Optimization of Secondary metabolite production and antibacterial assay of isolated Streptomyces sps

Quasim Turiki, Merakanapalli Kishore Babu, Kunchala Rajyalakshmi, V. E. Vijaya Sekhar, Syed Shabana, Alapati Krishna Satya.

Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur,
Andhra Pradesh, India

Abstract: The selected potent actinomycetes isolates, Streptomyces rochei, Streptomyces cavourensis and Streptomyces alfalfa from the primary screening were tested for antimicrobial activity against both gram positive and gram negative bacteria and several parameters for biomass and secondary metabolite production were optimized. The results obtained in the present investigation revealed that both the growth and bioactive metabolite production by the isolated strains were mainly influenced by several environmental and cultural conditions. The optimum conditions for in vitro production of antimicrobial metabolites could be achieved in medium supplemented with 2% glucose as carbon source and tryptone at 2.5g/ L as nitrogen source. Four days of incubation at 35°C and pH 7 were found to be optimum for bioactive metabolite production. The optimal conditions for the production of bioactive metabolites by the isolated strains were determined, and metabolites showed good antimicrobial activity against Gram positive and Gram negative bacteria.

Keywords: Actinomycetes, Streptomyces sps, Antimicrobial activity, growth, bioactive metabolites

Introduction

Prominent sources of antibacterial and anti-cancer drugs are natural products isolated from different sources in view of the urgent need and demand for the new drugs (Bull and Stach, 2007; Appendio and Banfi, 2011). The natural product discovery started with the discovery of Penicillin and stretched with a wide array of applications in the fields of medicine and agriculture. Till now commercially important bioactive compounds are isolated from Actinomycetes (Saleem et al., 2009) which have been traditionally rich in producing many important bioactive compounds (Parthasarathi et al., 2010). The list of novel actino-bacterial derived from different areas of the world stresses the importance of investigating all habitats. There is a need to bio-prospect untapped geographical sources and explore new groups of microorganisms to increase the discovery of novel bioactive metabolites (Nolan and Cross, 1988; Faulkner, 2000; Leal et al., 2012). In the present study 93 Actinomycetes isolates were obtained from different soil samples collected in the fields of Soybean, only 29 isolates were found to have antagonistic activity. These 29 Actinomycetes isolates were further screened for the production of bioactive metabolites and finally three species were tested for antibacterial activity against Proteus vulgaris, Salmonella sp, Serratia marcescens Klebsiella sp, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa. Several researchers have reported antimicrobial activity of actinobacteria against various human pathogens.
Materials & Methods

Extraction of antimicrobial agents by using different solvents

The selected potent actinomycetes isolates, *Streptomyces rochei, Streptomyces cavourens*is and *Streptomyces alfalfa* from the primary screening were inoculated in asparagine-glucose broth and incubated at 28°C in a rotatory shaker with 120 rpm. After 5 days of inoculation the crude culture filtrate was obtained with equal volumes of four solvents chloroform, ethyl acetate, methanol and acetone separately to extract antimicrobial compounds. The Extracts were dried in a water bath at 80°C to obtained dry powder of the compounds obtained from each solvent. They were further tested for its activity against the test microorganisms by agar well diffusion method (Cappuccino and Sherman, 2004).

Optimization of biomass production

The growth pattern and bioactive metabolite production of the *Streptomyces rochei, Streptomyces cavourens*is and *Streptomyces alfalfa* was studied for week days. One week old cultures of the strains were cultivated in asparagine-glucose broth at room temperature for 48 h. 10% of each of this culture was inoculated into the production medium and fermentation process was carried out for one week under 120 rpm agitation. Biomass was extracted at every 24 h interval, the flasks were harvested and biomass was separated from the culture filtrate and measured in terms of dry weight of the biomass collected. Antimicrobial metabolite production is measured in terms of antimicrobial spectrum recorded. The culture filtrates were extracted with ethyl acetate and evaporated to dryness in a water bath at 80°C. The solvent extracts were concentrated and tested for antimicrobial activity by agar well–diffusion method followed by Cappuccino and Sherman in 2004 against test organisms like *Proteus vulgaris, Salmonella sp, Serratia marcescens Klebsiella sp,Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*.

Optimization of Culture conditions for production of bioactive metabolites

Secondary metabolite production of the strains was studied by optimizing parameters such as pH, temperature, culture media, carbon sources, nitrogen sources and minerals.

Optimization of pH and incubation temperature

To determine the influence of pH on growth and bioactive metabolite production, the potent strains were cultured in the medium with different pH levels (4-10) and at different temperatures (25 - 50°C). The biomass and bioactive metabolite production were estimated to determine the optimal pH and temperature.

Optimization of culture media

In order to determine ideal conditions for the maximum production of antimicrobial metabolites, the strains were cultivated in different media such as asparagine-glucose broth, tyrosine broth, starch inorganic salts broth, glycerol-asparagine broth, yeast-starch broth, malt extract broth, tryptone yeast extract broth, Czapek-Dox broth, maltose-tryptone broth and soya-bean meal broth. The biomass and bioactive metabolite production in each medium
was evaluated. The medium in which the strains exhibit good levels of bioactive metabolites was used for further study.

**Optimization of carbon sources and nitrogen sources on biomass and bioactive metabolite production**

To determine the effect of carbon sources on biomass and bioactive metabolite production of the strains, different carbon sources like maltose, lactose, fructose, sucrose, dextrose, starch, mannitol, arabinose, xylose, glycerol and inositol @1% were added separately to the optimized medium at different concentrations (0.5 - 5%) of the best carbon source.

**Optimization of nitrogen sources**

Influence of various nitrogen sources on bioactive metabolite production was evaluated by supplementing inorganic and organic nitrogen sources like sodium nitrate, ammonium sulfate, ammonium oxalate, peptone, yeast-extract, tryptone, casein, tyrosine, phenyl alanine, glycine and glutamine at 0.5% to the medium containing optimum level of the good carbon source.

**Optimization of minerals**

Impact of minerals on the production of biomass and bioactive metabolites was studied by supplementing minerals like K2HPO4, MgSO4.7 H2O, FeSO4.7 H2O, KH2PO4 and ZnSO4.7 H2O each at a concentration of 0.05% (w/v) to the optimized medium.

**Antimicrobial activity against test organisms**

The antimicrobial metabolites produced by the strain under optimized conditions were tested against bacteria like *Proteus vulgaris*, *Salmonella sp*, *Serratia marcescens* *Klebsiella sp*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*. Bacteria were grown in Nutrient Agar broth for 24 hours at 37°C prior to use (Ginovyan M et al., 2015). Nutrient agar plates were prepared, sterilized and after solidification bacterial cultures were streaked. Using a cork borer 6mm diameter well was made. The wells were loaded with crude extracts (20μl-50μl) and streptomycin was used as control with a concentration of (10μg/mL⁻¹). The plates were incubated at 37°C ± 2 for 16-24hours. After the incubation the plates were observed, the zone of inhibition around the wells was measured in mm. All the experiments were carried out in triplicates and mean values were represented in results (Ginovyan M et al., 2017).

**Biological assay**

**MIC assay**

The antimicrobial spectra of the bioactive compounds produced by the strain were determined in terms of minimum inhibitory concentration (MIC) against a wide variety of Gram-positive and Gram-negative bacteria by using agar plate diffusion assay (Cappuccino and Sherman, 2004). Nutrient agar media was prepared for the
growth of bacteria. The metabolites dissolved in DMSO (dimethylsulfoxide) at concentrations ranging from 0 to 1000µg/ml were used to assay against the test bacteria such as Proteus vulgaris, Salmonella sp, Serratia marcescens Klebsiella sp, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa. The inoculated plates were examined after 24-48 h of incubation at 37˚C for bacteria. The lowest concentration of the bioactive metabolites exhibiting significant antimicrobial activity against the test microbes was taken as the MIC of the compound.

**Results**

The antimicrobial efficacy of the isolates was evaluated by using four different solvents such as methanol, acetone, chloroform and ethyl acetate. Among the solvents used, ethyl acetate extract exhibited maximum antimicrobial activity where as the other solvent extracts showed moderate to minimum activity against all the pathogens tested. The ethyl acetate extract of the isolates showed maximum activity against Salmonella sp, Serratia marcescens and Staphylococcus aureus. The strains inhibited the growth of Gram Positive, Gram negative bacteria, suggesting a broad spectrum nature of the active compounds. Following figure 1 and table 1 clearly explains the antimicrobial activity of 3 isolates.

The growth pattern of the isolates was studied on asparagine glucose broth. The stationary phase of the strain was extended from 72 h to 120 h of incubation. The bioactive metabolites obtained from four day old culture exhibited high antimicrobial activity against the test microorganisms.

The influence of different media on the production of biomass and bioactive metabolites was recorded. Among the media tested, modified YMD broth supported the production of bioactive metabolites followed by maltose-tryptone broth and Czapek Dox broth, where as the production of biomass was high in Soya bean meal broth followed by Starch inorganic salts broth and modified YMD (ISP-2) broth.

Among the various carbon sources tested, glucose was found to be the best carbon source for both biomass and bioactive metabolite production. 2% glucose supplemented in the medium promoted highest biomass production.

Different nitrogen sources were found to have significant effect on growth and secondary metabolite production. Maximum antimicrobial activity was obtained in culture filtrates supplemented with tryptone and asparagines.

The impact of minerals on biomass and bioactive metabolite production was studied. K2HPO4 enhanced the production of biomass and bioactive metabolites. Maximum growth was obtained at pH 7, suggesting the neutrophilic characteristics of the strains.

The influence of temperature on the biomass and bioactive metabolite production of the strain was studied. Highest growth as well as anti-microbial compound production was obtained at 35°C. In terms of its optimum temperature for growth, the organisms appeared to be mesophilic.
Table: 1 Mean value of zone of Inhibition of Antimicrobial activity

<table>
<thead>
<tr>
<th>Pathogenic culture</th>
<th>Isolated <em>Streptomyces</em> sps Ethyl acetate extracts</th>
<th></th>
<th></th>
<th>Control (Streptomycin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Streptomyces rochei</em></td>
<td><em>Streptomyces cavourens</em></td>
<td><em>Streptomyces alfalfa</em></td>
<td></td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>24 mm</td>
<td>24 mm</td>
<td>26 mm</td>
<td>30 mm</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>24 mm</td>
<td>25 mm</td>
<td>24 mm</td>
<td>30 mm</td>
</tr>
<tr>
<td><em>Salmonella sp</em></td>
<td>26 mm</td>
<td>26 mm</td>
<td>25 mm</td>
<td>30 mm</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>28 mm</td>
<td>29 mm</td>
<td>30 mm</td>
<td>32 mm</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>28 mm</td>
<td>26 mm</td>
<td>29 mm</td>
<td>32 mm</td>
</tr>
<tr>
<td><em>Klebsiella sp</em></td>
<td>26 mm</td>
<td>27 mm</td>
<td>26 mm</td>
<td>32 mm</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>25 mm</td>
<td>26 mm</td>
<td>26 mm</td>
<td>30 mm</td>
</tr>
</tbody>
</table>

Figure 1: Illustrates the antimicrobial activity of 3 isolates against bacterial pathogens:
Conclusion

The results obtained in the present investigation revealed that both the growth and bioactive metabolite production by the isolated strains were mainly influenced by several environmental and cultural conditions. The optimum conditions for *in vitro* production of antimicrobial metabolites could be achieved in medium supplemented with 2% glucose as carbon source and tryptone at 2.5g/L as nitrogen source. Four days of incubation at 35°C and pH 7 were found to be optimum for bioactive metabolite production. The optimal conditions for the production of bioactive metabolites by the isolated strains were determined, and metabolites showed good antimicrobial activity against Gram positive and Gram negative bacteria.

References:


