

RRST-Biochemistry

Optimization of Cultural Conditions for the Production of Antibiotic by *Streptomyces* sp. VRY-1

Richa Jain*, Kamal Uddin Zaidi, Naima Parveen, Ankita Saxena

MBL, Centre for Scientific Research & Development, People's Group, Bhopal 462011, M.P., India

Article Info	Abstract
Article History <i>Received</i> : 18-05-2011 <i>Revised</i> : 05-09-2011 <i>Accepted</i> : 06-09-2011	<i>Streptomyces</i> sp. VRY-1 was screened for its bioactive potentials against various pathogenic microorganisms and was found to possess antibacterial activity against <i>Salmonella typhimurium</i> (drug resistant strain of bacteria) and various other pathogenic bacteria and fungi. Optimization of cultural conditions for production of antibiotic showed that maximum antibiotic production occurred on 10 th day in stationary cultural, 28° C, 8.0 pH, liver extract, 1.5%(w/v) glucose. The bioactive compound was found to be soluble in water and ethyl acetate.
*Corresponding Author <i>Tel</i> : +91-9754061697 <i>Fax</i> : <i>Email</i> : richa198@gmail.com	Key Words: Antibiotics, Optimization, <i>Streptomyces</i>
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Introduction

The new drug discovery processes have proved that novel skeletons of drugs come from natural sources in majority of cases [1]. This involves the screening of microorganism and plant extracts [2]. Microbial production of antibiotics is one of the rapidly expanding branch of industrial microbiology. Biotic potentials of actinomycetes are very wide as are capable of synthesizing many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, enzymes etc. Of these compounds, antibiotics predominate in therapeutic and commercial importance [3, 4, 5, 6]. Antibiotics of actinomycetes have extremely diverse chemical structures as acids, bases, amphoteric compounds, neutral compounds, polypeptides, amino sugar complexes, compounds with huge lactone rings, nitro compounds, guanido compounds, polyenic compounds, and acetylenic compounds. The exploration of new habitats plays a pivotal role in search of new microbes possessing potentials to produce novel metabolites. and are urgent to counter the threats posed by the fast emerging phenomenon of antibiotic resistance [7].

At present, resistance to the antibiotic used in the treatment of many infectious diseases is increasing, while microbial infections are being found to be responsible for more life threatening diseases than previously thought. The reasons for the increase in incidence of infectious diseases are not fully understood. One such reason is the emergence of multi-drug resistant pathogens [8]. The serious infections caused by bacteria that have become resistant to commonly used antibiotics has become a major global healthcare problem in the 21st century [9]. Gram negative and enteric organisms threaten patients in hospitals and communities with multi-drug resistance, including broad resistance to first, second, and third generations of penicillin's and cephalosporin's [10, 11, 12].

Drug resistance develops in bacteria through several mechanisms. Some bacteria develop the ability to neutralize the antibiotic before it can do harm, others can rapidly pump the antibiotic out, others can change the antibiotic attack site so it cannot affect the function of the bacteria, some bacteria acquire resistance through mutation of their genetic material, some acquire piece of DNA that code for the resistance properties from other bacteria through gene transfer phenomenon, transfer of multi-drug resistant plasmid of one bacteria to other through conjugation.

Rising numbers of antibiotic unresponsive infectious disease agents confront patients worldwide [13, 14]. and consensus has emerged that it is essential that novel antibiotic classes should be developed as part of the strategy to control the emerging drug-resistant pathogens [15,16,17]. The present study was carried out to evaluate the effect of antimicrobial compound of *Streptomyces* sp. VRY-1 on various pathogenic microbes, especially a drug resistant bacteria *Salmonella typhimurium* (MTCC 3214).

Materials and Methods

Streptomyces VRY-1

The culture of *Streptomyces* sp. VRY-1, an isolate of cultivated soil was maintained on ISP-2 medium containing (g L⁻¹) glucose, 4.0, malt extract, 10; yeast extract, 4.0; Agar agar, 20. The culture was identified based on its cultural, morphological and biochemical properties using standard protocol [18, 19].

Study of antimicrobial spectrum of Streptomyces sp. VRY-1 in a solid medium

Culture of pathogenic microorganisms were obtained from Microbial Type Culture Collection and Gene Bank, IMTECH, Chandigarh, India for testing the antimicrobial spectrum of

Streptomyces VRY-1. The cultures tested are as follow *Salmonella typhimurium* (MTCC 3214), *Salmonella typhi* (MTCC 733), *Klebsiella pneumoniae* (MTCC 4032), *Streptococcus pyogenes* (MTCC 1926), *Staphylococcus aureus* (MTCC 96), *Candida albicans* (MTCC 227), *Microsporium gypseum* (MTCC 6041), *Trichophyton rubrum* (MTCC 3272). Antimicrobial activity of the isolated *Streptomyces* sp. VRY-1 was tested using cross streak assay method [20, 21]. *Streptomyces* sp. VRY-1 was line streaked on the periphery of the Petri plate containing Sabouroud Dextrose Agar and incubated at 28±2°C for 5 days. Plates were re-inoculated equidistantly with test pathogenic microorganisms by streaking at right angles to growing actinomycetes and were further incubated at 37°C for 24-48 hours for bacteria and at 28°C for 4-5 days for fungi. The extent and type of inhibition (mm) was measured.

Study of drug resistant spectrum of *Salmonella typhimurium*

The drug resistance in bacteria *Salmonella typhimurium* (MTCC 3214) obtained from Microbial Type Culture Collection was studied against different antibiotics (10µgml⁻¹) i.e. Carbenicillin, Rifampicin, Streptomycin, Tetracycline, Ampicillin, Kanamycin, Neomycin using agar well diffusion technique. The stock solutions of antibiotics were prepared in methanol. The solutions of antibiotics were poured in agar well (8mm diameter) and allowed to diffuse in medium for 2 hours at 4°C. Methanol was used as control. Suspension of *S. typhimurium* (2x10⁶ cells ml⁻¹) was spread over the plates and inoculated at 37 ±2 °C for 24 hours.

Production of antibiotic by *Streptomyces* VRY-1

Production of antibiotic by *Streptomyces* sp. VRY-1 was done on glucose tryptone yeast extract broth. The media were inoculated with 2x10⁶ cells mL⁻¹ spores and kept for incubation at 28 ±2°C for 10 days. Culture broth was centrifuged at 5,000 rpm for 15 minutes at 4°C. Supernatant was filtered through 0.22 µm filter. The filtrate obtained was used as crude antibiotic sample, 100 µL of crude culture broth was poured in each well and allowed to diffuse. The culture of *S. typhimurium* was spread on plates and incubated at 37±2 °C for 24 hours.

Comparison of antimicrobial spectrum of bioactive compound produced by *Streptomyces* VRY-1 with known antibiotics

Standard antibiotic solution (100 µL) of concentration 10µg mL⁻¹ (made in methanol) and crude culture broth of *Streptomyces* sp. VRY-1 was tested for their anti- *S. typhimurium* activity using agar wells diffuse technique.

Optimization of culture conditions for the growth of *Streptomyces* sp. VRY-1 and antibiotic production.

Optimization studies were carried out in glucose tryptone yeast extract medium. The *Streptomyces* VRY-1 subjected to incubation period from 1-12 days, temperature 15 °C, 20 °C, 28 °C, 37 °C and 42 °C for 10 days in stationary culture, pH-6, 7, 8, 9 and 10. Medium was inoculated with spore suspension (2x10⁶ cells mL⁻¹) of VRY-1. Tryptone yeast extract medium (pH 8) having different glucose concentrations i.e., 0.1%, 0.5%, 1.0%, 1.5% and 2.0% (w/v) was used for determining optimum glucose concentration for production of

antibiotic. While for testing best nitrogen source Glucose tryptone yeast extract medium was supplemented with different nitrogen source like beef extract, malt extract, gelatin, urea, liver extract and peptone. In all sets of experiment the culture conditions were kept optimum as determined in the previous experiment. The optimization studies were carried out in triplicate set.

Extraction of antibiotic from culture broth

The extraction of antibiotic from crude culture broth was done using different solvents n-butanol, n-hexane, ethyl acetate, methanol and benzene. The solvents were mixed in 1:1 proportion. Aqueous layer and organic layers were separated and tested for presence of antibiotic using disc diffusion technique.

Thin layer chromatography and Bioautography

The partially purified antibiotic obtained after solvent extraction was spotted on Silica gel plate (Sigma-Aldrich) eluted using solvent ethyl acetate: methanol: water (70:20:10). For bioautography semisolid SDA medium seeded with *S. typhimurium* culture was poured over the TLC plate and incubated for 2 days at 37°C.

Detection of plasmid in *Streptomyces* VRY-1

Log phase culture of *Streptomyces* VRY-1 (5mL) was taken into Eppendorf centrifuge at 6000 rpm for 5 minutes at 4°C. Supernatant discarded and pellet was washed with sterilized Millipore water 3-4 times, 40µL of cracking buffer was added into the pellet and cells were crushed with the help of micro pestle. Sample was then loaded on 0.8 % (w/v) agarose gel.

Plasmid isolation

Plasmid isolation was carried out by using GeNis puresol™ plasmid isolation kit. 1.5 mL of 4-5 days old culture broth of *Streptomyces* VRY-1 was taken into Eppendorf. Centrifuged it at 9000 rpm for 1 minute at 4 °C. Pellet obtained was dissolved in 200 µL of puresol solution A and 10 µL of lysozyme, kept it in boiling water bath for 1 minute, immediately cooled it for 5 minute. Spinned down the pellet again at 13,000 rpm for 10 minutes at 4 °C. Supernatant collected and equal volume of isopropanol was added into it. Mixed it at room temperature for 5 minute. Again centrifuged at 13,000 rpm for 10 minutes at 4 °C. Pellet so obtained was dissolved in 70% chilled ethanol and centrifuged at 8000 rpm for 5 minute, pellet was air dried and resuspended into 25 µL of puresol solution B and 2 µL of RNase. Kept it at room temperature for 5 minute. Run the plasmid DNA in 0.8% agarose gel.

Results

Cultural and morphological characterization

Spores of *Streptomyces* VRY-1 appeared violet on performing Gram's reaction indicating culture to be Gram positive. The cultural and morphological characteristics are described in Table-1. The spore chain in VRY-1 was found to be less spiral. On analyzing cell wall amino acid composition it showed greenish spot of LL-DAP, thus we tentatively identify the culture to be of *Streptomyces* sp.

Table 1: Cultural and Morphological Characteristics of *Streptomyces* VRY-1

S.No.	Cultural & Morphological Characteristics of <i>Streptomyces</i> VRY-1	
1.	Appearance of colony	Powdery & wrinkled
2.	Color of colony	White
3.	Reverse color of colony	Brown
4.	Elevation	Elevated
5.	Concentric rings	Present
6.	Diffusible Pigment	Light Brown
7.	Spore chain	Spiral
8.	Cell wall amino acid	LL-DAP

Primary screening

The culture of *Streptomyces* sp. VRY-1 was found to inhibit the growth of both Gram positive and Gram negative pathogenic bacteria. Also it was found to inhibit fungi *Candida*

albicans and *Microsporium gypseum*. Indicating that organism *Streptomyces* sp. VRY-1 is producing broad spectrum of antimicrobial compounds. The results of antagonistic studies conducted are shown in Table 2.

Table 2: Antagonistic Activity of *Streptomyces* sp. VRY-1

S.No.	Test pathogens	MTCC No.	Zone of Inhibition (mm)**
1.	<i>Streptococcus pyogenes</i>	1926	10
2.	<i>Salmonella typhimurium</i>	3214	15
3.	<i>Klebsiella pneumoniae</i>	4032	11
4.	<i>Streptococcus aureus</i>	96	16
5.	<i>Salmonella typhi</i>	733	25
6.	<i>Bacillus</i> sp. (Gram positive)	-	15
7.	<i>Candida albicans</i>	227	11
8.	<i>Microsporium gypseum</i>	6041	5
9.	<i>Trichophyton rubrum</i>	3272	No inhibition

Study of drug resistant spectrum of *S. typhimurium*

Among all antibiotics tested against drug resistant strain of *S. typhimurium*, kanamycin (10-40 µg/mL) and streptomycin (30 µg/mL and 40 µg/mL) was able to inhibit the growth of *S.*

typhimurium while other antibiotics i.e. neomycin, tetracycline, ampicillin, carbenicillin and rifampicin are not effective even at high concentration i.e. 40 µg/mL (Table 3).

Table 3: Effect of different antibiotics on the growth of *S. typhimurium*

Antibiotics(µg/mL)	Different Concentration Zone of Inhibition (mm)**				
	10	20	30	40	50
Carbiniciline	-ve	-ve	-ve	-ve	-ve
Tetracycline	-ve	-ve	-ve	-ve	-ve
Ampiciline	-ve	-ve	-ve	-ve	4
Neomycin	-ve	-ve	-ve	-ve	6
Streptomycin	-ve	-ve	6	11	17
Kanamycin	9	13	14	14	15
Rifampicin	-ve	-ve	-ve	-ve	-ve

Comparison of antimicrobial spectrum of bioactive compound produced by VRY-1 with known antibiotics

Only kanamycin was able to inhibit the growth of *S. typhimurium* at concentration of 10µg/mL and diluted as well

as concentrated sample of VRY-1 show inhibition which was measured in terms of zone of inhibition in mm (Table 4).

Table 4: comparison of antimicrobial spectrum of bioactive compound produced by *Streptomyces* VRY-1 with known antibiotics

S.No.	Antibiotics/sample	Zone of Inhibition (mm)**
1	Kanamycin	9
2	Streptomycin	-ve
3.	Tetracycline	-ve
4.	Ampicillin	-ve
5.	Rifampicin	-ve
6.	Neomycin	-ve
7.	Carbenicillin	-ve
8.	sample	18

** Excluding well diameter.

Optimization of culture conditions for antibiotic production by *Streptomyces* sp. VRY-1
Incubation period

After every 24 hours, antibiotic activity was noticed from 5th day of incubation which was found to increase up to 11 day and maximum residual activity was observed at 10th day, whereas it decreases from 11th day. Zone of inhibition shown in a Fig.1

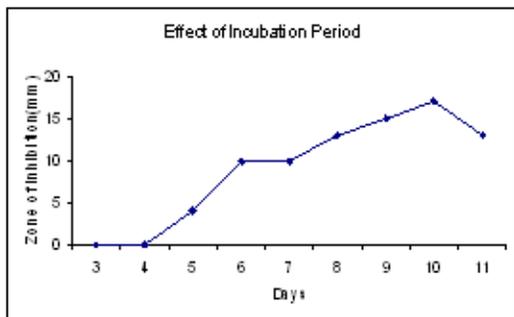


Fig.1. Effect of incubation period on growth of *Streptomyces* VRY-1 and antibiotic production

Temperature

The antibiotic production by VRY-1 was stable at different temperatures but growth of VRY-1 and its antibiotic production was more at temperature 28 °C which was shown in a Fig.2

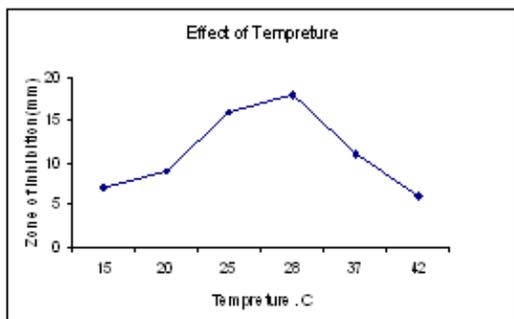


Fig..2: Effect of different temperature on growth of VRY-1 and antibiotic production

pH

The growth of VRY-1 was to be maximum at pH 8 but at pH 6 and 7 also the culture showed good growth. On increasing the pH to 9 the growth decreases. Maximum zone of inhibition i.e. 18 mm was observed at pH 8 which was shown in a Fig.3

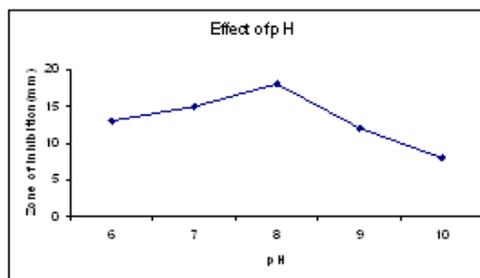


Fig.3: Effect of different pH on antibiotic production by *Streptomyces* VRY-1

Carbon source

At different glucose concentration, the growth and antibiotic production by VRY-1 varied but at 1.5% glucose concentration into the medium, the growth and antibiotic production was found more as shown in a Fig.4.

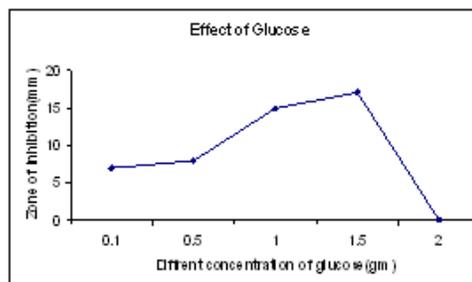


Fig.4: Effect of different glucose concentration on antibiotic production by *Streptomyces* VRY-1

Nitrogen source

Different nitrogen sources supplemented along with the medium composition have varying effect on growth of VRY-1 and activity of its antibiotic, shown in Fig.5

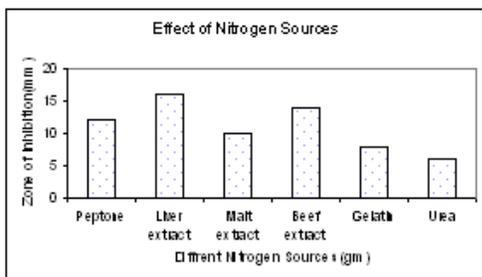


Fig 5: Effect of different nitrogen source on growth of Streptomyces VRY-1 and antibiotic production.

Direct concentration and ammonium sulphate precipitation

Specific centrifugation

In order to concentrate the antibiotic present in culture broth two methods i.e. direct concentration and ammonium sulphate precipitation were used. On analyzing the sample

(above 5 KD) the zone of inhibition against *S. typhimurium* increases, while no zone of inhibition was observed with ammonium sulphate precipitated sample.

Extraction of the antibiotic from culture filtrate using different solvents

The antibiotic produced by *Streptomyces* VRY-1 was subject to extraction using five solvents i.e. ethyl acetate, benzene, methanol, n-butanol, n-hexane. On testing antibacterial activity against *S. typhimurium* only ethyl acetate showed zone of inhibition, while with other solvent no inhibition was found, indicating that antibiotic is not soluble in pure organic solvents such as n-butanol, benzene, methanol, n-hexane. While it is soluble in ethyl acetate.

Thin layer chromatography

The developed chromatogram show 4 spots whose RF values were calculated by the formula:

$$RF = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}$$

Table 5 : TLC of antibiotic sample (above 5 KD)

S. NO	SPOT NO.*	Rf VALUE
1	1	0.14
2	2	0.25
3	3	0.39
4	4	0.57

*Starting from sample loading end of TLC

On performing autography after TLC the zone of inhibition was found around the third spot (Rf 0.39). It showed 0.8 cm of zone of inhibition.

Study of genetic material of Streptomyces VRY-1

Detection of plasmid using cracking buffer

Three bands each of genomic DNA, plasmid DNA and RNA were seen in an agarose gel (0.8%) under Gel Doc XR system [BIORAD]. As genomic DNA is heavier than plasmid DNA so its band was seen near the wells and band of plasmid was seen in between the bands of genomic DNA and RNA.

Plasmid isolation

The isolation of plasmid was carried out using GeNei Puresol™ plasmid isolation kit and its purity was checked using agarose gel (0.8%) electrophoresis. The molecular weight was determined to be 5148 bp.

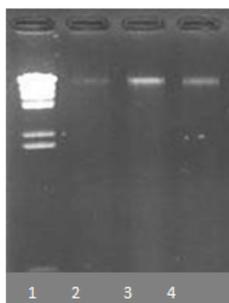


Fig 7: (i) Agarose gel showing four lanes in which lane-1 containing marker (NEcoRI + Hind III), lane-2, 3 and 4 contains plasmid isolated from *Streptomyces* VRY-1

Discussion

Actinomycetes are the group of Gram positive bacteria having branched filaments, which somewhat resemble the mycelia of the fungi. Actinomycetes perform significant biogeochemical roles in nature and are highly valued for their unparalleled ability to produce wide variety of biologically active secondary metabolites [22, 23]. The value of actinomycetes to society in terms of providing useful drugs for pharmaceutical industry is indisputable. Actinomycete products such as antibiotics are firmly cemented. They are chemically prolific bacteria in the centre stage of natural products drug discovery research [24]. By virtue of the plasmids present in them are known as highest producer of bioactive molecules. Many *Streptomyces* carry detectable extra chromosomal elements (plasmid) and in most cases, plasmid are present abundance in the form of covalently closed circular- DNA, but occasionally, linear elements are also found[25].

Most of the antibiotics are specific in their activity, some antibiotics are antagonistic to few bacteria only while other act against fungi. They are called as narrow spectrum antibiotic. Broad spectrum antibiotics repress the growth of both Gram positive and Gram negative bacteria. While certain other antibiotics are able to inhibit the growth of both bacteria and fungi or they are active against more than one microorganism [26].

Nowadays, antibiotic resistant pathogens pose an enormous threat in the treatment of infectious diseases. Appearance of antibiotic resistance in bacteria causes reduction in effectiveness of drugs in curing disease or improving a patient's symptoms. Since spread of antibiotic

resistance in microorganisms is very fast and exponential. Thus to prevent this exponential emergence of antibiotic resistance, a periodic replacement of the existing antibiotic with new and broad spectrum antibiotics is necessary [27]. Development of novel drugs having broad spectrum mode of action against drug resistant pathogen, is the need of an hour. Present study focused on the production of a broad spectrum antibiotic from *Streptomyces* VRY-1, which was found to be active against a drug resistant strain of bacteria *Salmonella typhimurium* (MTCC 3214). *S. typhimurium* causes a food borne disease salmonellosis. Multidrug resistant (MDR) strain of it are now encountered frequently and the rates of multidrug resistance have increased considerably in recent years. The emergence of MDR in *Salmonella* strains with resistance to Fluoroquinolones and third generation Cephalosporin is a serious problem, which results in severe limitation of the possibilities for effective treatment of human infections.

Waksman and Lechevalier [20] advised to have an idea of the stability of the antibiotic substance at various temperatures and at various pH values before attempting to devise a method of their extraction. So, the optimization of cultural conditions for the production of antibiotic from *Streptomyces* VRY-1 was done. Production of antibiotic by *Streptomyces* VRY-1 occurred at wide range of temperature, pH, glucose concentration, nitrogen source. This clearly indicates that the organism is producing a bioactive compound under different environmental conditions. The *Streptomyces* VRY-1 strain was found to produce a highly active antibiotic substance was tentatively identified as *Streptomyces* sp. based on their cultural characteristics, morphological, and its cell wall amino acid composition determination. The isolate was also found to contain a plasmid which might be responsible for the production of bioactive compounds by it. In order to reach the targeted area the antibiotic must be soluble in body fluids i.e., blood and lymph. Both of them contain large volume of water. Antibiotic substance produced by *Streptomyces* VRY-1 was found to be soluble in water and ethyl acetate but was not soluble in any other pure organic solvents tested, indicating its water solubility nature.

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