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### Isolation and identification of bioactive compound producing *Rhodococcus* spp. isolated from soil samples

Aghaei<sup>1</sup>, S. Nima Bahador<sup>2,\*</sup> and Majid Baserisalehi<sup>3</sup>

1. Department of Microbiology, Science and Research Branch, Islamic Azad University, Fars, Iran; Department of Microbiology, Shiraz Branch, Islamic Azad University, Shiraz, Iran

2. Department of Microbiology, Shiraz Branch, Islamic Azad University, Shiraz, Iran

3. Department of Microbiology, Kazeroun Branch, Islamic Azad University, Kazeroun, Iran

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#### ABSTRACT

The genus *Rhodococcus* is gram positive bacterium which was classified into the family of Nocardiaceae. The organism can be isolates from different sources, with capability to degrade diverse types of pollutant. Nowadays, scientists reported a few secondary metabolites which are produced by Rhodococci. Hence, the present study tried to isolate Rhodococci from different soil samples with the capability to produce antimicrobial metabolites. For this purpose 360 soil samples were collected from different types of soil. Then the isolates were identified and characterized using APi Coryne kit. Afterward antibacterial activity of the suspected bacteria was evaluated using well diffusion agar method against test bacteria. Furthermore, the extracted metabolites were analysed using GC-MS. The results obtained from this study indicated that 4 out of 9 isolates were able to produce antimicrobial metabolites. The most effective isolates were R87 strain with the maximum inhibition zone of 35 mm and the least effective were R85 strain with zone of inhibition 20 mm against *E. coli*. The GC-Mass chromatogram showed different special peaks and the identified compounds were cyclopentasiloxane, Azulene, 2-7 octadiene, docosane, cyclohexasiloxane, Bis (3,5,5 trimethylhexyl), Tetradecane – 2 methyl, Cycloheptasiloxane and Heptadecanol. In general, isolation of native bacteria from environmental samples with antimicrobial property is an interesting aspect which could help us to ignore antibiotic resistant bacteria from our environment.

#### 1. Introduction

The genus *Rhodococcus* is Gram positive to Gram variable, non-motile and non-sporulating, aerobic chemoorganotrophic bacteria. Depending on the strains and culture conditions with complexity in their life cycle and cell morphology the scientists found different shapes of the bacterium (Goodfellow and Maldonado 2006; Jones and Goodfellow, 2010; Alvarez, 2010). Indeed, the Rhodococci belonged to the

wider grouping of Actinomycets but presently, there are classified under the genus *Rhodococcus* (Euzéby, 2011). A few members of this genus have biodiversity in many different natural environments such as faecally contaminated soils, aquatic sediments (Jones and Goodfellow, 2010), and foaming activated sludge reactor (De los Reyes, 2009). Nowadays, Rhodococci have become a valuable tool in industry due to their ability to synthesize diverse compounds such as bio-surfactants, wax esters,

\*Corresponding author: Dr. Nima Bahador  
Tel: 989170913580  
E-mail address: bahador@iaushiraz.ac.ir

and oil that can be used for biotechnological purpose (Larkin *et al.*, 2005; Martinkova *et al.*, 2009). Furthermore, it has been supposed that they have an important role in degrading xenobiotic compounds (Larkin *et al.*, 2006; McLeod *et al.*, 2006) while, it was recently shown that only a few secondary metabolites characterized from Rhodococci including antibiotics, siderophores and carotenoid pigments (McLeod *et al.* 2006). Hence the present study tried to isolate different species of genus *Rhodococcus* from different agricultural soils in Qum city and evaluate presence of antimicrobial compounds against pathogenic bacteria.

## 2. Materials and Methods

### 2.1. Isolation of *Rhodococcus* from agricultural fields

Totally 360 soil samples were collected from different parts around the agricultural fields of Qum city, Iran. The samples were collected from 10 cm depth of soil using sterile polyethylene bag and transferred to the laboratory within one hour of sampling. All samples were subjected to detection of *Rhodococcus* instantly upon arrival in the laboratory. Hence, 0.1 ml of serially diluted ( $10^{-1} - 10^{-6}$ ) soil samples were poured on ISP5 agar and Bennet agar medium then the plates were kept at 30°C for 48-72 hours (Zhang *et al.*, 2005).

### 2.2. Phenotypic identification of the isolates using ApiCoryne kit

All suspected colonies were characterized using: microscopic examination, gram and acid fast staining, catalase, oxidase and motility tests (De Wever *et al.*, 1997). Then the suspected colonies with different pigment identified using ApiCoryne kit (bioMérieux). For this purpose the inoculums was prepared in distilled water with turbidity greater than 6 on the McFarland scale and they were used for enzymatic tests. Finally the strips were incubated at 30°C for 24-48 hours (Sotto *et al.*, 1994).

### 2.3. Test organisms

The pathogenic bacteria have been prepared from American and Persian type culture

collections including *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028), *Shigella dysenteriae* (PTCC 1188), *Klebsiella pneumoniae* (ATCC 700603), *Bacillus subtilis* (PTCC1715), *Bacillus cereus* (PTCC 1015), *Listeria monocytogenes* (PTCC 1295), *Enterococcus faecalis* (ATCC 51299) and *Corynebacterium glutamicum* (PTCC 1532). Then they were activated by nutrient broth medium.

### 2.4. Antibacterial activity of collected bacterial isolates

Antibacterial activity of the cultivated bacteria was evaluated using agar well diffusion method against test bacteria. For this purpose 0.1 ml of pathogenic bacteria was spread over the agar surface of the plates. Then two equally spaced wells of 6 mm diameter were made in the agar with sterilized core borer. The isolates were cultivated in broth culture medium, then centrifuged at 9000 rpm for 15 min subsequently the supernatant were poured into the well and the other well were used as control. The plates were kept at 37° C for 24 hrs (Bamzadeh *et al.*, 2013).

### 2.5. Extraction of antibacterial compounds from culture supernatant

The bacterial isolates were cultivated in TSB medium, and then the culture broths were centrifuged at 9000 rpm for 15 min to separate bacterial cells. Then the supernatant were acidified to pH 2.5 with concentrated HCl and ammonium sulfate (40%) was added up to saturation to precipitate the antibacterial metabolites. Ethanol, methanol, chlorophorm and ethyl acetate were used to determine the best solvent for extraction (Augustine *et al.*, 2005). Afterward the solvents were (1:1 ratio) added to the supernatant. The mixture of solvent and supernatant were agitated for 90 min with homogenizer and the solvent was separated from the supernatant using separating funnel. Subsequently all the solvent extract was assayed for their antibacterial activity by well agar diffusion method (Kumar *et al.*, 2009).

### 2.6. GC Mass analysis of the extracted microbial metabolites

The gas chromatography combined with mass spectrometry detection technique is a qualitative and quantitative analysis of the crude extracts with high sensitivity even with trace amount of constituents. Therefore, the GC-MS analysis was done by standard specification by dissolving 10mg of crude extracts in one milliliter of ethyl acetate. The aliquot of 0.1  $\mu$ l was injected automatically into 0.25 mm x 30 m column of GC-MS model (Agilent 5973N, HP-5MS) 5% methyl phenyl poly siloxane as stationary phase and ionization energy 70eV. Helium was used as a carrier gas at 17.69 psi pressure with the flow of 1.5ml/min at the flow rate of 0.4m/min. The temperature gradient program was implemented for the evaporation of organic solvent to identify the chemical constituent. The initial temperature was 60°C and gradually accelerated to 246°C at a rate of 3°C per minute. The sample was injected after 18 minutes at 250°C. The maximum peaks representing mass to charge ratio characteristics of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds (pandy *et al.*, 2010). The concentration of such compound was calculated by the following formula:

Compound concentration percentage =  $[P1/P2] \times 100$

Where, P1 is the peak area of the compound and P2 is whole peak areas in the fractionated extracts.

### 3. Results

#### 3.1. Isolation and identification of the isolates

Out of 87 coryne form organisms studied using the API system, 9 of them were correctly and completely identified within 24 hours to species level which were belong to the genus *Rhodococcus*.

#### 3.2. Antibacterial effect of the microbial metabolite

The results obtained from this study indicated that out of nine isolates, 4 of them were capable to produce antimicrobial product including R51, R65, R85, and R87. The most effective isolates were strain R87 with maximum inhibition zone of 35 mm and the least effective were isolate 85 with inhibition zone of 20 mm against *E. coli*

(ATCC 8739). Figure 1 shows synergistic effect of all extracted metabolites on *E. coli* (ATCC 8739).

#### 3.3. GC Mass analysis of the extracted microbial metabolites

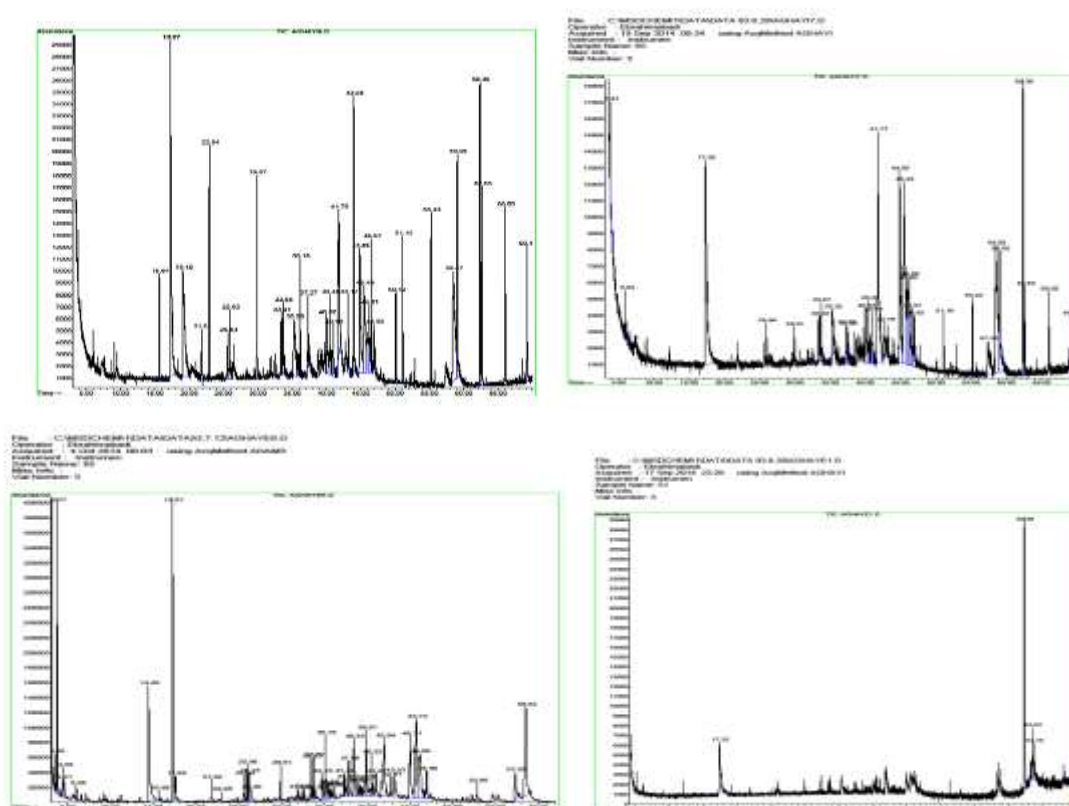
Analysis was carried out to identify the chemical compounds presented in it. The chromatogram showed different special peaks and mass spectrum detected the compounds present in the respective peak areas (Figure 2). The identified compounds were cyclopentasiloxane, Azulene, 2-7 octadiene, docosane, cyclohexasiloxane, Bis (3,5,5 trimethylhexyl, Tetraedacane – 2 methyl, Cycloheptasiloxane and Heptadecanol.

### 4. Discussion

*Rhodococcus* is a bacterial genus that could be isolated from different sources including soil, marine sediments, rocks, groundwater, boreholes, animal dung, insect guts, healthy and diseased plants and animals (Goodfellow, 1989a; Ivshina *et al.*, 1994). Furthermore, the organism has been isolated from high-level nuclear waste plume (Fredrickson *et al.*, 2004) and even a medieval grave (Takeuchi *et al.*, 2002). The organism is a gram positive, aerobic, non-motile, mycolate-containing nocardio form actinomycetes (Goodfellow, 1989b) which demonstrate a large metabolic diversity with capability to degrade different types of pollutants. The genus has the ability to produce some metabolites including surfactants, polymers, amides and flocculants, therefore, these capacities make them useful for biotechnological research (Carvalho and da Fonseca 2005; Rzeznicka *et al.*, 2010). Hence the aim of this study was to isolate the *Rhodococcus* from soil samples with the capability to produce antimicrobial metabolite. For this purpose nine coryneform bacteria were isolated and four of them were able to produce antimicrobial metabolite. Along with our research Chiba and his colleague were isolated *Rhodococcus* sp. from soil sample at Mt. Hayachine, Iwate Prefecture, Japan. The rhodopeptins were isolated as either colorless solids or white powders and were soluble in acetic acid, dimethylsulfoxide, methanol, and



**Fig 1.** Synergistic effect of extracted metabolites from isolated *Rhodococcus*



**Figure 2.** The extracted compounds from isolated *Rhodococcus* using GC/MS

slightly soluble in water but insoluble in chloroform and ethyl acetate (Chiba *et al.*, 1999), while, our extracted antimicrobial metabolite were more soluble in ethyl acetate. Furthermore, in 2006, Iwatsuki *et al.* isolated a strain of *Rhodococcus*, *R. jostii* K01-B0171 from a soil sample in Yunnan, China and

discovered that this strain is capable of producing 2 antibacterial compounds including lariatins A and B. Both lariatins had antimycobacterial properties against *Mycobacterium smegmatis*; however, lariatins A also inhibited the growth of *Mycobacterium tuberculosis*. Lariatins A and B also were

soluble in water, methanol, and DMSO while insoluble in chloroform and ethyl acetate (Iwatsuki *et al.*, 2007).

In this study the extracted metabolites from isolated *Rhodococcus* were examined against gram positive and negative bacteria including *S.aureus* (ATCC 25923), *E.coli* (ATCC 8739), *P.aeruginosa* (ATCC 27853), *S.typhimurium* (ATCC 14028) *S.dysenteriae* (PTCC 1188), *K.pneumoniae* (ATCC 700603), *B.subtilis* (PTCC 1715), *B.cereus* (PTCC 1015), *L.monocytogenes* (PTCC 1295), *E.faecalis* (ATCC 51299) and *C.glutamicum* (PTCC 1532).

The results obtained indicated that *Esherichia coli* was more sensitive to the extracted metabolite. The GC-Mass analysis confirmed the presence of different metabolites. Indeed along with our results Kitagawa and Tamura illustrated that 80 *Rhodococcus* strains from Japanese and German culture collections had antibacterial activity and they were classified in 3 groups based on the type of antibiotic they produced (Kitagawa and Tamura, 2008a; Kitagawa and Tamura, 2008b).

At the same time Kurosawa and his colleague worked on horizontal gene transfer from *Streptomyces padanus* to *R. facians* and they allowed the new *Rhodococcus* strain independently begin producing 2 new antimicrobial compounds (Kurosawa *et al.*, 2008). In addition, Narayana and his colleague isolated *Streptomyces* sp. from soil samples with capability to produce antimicrobial compound. They showed that the GC-Mass spectrometry monitored four active fraction against Gram positive, Gram negative and fungi, which among that hydroxyquinoline exhibited strong antibacterial and antifungal activity (Narayana *et al.*, 2008), while in our study 9 fraction have been screened and it was shown that Azuelene is the strongest. Although the isolates had antimicrobial properties, it seems that additional studies would clarify to assess the potential of the extracts for introduction of novel antibiotics from this geographical area of investigation.

## Refereces

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