	Cotton seed	-	-
4	Flaxseed	-	-
5	Groundnut	-	08
6	Mustard seed	-	-
7	Safflower seed	-	-
8	Sesame	30	20
9	Soybean	-	08
10	Sunflower	-	-
11	Pablum	08	15

*Antimicrobial activity assayed by bioautography. Fermentation of medium containing oil cake by *Microbispora*V2 was carried out at 40^0C for 10 days. Antimicrobial compound was extracted using chloroform and cell free broth in 1:1 proportion.

Conclusion

There is a need for isolation, characterization, determination of antimicrobial activity of the lead compound for its applications in various fields. Analytical methods play important roles in the discovery, development and production of antimicrobial compounds. The common methods employed in studying production of antimicrobial compounds include the, separation and isolation of the individual components from partially purified extracts derived from fermented media. Further screening of partially purified extracts is done for targeted isolation of new or useful types of compounds with potential antimicrobial activities. The bioautography is a preferred tool in detecting the presence of antimicrobial compounds in extracts at the earliest stages of down streaming. This makes the screening programme not only economic but also saves the time required for whole screening process. Thus bioautography guided screening of antimicrobial compounds produced by *Microbispora* V2 lead to the findings that the isolate produces antimicrobial compounds, phenazines-1-carboxylic acid and 2-hydroxyphenazine when sesame oilcake medium was employed as fermentation medium. These compounds were further purified by preparative TLC and structural elucidation was carried out by spectrophotometric methods along with the standards (data not shown).

References

1. Gerber N.N. and Lechevalier M.P. Phenazines & phenoxazinones from *Waksmania aerata* sp.nov. and *Pseudomonas* sp., *Biochemistry*, **3(4)**, 598–602 **(1964)**

2. Gerber N.N. and Lechevalier M.P., 1, 6-Phenazinediol-5-oxide from Microorganisms, *Biochemistry*, **4(1)**, 176–180 **(1965)**

3. Tanabe I., Kuriyama M. and Nonomura H., Production of C2 – symmetrical phenazines by some actinomycetes, *Journal of fermentation and bioengineering*, **l79(4)**, 384-386 **(1995)**

4. Cooper R., Horan A.C., Gunnarsson I., Patel M. and Truumees I., Nebularine from a novel *Microbispora* sp., *Journal of Industrial Microbiology and Biotechnology*., **19(4)**, 275-276 **(1986)**

5. Igarashi Y., Takagi K., Kajiura T., Furumai T. and Oki T. Glucosyl questiomycin, a novel antibiotic from *Microbispora* sp. TP-A0184: fermentation, isolation, structure determination, synthesis and biological activities, *J Antibiot* (Tokyo), **51(10)**, 915-920 **(1998)**

6. Patel M.G., Horan A.C., Marquez J.A. and Waitz J.A. Antifungal triacetylene dioxolone from *Microbispora* sp SCC1438 ATCC 53620, U.S. Patent 4, 956, 383 **(1990)**

7. Ivanova V., Graefe U., Schlegel R., Schlegel B., Gusterova A., Kolarova M. and Aleksieva K., Isolation and structure elucidation of tyramine and indole alkaloids from antarctic strain *Microbispora aerata imbas*- 11A, *Biotechnology and Biotechnological Equipments*, **17(2)**, 128-133 **(2003)**

8. Ivanova V., Kolarova M., Aleksieva K., Gräfe U., Dahse H.M. and Laatsch H., Microbiaeratin, a new natural indole alkaloid from a *Microbispora aerata* strain, isolated from Livingston Island, Antarctica. *Prep Biochem Biotechnol,* **37(2)**, 161-168 **(2007)**

9. Okujo N., Iinuma H., George A., Eim K.S., Li T.L., Ting N.S., Jye T.C., Hotta K., Hatsu M., Fukagawa Y., Shibahara S., Numata K. and Kondo S. Bispolides, novel 20-membered ring macrodiolide antibiotics from *Microbispora*. *J. Antibiot*., **60(3)**, 216–219 **(2007)**

10. Foulston L.C. and Bibb M.J., Microbisporicin gene cluster reveals unusual features of lantibiotic biosynthesis in actinomycetes, Proceedings of National Academy of Sciences of the United States of America, **107(30)**, 13461–13466 **(2010)**

11. Burkhead K.D., Schisler D.A. and Slininger P.T., Bioautography shows antibiotic production by soil bacterial isolates antagonistic to fungal dry rot of potatoes, *Soil Biology and Biochemistry,* **27(12)**, 1611- 1616 **(1995)**

12. Thomshow L.S., Bonsall R.F. and Weller D., Detection of Antibiotics Produced by Soil and Rhizosphere Microbes In

Situ. Book- Secondary Metabolites in Soil Ecology Book Series -Soil Biology, ISSN-1613- 3382 Volume 14 Publisher- Springer Berlin Heidelberg **(2008)**

13. Choma I.M. and Grzelak E.M., Review- Bioautography detection in thin-layer chromatography, *Journal of Chromatography A*., **1218**, 2684–2691 **(2011)**

14. Hostettmann K., Strategy for the Biological and Chemical Evaluation of Plant Extracts, www.iupac.org/symposia/proceedings/phuket97 **(1999)**

15. Goodall R.R. and Levi A.A., A microchromatographic method for the detection and approximate determination of the different penicillins in a mixture, *Nature,* **158**, 675-676 **(1946)**

16. Betina V., Bioautography in paper and thin-layer chromatography and its scope in the antibiotic field, *J. Chromatography,* **78**, 41-51 **(1973)**

17. Cos P., Vlietinck A.J., Berghe D.V. and Maes L., Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept', *J. Ethnopharmacol*., **106**, 290-302 **(2006)**

18. Rios J.L., Recio M.C. and Villar A., Screening methods for natural products with antimicrobial activity: A review of the literature, *J. Ethnopharmacol,* **23**, 127–149 **(1988)**

19. Moreno M.I.N., Isla M.I., Cudmani N.G., Vattuone M.A. and Sampietro A.R., Screening of antibacterial activity of *Amaicha del Valle* (Tucumán, Argentina) propolis, *J. Ethnopharmacol*., **68**, 97-102 **(1999)**

20. Arun D. and Dharmalingam K., *Streptomyces peuticus* converts anthracycline intermediates efficiently in culture media containing oil cake as carbon source, *World Journal of Microbiology and Biotechnology,* **15(2),** 333-334 **(1999)**

21. Beattie S.H. and Williams A.G., Detection of Toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. with an improved cytotoxicity assay, *J. Appl. Microbiol*., **82**, 677-682 **(1999)**

22. Rusul G. and Yaacob N.H., Prevalence of *Bacillus cereus* in selected foods and detection of enterotoxin using TECRA-VIA and BCET-RPLA, *Int. J. Food Microbiol*., **25**, 131-135 **(1995)**

23. Kashid S.G. and Ghosh J.S., Production, Isolation and Characterization of Exotoxin Produced by *Bacillus cereus* NCIM-2156 and *Bacillus licheniformis* NCIM-5343, *British Journal of Pharmacology and Toxicology,* **1(1)**, 50-55 **(2010)**

24. Khond M., Bhosale J.D., Arif T., Mandal T.K., Padhi M.M. and Dabur R., Screening of Some Selected Medicinal Plants Extracts for In-vitro Antimicrobial Activity, *Middle-East Journal of Scientific Research,* **4(4)**, 271-278 **(2009)**

25. Punja Z.K., The biology, ecology and control of *Sclerotium rolfsii*, *Annual Review of Phytopathology,* **23,** 97-127 **(1985)**

26. Singh R.K. and Dwivedi R.S., Ecology and biology of *Sclerotium rolfsii* Sacc, *International Journal of Tropical Plant Diseases,* **9**, 161-171 **(1991)**