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Optimization for Enhanced Production of Antibacterial Metabolites by Marine Actinomycetes *Kocuria* sp. strain rsk4

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Abstract

A new halophilic actinomycetes strain was characterized and on the basis of physiological and 16S rDNA sequence, it was designated as *Kocuria sp*. strain rsk4. It was able to produce antibacterial metabolites and shows broad spectrum antibacterial activity against several gram-positive and gram-negative bacteria. Rsk4 showed highest antagonistic effect against multi drug resistant bacteria *Staphylococcus aureus*. Cultural and nutritional conditions of strain have been optimized under shake-flask conditions for the efficient production of antibacterial metabolites. Among the various factors, starch casein medium, 30ºC temperature, neutral pH, 5% salinity, 7 days incubation period and 180 rpm agitation was found to be optimal for antibacterial metabolite production. 1% starch, yeast extract, casein and magnesium were found to be best carbon source, nitrogen sources and mineral respectively for improved antibacterial activity. Among various combinations of nitrogen source, yeast extract and casein in equal ratio showed enhanced antibacterial activity. This is the first report on the optimization of antibacterial metabolites by *Kocuria* sp. The study suggests that the *Kocuria* sp. could scale up by traditional method for production of antibacterial metabolites effective against multiple drug resistant *S. aureus.*

Key words: Actinomycetes, Antibacterial metabolites, Culture condition, *Kocuria*, Optimization

Introduction

Actinomycetes are potential source for production of valuable bioactive compounds and continue to be routinely screened for novel bioactive substances (1). They are well known for prolific production of commercially important secondary metabolites such as antibiotics (2), antifungal (3), antitumor (4) and immunosuppressive agents (5). Actinomycetes are responsible for production of various antimicrobial substances such as aminoglycosides, anthracyclines, glycopeptides, beta-lactams, macrolides, nucleosides, peptides, polyenes, polyesters, polyketides, actionomycins and tetracyclines (6). Emerging problem of multi drug resistance and new phyto-pathogens in the few decades leads to increasing interest for isolation of actinomycetes from relatively unexplored region (7). Extremophilic actinomycetes are always proved to be promising source for antimicrobial compounds due to adaptation under extreme conditions (8). They have diverse community structure and various unexplored metabolic pathways existing in various genera. Exhaustible study of terrestrial actinomycetes leads to re-isolation of known antimicrobial compounds; hence alternatively marine ecosystems are viewed as a rich source of microorganisms capable of producing unknown novel antimicrobial compounds. Actinomycetes derived from marine habitats provide industrially important secondary metabolites and still considered as a potential source of unique and novel chemical structures (9). Most of the

antibiotics are extracellular metabolites which are normally secreted in culture media (10) and have been used as various drugs. It has been observed that production of antibiotics is not a fixed property of organisms as it is variably affected by various factors such as physical, chemical or genetic composition of organism. Productions of secondary metabolites by microorganisms are dependent on the species and strains of microorganisms and their nutritional and cultural conditions (11). Minor manipulation in media composition exerts a huge impact on quantity and quality of secondary metabolites and general metabolic profile of microorganisms (12). Therefore, designing an appropriate fermentation medium is of critical importance in the production of secondary metabolites (13). Complete knowledge of optimal conditions with particular reference to the strain must be required for maximum fermentation activity leading to production of antimicrobial compounds by actinomycetes (14).

Several statistical and non statistical methods are available for optimization of medium constituents. The problem of statistical approach is selections of media components which are either decided by borrowing or by random selection and too many options are available if the organism under analysis has not been previously studied for production of desired product (15). Optimization of culture medium and physical parameter is efficiently done by non statistical one factor at a time method (16). The aim of the present study was to optimize the conditions for improved antibacterial compound production in the means of antibacterial activity.

Materials and Methods

***Collection and identification of organism*:** The strain rsk4 was isolated from marine water sample collected from Porbandar region (21°37'48"N 69°36'0"E) at Gujarat, Western India. The identification of the strain was carried out on the basis of cultural, morphological and biochemical characteristics. 16S rRNA gene sequencing was also carried out for the molecular identification of microorganism.

Antibacterial activity of strain rsk4: Strain rsk4 was grown in starch casein media with 5% NaCl and antibacterial activity of the ethyl acetate extract was assayed for 10 days by agar well diffusion method on Mueller Hinton agar. Ten pathogenic gram positive and gram negative bacteria were tested: *Staphylococcus aureus* MTCC 96, *Mycobacterium smegmatis* MTCC 6, *Bacillus subtilis* MTCC 441, *Bacillus megaterium* MTCC 2444, *Enterococcus faecalis* MTCC 439, *Klebsiella pneumoniae* MTCC 109, *Proteus vulgaris* MTCC 1771, *Salmonella typhimurium* MTCC 1251, *Pseudomonas aeruginosa* MTCC 2453 and *Escherichia coli* MTCC 739. Zones of inhibition were measured after 24 h incubation at 37ÚC using Hi-Antibiotic ZoneScale (HiMedia). Antibacterial assays were repeated in triplicates to confirm the consistent production of antibacterial metabolites along with media controls. *Staphylococcus aureus* MTCC 96 was found to be more sensitive to the antibacterial agent; hence further studies was carried out with it.

Optimization for production of Antibacterial compound

Effect of Media: Six different media namely starch casein medium (SC), glucose yeast extract malt extract broth (ISP2), inorganic salts starch broth (ISP4), glycerol asparagines broth (ISP5) (17), glucose yeast extract broth (GYEA) (18) and Bennett medium (BM) (19) were inoculated with culture in conical flasks and incubated at 30°C in rotary shaker for 10 days. After incubation, 30 µl ethyl acetate extract was evaluated for antibacterial activity by agar well diffusion method against *Staphylococcus aureus*.

Effect of Incubation Periods: The optimized media was inoculated with actinomycetes strain and incubated for 10 days. 1ml aliquots were withdrawn after every 24 hours and subjected to centrifugation and filtration followed by ethyl acetate extraction. The growth was measured as dry weight per ml of the sample withdrawn to know the relation between growth and product. 30 µl extracts were tested for antibacterial activities by agar well diffusion method against test pathogens.

Effect of Temperature: Rsk4 strain of the actinomycetes was inoculated into optimized media and incubated at different temperature viz. 20, 25, 30, 35, 40, 45 and 50°C for 10 days in rotary shaker. After incubation, ethyl acetate extract were analyzed for antibacterial activities by agar well diffusion method against *S. aureus*.

Effect of agitation: Effect of agitation speed for antibacterial product formation has been studied by inoculation of strain in the optimized media and kept at different agitation speed to achieve high rate of antibiotic production. The different agitation speeds were 60, 100, 140, 180, 220, 240 and 280 rpm. A control flask was maintained at static condition and all the flasks were incubated for 10 days. Antibacterial activity was evaluated by above mentioned method.

Effect of Salinity: Salinity was considered as one of the important parameter for metabolite production. Strain rsk4 was halophilic and isolated initially at 5% NaCl. Optimized media was prepared with different salinity concentrations (0, 2.5, 5, 7.5, 10, 12.5 and 15%) by adding NaCl. The strain was incubated at 30°C for 10 days, growth was measured and antibacterial activity of the ethyl acetate extract was tested against test pathogen *S. aureus*.

Effect of pH: Antibacterial activity of strain at different pH value was measured by agar well diffusion method. The pH of optimized media was adjusted from 5 to10 and incubated at 30°C in rotary condition. 30 µl ethyl acetate extract were used evaluated for its antagonistic effect against *Staphylococcus aureus*.

Effect of Carbon Sources: The effect of different carbon sources on antibacterial metabolite production was studied by replacing carbon source in the optimized medium with other carbon sources namely, dextrose, glucose, sucrose, maltose, mannitol, lactose, glycerol and starch at the concentration of 10 g/L. A flask without any carbon source was kept as a control. The optimized carbon source was varied at different concentration viz. 0.5%, 1%, 1.5%, 2% and 2.5% (W/V) in order to get the high rate of antibiotic production. Antibacterial activity of 30 µl ethyl acetate extract was evaluated by agar well diffusion method against *S. aureus*.

Effect of Nitrogen Sources: Effect of various nitrogen sources was assessed by replacing nitrogen source in the optimized medium with peptone, Beef extract, Yeast extract, Malt extract, casein, ammonium sulphate and potassium nitrate at the concentration of 10 g/L for the maximum antibacterial compound production. A flask without any nitrogen source was kept as a control. Different combination of optimized nitrogen source was combined to get highest antibacterial activity. Antibacterial activity was evaluated by above mentioned protocol.

Effect of Minerals: Effects of minerals on antibacterial metabolite production was evaluated by addition of $MgCl_2$, $CuSO_4$, $MnSO_4$, $FeCl_3$, $CoCl_2$, K_2HPO_4 and $ZnCl_2$, each at a concentration of 0.05% (w/v) in the optimized medium with superior carbon and nitrogen sources.

Results and Discussion

Rsk4 was halophilic, reddish orange colored, gram-positive, aerobic, catalase-positive, non-encapsulated coccoid and non-endosperm forming bacteria. The 16S rDNA sequence (1361 bp) of the strain rsk4 was determined and submitted to GenBank under the accession number HQ258887. On the basis of morphological, physiological, biochemical and analysis of the 16S rDNA sequence, the strain rsk4 was designated as *Kocuria* sp. strain rsk4.

Antibacterial activity of strain rsk4 was detected on starch casein (SC) medium using agar well diffusion method against several pathogenic gram-positive and gram-negative bacteria (Fig. 1). Antimicrobial activity of *Kocuria* sp. was recently reported by Palomo et al. (20) and Ballav et al. (21). It was showing broad spectrum activity and highest antagonistic effect against multiple drug resistance bacteria *S. aureus*. The similar antibacterial activity against *S. aureus* has been reported in halotolerant alkaiphilic *Streptomyces* EWC 7(2) and *Streptomyces* strain CH54-4 by Vijay et al. (22) and Rattanaporn et al. (23). *S. aureus* is multiple

drug resistance human pathogens which can cause various infections. Pediatric patients are frequently affected by these infections and healing options are also limited (24). Therefore, antibacterial metabolites isolated from rsk4 strain could be an important therapeutic against *S. aureus*.

***Optimization for production of Antibacterial compound*:** Optimization of antibiotic production requires complete knowledge on optimal fermentation conditions (25). In the present study, all required conditions and media components had been optimized for the production of antibacterial compound using strain rsk4.

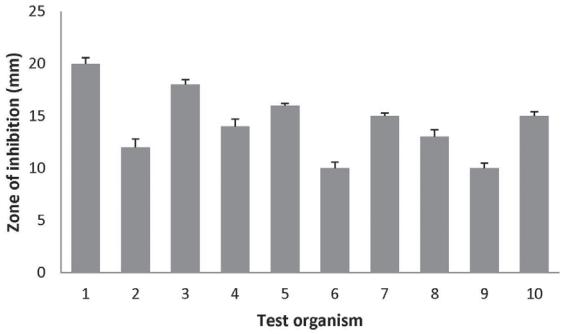

Fig. 1 Production of antibacterial metabolite by rsk4 strain against test organisms such as 1, *Staphylococcus aureus*; 2, *Mycobacterium smegmatis*; 3, *Bacillus subtilis*; 4, *Bacillus megaterium*; 5, *Enterococcus faecalis*; 6, *Klebsiella pneumoniae*; 7, *Proteus vulgaris*; 8, *Salmonella typhimurium*; 9, *Pseudomonas aeruginosa* and 10, *Escherichia coli*.

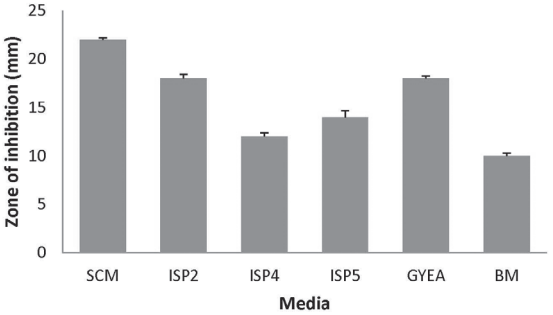

Fig. 2 Effect of different media on antibacterial metabolite production

Effect of media: All media are found to be suitable for antibacterial metabolite production by strain rsk4 but amount of zone of inhibition was varied. Among the six selected media SC medium showed good antibacterial activity against *S. aureus* (Fig. 2). Variation in the antibacterial metabolite production among media could possibly be related to the composition of the medium in which the strain was grown. SC contains three nitrogen sources which might be playing important role in metabolite production. Similar effect of improved antimicrobial metabolite production in starch casein media was shown by marine actinomycetes *Streptomyces afghaniensis* (26).

Effect of incubation periods: The antibacterial metabolite production by rsk4 was monitored over a period of 10 days. The bioactive metabolite production started from 5th day and reached optimum on 7th day (22 mm) but decreased gradually from 8th day. Growth patterns of strain showed that trophophase to idiophase shift occurs during 5th to 6th day (Fig. 3). Antibacterial metabolites production started during stationary phase that confirmed the compound to be a secondary metabolite. Conditions for secondary metabolite production are more restricted than the growth conditions, thus the efficient conversion from the trophophase to the idiophase is important for the production of antibiotics (27). The active metabolic product was not stable and decreases with respect to increasing incubation time. Similarly Jose et al. (28) optimized the required time courses for antibiotic production which was suppressed with the increase in incubation period in the production medium.

Effect of Temperature and agitation: Temperature has impressive effect on the physiology, biochemistry and metabolites production of organisms. It was observed that the culture filtrate of rsk4 had highest antagonistic effect in the range of 25ÚC-30ÚC shown in Fig. 4. There was no antibacterial activity after 40ÚC which states that deviation from optimum temperature affects the efficiency of metabolite. These results accord with a fact, that extreme

temperature are not suitable for antibiotic production (29).

Agitation speed affect oxygen supply and increases contact time of organism with media components. Fig. 5 shows significant differences of metabolite production in different range of agitation. The optimum antibacterial compound production was observed at 180 rpm. Production of antibacterial compound is decreases gradually with increasing rotary speed beyond 180 rpm. This result shows similarity with Bhavana et al. (30) which showed maximum antibiotic activity at 180 rpm and decreases gradually with increasing speed.

***Effect of Salinity and pH*:** Salt concentration effects on the osmotic pressure to the medium and hence marine organisms respond to different salinity conditions to produce secondary metabolites. Rsk4 is halophilic actinomycetes and able to grow at high salt concentration up to 15%, but produces maximum antibacterial compound at 5% NaCl (Fig. 6). Low salinity may effectively create stressful environment condition for halophilic strain, and a response to such stress may include alterations in the secondary metabolite spectrum (31).

pH affect cellular developments such as regulation of the biosynthesis of microbial bioactive metabolites (32) and hence change in pH of the

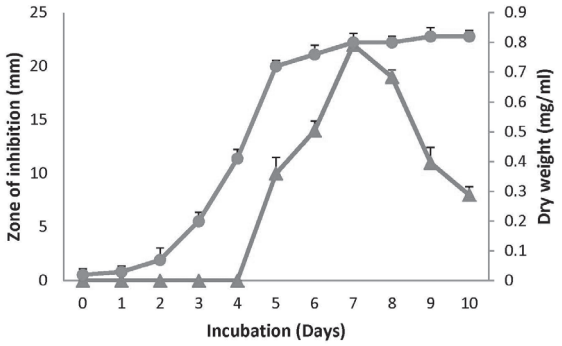

Fig. 3 Effect of incubation period on growth (circles) and antibacterial metabolite production (triangles)

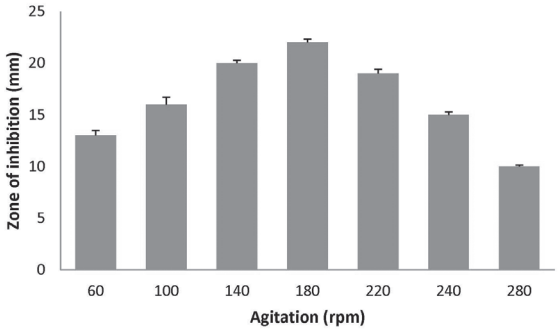

Fig. 5 Effect of agitation on production of antibacterial metabolite

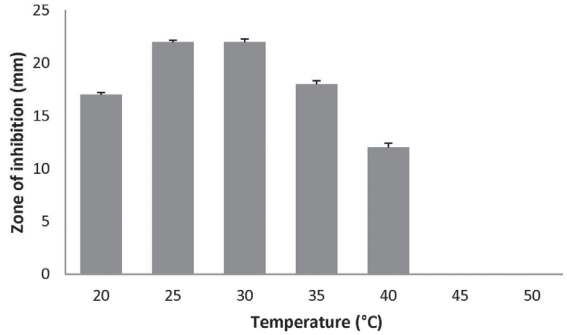

Fig. 4 Effect of temperature on production of antibacterial metabolite

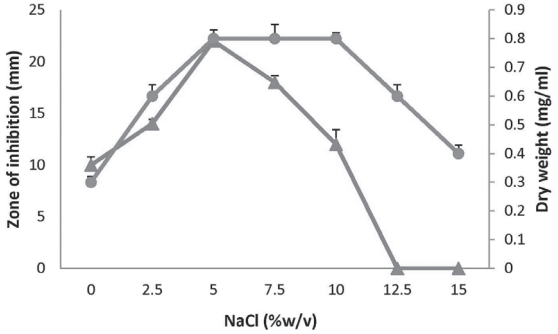

Fig. 6 Effect of salinity on production of antibacterial metabolite

culture medium induces the production of new products that adversely affect antibiotic production. Present study revealed that the optimal pH for production of antibacterial compound by rsk4 strain is neutral pH (Fig. 7). Similar report was shown by Vijay et al. for maximum antimicrobial metabolite production at pH 7 by *Nocardiopsis* sp (33).

***Effect of Carbon and Nitrogen sources*:** Addition of carbon and nitrogen sources in the production media optimally effects the production of antimicrobial compounds (34). Starch was found to be most suitable carbon source for production of antibacterial metabolites by rsk4 shown in Fig. 8. Various studies suggest that the continuous and gradual hydrolysis of starch can avoid inhibition in the production of antibiotics that are normally triggered by simple sources and more easily metabolized by the microorganism (35-36). Among the variable concentration of starch, 1% starch exert more effect on antibacterial metabolite production (Fig. 9).

The nature and concentration of nitrogen sources are considered as direct precursors for the synthesis of antibacterial compound. The effect of various nitrogen sources on antibacterial metabolites production by rsk4 is shown in Fig. 10. Organic nitrogen sources produced better amount of antimicrobial metabolites compared to inorganic nitrogen. The highest activity was obtained with both casein and yeast extract

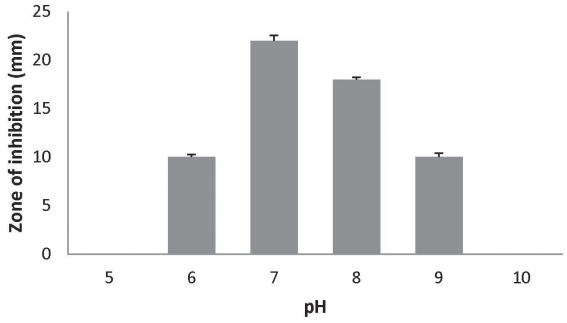

Fig. 7 Effect of pH on production of antibacterial metabolite

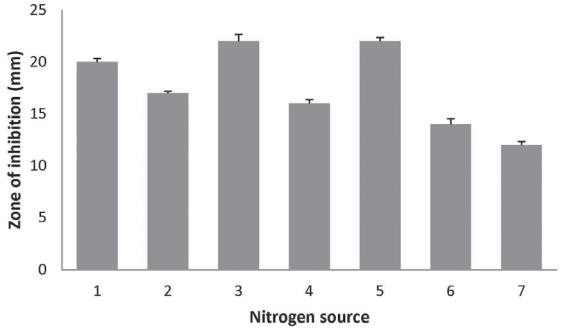

Fig. 9 Effect of various concentration of starch on production of antibacterial metabolite

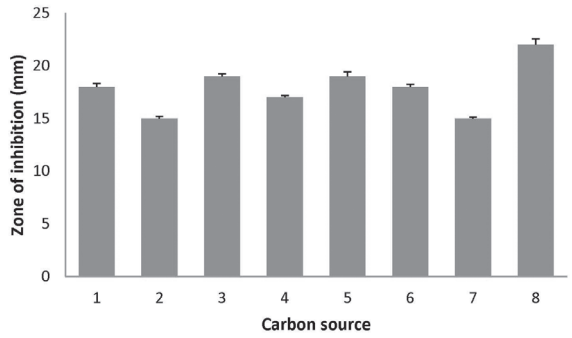

Fig. 8 Effect of carbon source on production of antibacterial metabolite 1, Dextrose; 2, Glucose; 3, Sucrose; 4, Maltose; 5, Mannitol; 6, Lactose; 7, Glycerol; 8, Starch

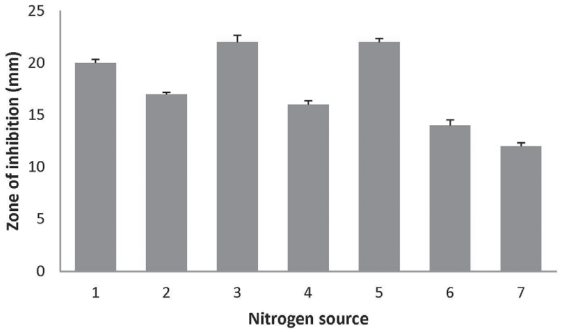

Fig. 10 Effect of nitrogen source on production of antibacterial metabolite 1, Peptone; 2, Beef extract; 3, Yeast extract; 4, Malt extract; 5, Casein; 6, Ammonium sulphate; 7, potassium nitrate

followed by peptone. This study suggests that antibacterial metabolite production is inhibited by a rapidly utilized nitrogen source. Among the various combination of organic nitrogenous source studied, casein + yeast extract showed maximum yield of antibacterial metabolites.

***Effect of Minerals*:** The effect of minerals is represented in Figure 11 which suggests that $MgCl_2$ exert best impact on production of antibacterial metabolites by strain rsk4. $CuSO_4$ did not show any increased metabolite production. This result shows similarity with Sujatha et al. where addition of 0.5 g/l of magnesium sulfate to the culture medium was optimal for antibiotic production by *Streptomyces sp* (37).

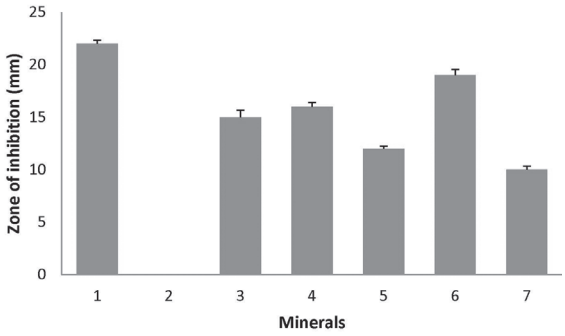

Fig. 11 Effect of minerals on production of antibacterial metabolite 1, $MgCl_2$; 2, $CuSO_4$; 3, $MnSO_4$; 4, $FeCl_3$; 5, $CoCl_2$; 6, K_2HPO_4; 7, $ZnCl_2$

Conclusion

Present investigation involved antibacterial activity of halophilic actinomycetes *Kocuria* sp. strain rsk4 and optimization of culture conditions for maximal metabolite production. Strain rsk4 showed broad spectrum activity and highest antagonistic effect against multi drug resistance *S. aureus*. It exhibited optimum antibacterial metabolite production in starch casein medium at 30ºC temperature, pH 7 and salinity 5% with agitation rate 180 rpm. Optimization of media components suggest that starch, yeast extract, casein and magnesium can increase the production of antibacterial metabolite. This study proves that non-statistical one factor at a time method is efficient, relatively simple and cost effective method and it can significantly increased antibacterial metabolite production.

Table 1 Effect of different combination of nitrogen source on production of antibacterial metabolite

Source	Zone of inhibition (mm)
Peptone + Yeast extract	21.5 ± 1.4
Peptone + Casein	21 ± 1.72
Peptone + Beef extract	18.2 ± 1.4
Peptone + Malt extract	17.3 ± 1.6
Yeast extract + Beef extract	20.2 ± 1.9
Yeast extract + Malt extract	18.5 ± 1.1
Casein + Yeast extract	25.2 ± 0.9
Casein + Beef extract	20.3 ± 1.1
Casein + Malt extract	20.4 ± 1.4
Beef extract + Malt extract	15.3 ± 0.9

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