Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

ISSN: 0972-6268	Vol. 13	No. 3	pp. 641-644	2014

Original Research Paper

Evaluation of Antimicrobial Pigment Produced by *Streptomyces coeruleorubidus*

S. Azimi, M. Baserisalehi and N. Bahador*
Department of Microbiology, Islamic Azad University, Kazerun Branch, Kazerun, Iran
*Department of Microbiology, Islamic Azad University, Fars Science Research Branch, Fars, Iran
Corresponding Author: Saba Azimi

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com
Received: 26-11-2013
Accepted: 18-12-2013

Key Words:
Antimicrobial pigment
Actinomycetes
Streptomyces coeruleorubidus

ABSTRACT

The bacterial pigments are secondary metabolites that help the producer to survive in different environments. The bacterial pigments have shown different properties such as antitumor, anticancer and antimicrobial effects. The present study was conducted to isolate soil origin Actinomycetes and to evaluate their antimicrobial pigments. In total, eighty seven soil samples were collected from different areas in Kazeroun city, Iran. The samples were serially diluted (10^{-1} to 10^{-7}) and 0.1 mL of each dilution was streaked on SCA and ISP2 media and incubated at 30C° for 5 days. Then pigmented Actinomycetes were selected and subjected for propagation and pigment extraction using different solvents. To continue the experiments, the antimicrobial property of each pigment was evaluated against *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Shigella dysentriae, Citrobacter ferundii, Klebsiella peumoniae, Serratia marcecens*, *Aspergillus* sp. and *Candida albicans*. Furthermore, identification of the promising strain was verified by 16srDNA gene sequencing method and finally different groups in the pigment structure were determined by TLC and reagent spray tests. The results obtained indicated that out of all the isolated Actinomycetes strains, *Streptomyces coeruleorubidus* could produce pigment with antimicrobial property. Of all the microorganisms tested *Staphylococcus aureus* was more sensitive and *Aspergillus* sp., *Candida albicans* and *E. coli* were more resistant. Our finding concerning to structure of the pigment illustrated existence of alcohols, phenols and steroid groups in the pigment. Overall, soil origin Actinomycetes could produce pigments with antimicrobial property. Therefore, Actinomycetes pigments must be considered a special remedy for investigation in order to eliminate occurrence of antibiotic resistant microorganisms.

INTRODUCTION

The genus of Actinomycetes includes rod and filamentous Gram-positive bacteria with GC content 63-78%. These bacteria are nonmotile, heterotrophic and could produce exospores (Goodfellow et al.1993). Actinomycetes produce several Actinomycetes metabolites, viz., antibiotics, antitumor, different enzymes and pigments (Aust 2009). These bacteria produce sticky pigmented colonies with various colours and physiological properties (Conn & Jean 1941).

Nowadays, more than 23000 secondary metabolites have been documented, of which most of them are produced by Actinomycetes. Though, several papers have been published on different properties of Actinomycetes metabolites, but sufficient information on the pigments produced by these bacteria is not available (Woznicka 1964). Although, application of synthetic pigments is strongly eliminated because of their hazardous effects, natural pigments and their application in food, cosmetic and drug industries has been recommended. Hence, production and application of pigments produced by plants and microorganisms have been suggested as special area of investigation (Mizukami et al. 1978). On the other hand, antimicrobial activities of the microbial pigments are noted for investigation in order to eliminate occurrence of antibiotic resistant bacteria. Therefore, the present study was undertaken to evaluate antimicrobial effect of the Actinomycetes pigments to introduce the new source of remedy.

MATERIALS AND METHODS

Isolation and identification of Actinomycetes**:** Eighty seven soil samples were collected from different fields of Kazerun city, Iran. All the samples were serially diluted (10^{-1} to 10^{-7}) and 0.1 mL of diluted samples(10^{-5} to 10^{-7}) were streaked on International Streptomyces Project (ISP2) and Starch casein agar (SCA) media and incubated at 30°C for 5 days (Radhakrishnan et al. 2007). Afterwards, the pigmented colonies were purified and subjected to phenotypic identification using microscopic and macroscopic analysis and Api kit (coryne, biomerix).

Extraction of the Actinomycetes **pigments:** All the pigmented Actinomycetes isolates were transferred into 250 mL nutrient broth in conical flask (500mL) and incubated

in a shaker incubator (150 rpm) at 30°C for 5 days.

Each culture was centrifuged at 4500 rpm for 15 min and distributed into four conical flasks (100mL). Then 5 mL of the solvents (chloroform, acetone, methanol and ethyl acetate) separately were added to each conical flask. The suspensions were kept in shaker at 250 rpm for 2 hr, and then centrifuged at 3500 rpm for 15min. The colour phase of each flask was withdrawn and the solvent volatilized at 50°C (Mayuran et al. 2006).

Antimicrobial spectrum of the extracted Actinomycetes pigment against pathogenic microorganisms: Antimicrobial spectrum of the extracted Actinomycetes pigment was carried out against pathogenic organisms viz., *Staphylococcus aureus (*PTCC 1112*), Bacillus cereus* (PTCC1015*), Escherichia coli* (PTCC.1330*), Pseudomonas aeruginosa* (PTCC 1074*), Shigella dysentriae* (PTCC1183*), Citrobacter ferundii* (ATCC 8090*), Klebsiella peumoniae (*PTCC 1053*), Serratia marcecens* (ATCC 14756*), Aspergillus niger* (PTCC 5011) and *Candida albicans* (PTCC5027). To perform the test, each bioassay strain was full streaked on Mueller Hinton agar and well was made in the plate agar using sterile sharp borer. Then, 100 µL of the extracted pigment was added into the well and the plate was incubated at 35°C. After 24h, clear zone of growth inhibition was measured and considered bioactivity of the pigments.

Optimization of the pigment production: Optimization for maximum production of pigments was carried out by carbon and nitrogen sources. To determine the best carbon and nitrogen sources, glucose, lactose, maltose, sucrose, fructose, starch, peptone, yeast extract and tryptone were used. To perform the experiment all the carbon and nitrogen sources were sterilized and at the concentration of 1% separately added to nutrient broth medium. Then, Actinomycetes isolates were separately inoculated into the medium containing different carbon and nitrogen sources and incubated in a rotator shaker incubator at 150 rpm at 35°C for 5 days (Hemashenpagam 2011). Afterward, optimum production of each pigment was measured by optical density at respective wavelength for each colour.

Verification of the pigmented Actinomycetes: Authentication of the pigmented Actinomycetes strain was performed by 16SrRNA gene DNA sequencing. DNA extraction was carried out using DNA PCR kit (Roche-Germany). Then the purity of extracted DNA was evaluated by absorbance at 260 and 280 nm. The extracted DNA with ratio (260/280nm) of 1.9 ≤ corresponding to 121 µg DNA mL^{-1} was used for polymerase chain reaction (PCR). Amplification of 16SrRNA gene was performed using forward and reverse primers with sequences of 5' AGAGTTTGATCMTGGCT AG 3') and 1492R (sequence5' ACGGYTACCTTGTTACG ACTT 3'). Each reaction tube was containing 14.5 µL of water (Sigma Aldrich Company Ltd.), 2.5 µL of 10×PCR buffer (Cinagen- Iran), 1 µL of each forward and reverse PCR primers, 1 µL of a 10 mM dNTPs (Cinagen-Iran), 0.5 µL of Smar taq polymerase (cinagen-Iran), 1µL of 50mM $MgCl_2$ (Cinagen-Iran) and 5 µL of DNA template. PCR conditions of thermocycler (Cleaver, England) were as follows: 94°C for 1min, followed by 35 cycles of 94°C for 1min, 52°C for 30 s, and 72°C for 1min, with a final extension at 72°C for 7 min and storage at 4°C. All the PCR products were run on a 1% (w/v) agarose gel. PCR products were electrophoresed at 70V for 60 min and then DNA bands were virtualized after staining with ethidium bromide. A 100bp DNA ladder (Fermentase GenRuller SM0373) was used to compare the length of the isolated amplicons. Finally, the PCR products with pure DNA bands have been sent to Macrogen in South Korea (http://www.macrogen. com/) for DNA sequencing. The 16S rRNA sequenced data were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/ BLAST/) to identify each respective 16SrRNA gene amplicon.

Purification of crude pigment by chromatography: The crude pigment was purified by thin layer chromatography (Balagurunathan & Subramanian 2001). To find the best solvent system for purification of the crude compound, various solvents viz., methanol, chloroform, acetic acid, n-butanol, n-hexane and water were used in different proportions. n-hexane:chloroform (40:60, 50:50, 60:40), n-butanol:acetic acid:water (70:20:10 and 60:30:10) and chloroform: methanol (60:40, 50:50, 70:30, 30:70, 80:20 and 20:80).

To perform the test, the crude pigment was dissolved in 200 µL of ethyl acetate and then spotted at the bottom sheet and placed in the beaker containing mobile phase. The solvent was allowed to run till reaches about a centimetre below the top of the sheet. The sheet was kept on hotplate for the complete drying. Of all solvent systems, chloroform: methanol (30:70) and n-butanol:acetic acid:water (70:20:10) exhibited good efficiency for using as the mobile phase.

Spraying reagents: The colour of spots on the sheets was observed after drying. The dried sheets were sprayed with the following reagents for detection of different groups in the pigment.

For detection of amines and alkaloids, the sheets were sprayed with a solution of 50 drops 65% nitric acid in 100mL ethanol (higher acid concentrations are also possible). The sheets were dried at 120°C (Jork et al. 1994). For detection of alcohols, phenols, steroids groups, 1 g vanillin was mixed with 100 mL sulphuric acid. The sheets were heated at 120°C for few minutes till maximal coloration. Coloured zones produced on a pale background indicated as a positive result

(Mayuran et al. 2006). For detection of sugars, the sheets were sprayed with a solution of 5g urea in 20mL 2M hydrochloric acid and 100mL ethanol. The mixture was heated at 100°C and sprayed on the impregnated sheet. Observation of blue colour indicated the existence of ketoses and oligosaccharides in the pigment (Jork et al. 1994).

RESULTS AND DISCUSSION

Actinomycetes isolates: In total 25 strains of Actinomycetes were isolated from the soil samples. Of all, the 17 strains exhibited pigmented colonies on ISP2 and SCA medium.

Extraction of Actinomycetes pigments and evaluation of their antimicrobial property: The results obtained from the extraction of Actinomycetes pigments indicated that ethyl acetate relatively was the best solvent. The colour of the extracted pigments by ethyl acetate was light and dark brown, red, white, dark green and black. As given in Table 1, extracted pigment of Actinomycetes A2 and A9 exhibited relatively more effects, however, the antimicrobial effect of extracted pigments Actinomycetes A25, A24, A6, A7 and A17 was nil. *Staphylococcus aureus* and *Bacillus cereus* were more sensitive and *Escherichia coli* and *Serratia marceccens* were relatively less sensitive. In addition, *Candida albicans* and *Aspergillus niger* were resistant to the extracted Actinomycetes pigments (Fig. 1).

Optimization of the pigment production: The results obtained indicated that growth of isolated Actinomycetes strains in presence of glucose and yeast extract was relatively more. In addition, glucose and maltose promoted production of the pigments, however, the strains exhibited different responses in present yeast extract as nitrogen sources.

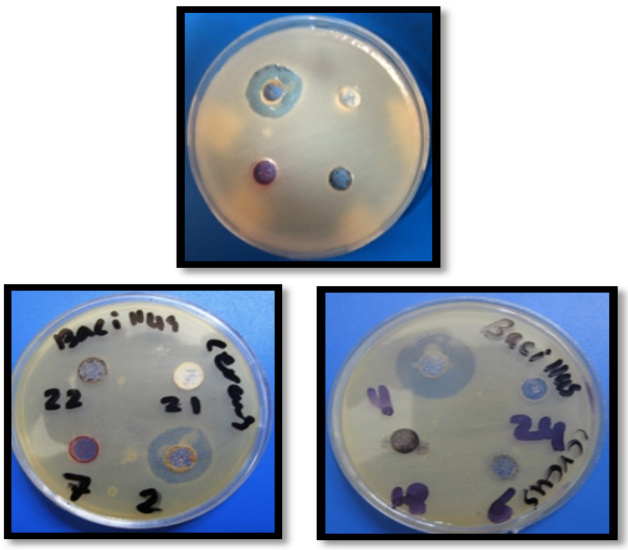

Fig.1: Antibacterial activity of pigments against *Bacillus cereus.*

16S rRNA gene sequence analysis: Alignment analysis of 16S rRNA gene sequence of Actinomycetes No. 9 exhibited 98/88% identical with *Streptomyces coeruleorubidus* strain NBRC 12844 with accession number AB184849.

Structure analysis of *Streptomyces coeruleorubidus* pigment: Our finding showed chloroform:methanol (30:70) and n-butanol:acetic acid:water (70:20:10) were the best solvent systems for TLC. The result obtained from structural analysis of pigmented Actinomycetes isolates exhibited existence of alcohols, phenols and steroids groups in the extracted pigment. Therefore, based on foregoing evidence and comparison of the extracted pigment with pigment structures in www.researchgate.net (natural pigments), probably quiones is a main compound of the *Streptomyces coeruleorubidus* pigment.

Nowadays, frequency of antibiotic resistant microorganisms is increasing therefore, treatment of the patients suffering from the infectious disease is considered problematic. To solve the problem, the new drugs must be found and used as remedies (Ilic et al. 2005). Although, several secondary metabolites are produced by microorganisms, the most important antimicrobial metabolites belong to the genera of Actinomycetes (Jensen et al 2003). It must be noted that secondary metabolites are the compounds that are not directly involved in the growth and reproduction. Therefore, unlike primary metabolites, the existence of the secondary metabolites does not depend on the life of microorganisms. However, antibiotics are secondary metabolites, but some secondary metabolites produced by Actinomycetes with potent antimicrobial property. One of the secondary metabolites produced by bacteria is pigment. Bacterial pigments due to their biodegradability and higher compatibility with the environment offer promising use for various applications. In this regard Mayuran et al. (2006) reported antimicrobial property of the pigments produced by *Streptomyces hygroscopics.* In addition, Sathi and his colleagues identified 4-hydroxy nitrobenzene pigment with brown colour (Sathi et al. 2002). Antifungal and antibacterial properties of violet pigment produced by *Streptomyces* have been reported by Zhirong et al. (2009).

In the present study, brown pigment of *Streptomyces coeruleorubidus* was isolated and identified. The pigment was assessed against Gram positive and Gram negative pathogenic bacteria. The results obtained indicated that *Staphylococcus aureus* and *Bacillus cereu*s were sensitive to the pigments. To continue the study, spray reagents were prepared and different groups of the structural pigment were determined. Our findings exhibited steroid and phenolic groups along with aromatic cycles and ester bond in the structure. Therefore, according to our finding probably struc-

Table 1: Antimicrobial effect of Actinomycetes pigment against pathogenic microorganisms.

Isolated strains	Colour of pigment	*Serratia marcecens*	*Klebsiella peumoniae*	*Citrobacter freundii*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Bacillus cereus*	*Staphylo-coccus aureus*	*Aspergilus niger*	*Candida albicans*
A2	Light Brown	-	+		+	-	+	+	-	-
A3	Dark brown	+	+		+	+	+	-	-	-
A4	Light brown	-	+	+	+	-	+	+	-	-
A5	yellow	-	-	+	-	+	+	+	-	-
A6	Brownish Green	-	-	-	-	-	-	-	-	-
A7	Red	-	-	-	+	-	-	-	-	-
A9	Light brown	+	+	+	+	-	+	+	-	-
A14	White	-	-	-	-	-	-	-	-	-
A15	Light brown	-	+	-	-	-	-	+	-	-
A16	Light brown	-	-	+	+	-	-	+	-	-
A17	brown	-	-	-	-	-	-	-	-	-
A18	black	-	+	-	+	-	+	-	-	-
A21	Yellow	-	-	+	-	-	-	-	-	-
A22	brown	-	-	-	+	-	-	-	-	-
A23	Dark green	-	-	-	+	-	+	+	-	-
A24	Yellow	-	-	-	-	-	-	-	-	-
A25	Dark green	-	-	-	-	-	-	-	-	-

tural pigment produced by *Streptomyces coeruleorubidus* is quinones.

REFERENCES

Aust, J. 2009. Taxonomy, fermentation, purification and biological activities produced by *Streptomyces* sp. AZ-AR-262. 3(1): 126-135.

Balagurunathan, R. and Subramanian, A. 2001. Antagonistic *Streptomycetes* from marine sediments. Adv. Biosci., 20: 71-76.

Conn, H.J., Conn, J. E. 1941. A preliminary note classifying Actinomycetes: Value of pigmentation. Journal of Bacteriology, 42(6): 791-799.

Goodfellow, M., O'Donnell, A.G. and Embley, T.M. 1993. Future of Bacterial Systematics. In: Handbook of New Bacterial Systematics, Academic Press, London, pp. 513-524.

Hemashenpagam, N. 2011. Purification of secondary metabolites from soil Actinomycetes. International Journal of Microbiology Research, 3(3): 148-156.

Ilic, S.B., Kontantinovic, S.S. and Todorovic, Z.B. 2005. UV/Vis analysis and antimicrobial activity of *Streptomyces* isolates. Med. Biol., 121: 44-46.

Jork, H., Funk, W., Fishcer, W., Wimmer, H. and Wiley, N.Y. 1994. TLC Reagents and Detection Methods-Physical and Chemical Detection Methods: Activation Reactions, Sequences, Reagents, II, Vol 1b, VCH, Weinheim.

Jensen, P.R., Mincer, T.J. and Fenical ,W. 2003. The true potential of the marine microorganism. Curr. Drug Discov., 17-19.

Mayuran, S., Radhakrishnan, M. and Balagurunathan, R. 2006. Antimicrobial pigments from desert soil actinomycetes. In: Proceeding of the National Symposium on "Recent Trends in Microbial Biotechnology". Sri Sankara Arts and Science College, pp. 35-51.

Mizukami, H., Konoshima, M. and Tabata, M. 1978. Variation in pigment production in *Lithospermum erythrorhizon* callus cultures. Phytochem., 17: 95-97.

Radhakrishnan, M., Balaji, S. and Balagurunathan, R. 2007. Thermotolerant actinomycetes from the Himalayan mountain-antagonistic potential, characterization and identification of selected strains. Malaysian Applied Biology, 36(1): 59-65.

Sathi, Z.S., Sugimoto, N., Khalil, M.I. and Gafur, M.A. 2002. Isolation of yellowish antibiotic pigment 4-hydroxy nitrobenzene from a strain of *Streptomyces*. Pak. J. Biol. Sci., 52: 201-203.

Woznicka, W. 1964. The significance of variation of some antibiotic actinomycetes for the taxonomy of microorganisms of this genus. Arch. Immunol. Ther. Exp. (Warsz), 12: 37-54.

Zhirong, Y., Li, W., Juan, L., Wenliang, X. and Siyuan., J. 2009. Identification and characterization of purple pigment-Producing actinomycete strain. College of Life Sciences, Sichuan University, Chengdu, 610064, China.