Research Journal of Pharmacy and Pharmaceutical 2021 Vol.5

Optimization of fermentation parameters for enhanced APHE antibiotics production from Streptomyces griseocarneus through submerged fermentation

ABIDA RAFIQUE
Institute of Industrial Biotechnology, GC
University Lahore 54000, Pakistan

Abstract:
The present study is concerned with the production of antitumor antibiotics from *Streptomyces griseocarneus* NRRL B1068 by submerged fermentation technique. The antibiotic activity was tested against *A.niger, E.coli* and *B.subtilis* by agar well diffusion method. Different culture media were screened and M1 medium consisting of (g/L), potassium dihydrogen phosphate, 3.24; dipotassium hydrogen phosphate, 5.65; hydrated magnesium sulphate, 1.0; and 1 ml stock solution of salts (ferrous sulphate, 0.1; manganese chloride, 0.1; and zinc sulphate, 0.1) supplemented with 7.5% glucose and 2.0% lysine was found to be best medium for fermentation. Optimum temperature, pH and incubation period for the production of antitumor antibiotic were found to be 30 ºC, 7.2 and 7 days, respectively.7 days old inoculum at a concentration of 8% (v/v) were found to be the best for antitumor antibiotic production by *Streptomyces griseocarneus* NRRL B1068.

Introduction: *Actinomyces* are profile producers of natural products with a broad range of biological actions. They are also a source of novel and potentially valuable molecules. An improved understanding is required to enhance the biosynthetic potential of their natural product biosynthesis (Bibb, 2013). Waksman and Henrici, originally coined the term *Streptomyces* in 1943 (Williams *et al.*, 1983). Being in the family of *Streptomycetaceae* (Arai, 1997), they can be differentiated on the basis of several characteristics such as their physical and phonological properties, cell walls constituency, nature of peptidoglycan present in the cell wall, composition of phospholipids and chains of fatty acids, GC contents, analysis of 16S ribosomal RNA and self hybridization of DNA (Kutzner, 1992).

In spite of the invention and synthesis of novel antimicrobial compounds, contagious infections remain the subsequent leading cause of deaths around the globe due to microbial opposition (Nikaido, 2009). It is mainly because of the genetic changes in the genes, mutations, and acquisition of resistance through horizontal and vertical gene transfer (Wright, 2010). The physiological conditions of bacteria such as formation of the biofilm also stimulate the resistance of antibiotics. Consequently, scientists and researchers are trying to modify the existing antibiotics and discover the newest antimicrobial agents that are more efficient, stable and effective to inactivate the enzyme and toxins produced from the resistant bacteria (Garza *et al.*, 2009).

The fermentation of *Streptomycetes* is a complex process; it not only depends upon the performance of the fermentation medium, but also requires the favorable environmental conditions such as volume of inoculum, medium capacity, time of fermentation, temperature of fermentation, rate of agitation and the initial pH of the medium(Kiers *et al.*, 2000). These factors may influence the production of antibiotics. Fermentation has three obvious phases. Firstly, pre-fermentation is cell growth phase, nutrients are progressively consumed and begin to produce antibiotics. Secondly, a large number of antibiotics are produced quickly. Lastly, in post-fermentation there is a slow accumulation of metabolites (Wang, 2011). During this stage, *Streptomycetes* even produce toxic metabolites.

The real antibiotics production is improved by the optimization of the fermentation conditions and determination of the best composition of the medium and cultural parameters. The fermentation levels are interrelated with time of fermentation, aeration and agitation, incubation temperature and initial pH of the medium etc. The laboratory and industrial scale fermentations are significantly affected by the medium components. The reduction in fermentation costs and increase in the production of antibiotics is greatly improved by the technical, methodological and balanced optimization of cultural parameters (Jia, 2010).

Streptomyces griseocarneus NRRL B1068 was first isolated from the Spainish soil and was previously known as *Streptoverticillium griseocarneum* (Soliveri *et al.*, 1987). It produces pyrazoloisoquinolinones (APHE), rotaventin, alboverticillin, hydroxystreptomycin, and a number of important metabolites like sphingomyelinase. APHE antibiotics are present in almost all the *Streptomycetes* which are the wealth of various bioactive secondary compounds with significant activities; for instance, antimicrobial, antiviral and anticancer. It is of high viable value and continues to be regularly screened for new bioactive substances (Takahashi, 2004). It produces a novel class of antimicrobial compounds named as pyrazoloisoquinolinones antibiotics (APHE-1 to APHE-4) (Cruz, 2000).

Pyrazoloisoquinolinones (APHE antibiotics) are the condensed heterocyclic aromatic compounds that can be differentiated on the basis of their aliphatic sequence at the carbon 3 (C-3) position of the pyrazole chime. It is an ethyl group in APHE-1, n propyl group in APHE-2 and a methyl group in APHE-3. APHE-1 and APHE-2 are the main antibiotics in the mycelium while APHE 3 is generally present in spores; during spore formation. APHE-4 is the fourth type of the antibiotic class, is produced only in minute quantity (Fidalgo *et al.*, 1992). They possess *in-vitro* cytostatic and immune modulatory properties against diverse tumour cell lines e.g. mouse, monkey and human. These antibiotics are also active against different bacteria (Gram +ve and Gram -ve), filamentous fungi and yeasts (Cruz *et al.*, 1996).

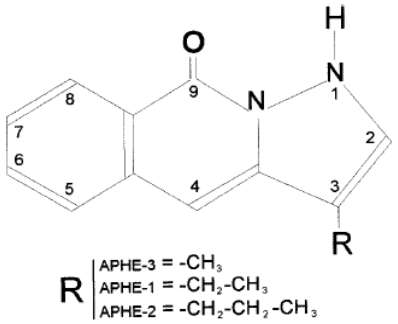


Fig.1: Structure of pyrazoloisoquinolinone antibiotics (Cruz *et al.*, 1996).

The *in-vitro* antimicrobial behavior of the pyrazoloisoquinolinone antibiotics is evaluated by serial dilution

technique. Immuno-modulatory and antimicrobial activity assays reveal a link between the biological actions of these compounds and the length of their aliphatic chains. Thus, antibiotics with longer aliphatic chains exhibited improved antimicrobial and immune modulatory activities. APHE-3 inhibits the proliferation of lymphocyte in the presence of phytohemagglutinin by more than 50 % at a concentration of 10^{-4} M while APHE-1 and APHE- 2 are less effective. This is due to the smaller size of APHE-3 being better able to bypass the membrane barrier of the cells (Fildago *et al.*, 1992).

The present study is aimed at the optimization of some critical parameters for the production of antitumor antibiotics from *Streptomyces griseocarneus* and having an insight into the production process for obtaining maximum titer of antibiotic for commercial productions**.**

Materials and Methods

Microorganism: A strain of *Streptomyces griseocarneus* NRRL B1068 was obtained from the culture bank of the Institute of Industrial Biotechnology, GC University Lahore. The culture was provided by the Agriculture Research Service (ARS) of U.S. Department of Agriculture. Stock culture was sub cultured in a 250ml flask containing 50ml of the malt yeast extract medium consisting of (g/L): yeast extract, 0.4; malt extract, 0.4; and glucose, 1.0. (Reddi & Rao, 1971) and maintained on solid medium in slants. The medium was sterilized at 121 ºC and 15 psi for 15 min. The inoculum was transferred to medium in aseptic conditions and flasks were incubated at 30°C and 200 rpm in shaking incubator (Vision Scientific CO, LTD, Korea MODEL: VS-8480) for 72 hrs.

Inoculum Preparation: The inoculum of the *Streptomyces griseocarneus* NRRL B1068 was prepared by adding 10 ml of sterilized distilled water to fully grown slants. The growth of the microorganism was scratched with the help of inoculating loop and mixed thoroughly with the added distilled water. Then, the inoculum was poured aseptically into 250mL Erlenmeyer flasks containing minimal basal medium (Cruz *et al.*, 1999).

Fermentation Experiments: For the production of APHE antibiotics, fermentation was carried out in 250 ml Erlenmeyer containing 50ml of modified minimal basal medium (Cruz *et al.*, 1999). The flasks were cotton plugged and sterilized in an autoclave at 121 ºC and 15 psi pressure for 20 min. The flasks were inoculated with 1 ml spore suspension after cooling at room temperature. After inoculation, the flasks were incubated at 30 ºC in shaking incubator for 10 days. The fermentation medium was filtered primarily by using filter paper and then centrifuged at 10,000 rpm for 10 min at 4 ºC. The pellet was removed and supernatant was used for antimicrobial activity.

Determination of biomass: The biomass of the fermentation broth was calculated as dry cell mass (DCM). The fermentation broth was centrifuged at 6000 rpm for 10 min. After centrifugation, the mycelial pellet was collected on the filter paper and dried at 70°C overnight (Bizukojc and Ledakowicz, 2008). The dry weight of the pellet was calculated by using the following formula:

Dry cell mass = (weight of filter paper + mycelium) - (weight of filter paper)

Determination of antimicrobial activity

Test Microorganisms: The bacterial and fungal test strains used in the present study were the clinical isolates obtained from Chughtais Lab Lahore (CLL). The test microorganisms used for antimicrobial activity included *Aspergillus niger*, *Escherichia coli* and *Bacillus subtilis.* The bacterial cultures were grown at 37°C on nutrient agar (NA) medium and fungi were grown at 30°C on Potato dextrose agar medium (PDA). All the cultures were preserved at 4°C and sub-cultured regularly.

Antimicrobial Assay: The antimicrobial activities of different bacterial and fungal strains were assayed by the agar well diffusion method (Malina, 2003). Petri plates were prepared with 15 ml sterile nutrient agar (NA) and potato dextrose medium (PDA) medium. The microbial cultures were spreaded on the surface of the solidified agar media. A 5 mm diameter well was made into the agar plates and 50µL broth from was poured into each well. The plates were left for 30 min at room temperature for diffusion. Negative control was prepared using the respective fermentation medium. The bacterial plates were incubated at 37°C for 24 hrs whereas the fungal plates were incubated at 30°C for 72 hrs. A zone of inhibition was measured in millimeters.

RESULTS AND DISCUSSIONS

Screening of fermentation media: Five different media were tested for the growth of *Streptomyces griseocarneus* NRRL B1068 and production of antitumor antibiotic (Fig. 1). The composition of these media is as follows:

M1 (g/L): potassium dihydrogen phosphate, 2.38; dipotassium hydrogen phosphate, 5.65; hydrated magnesium sulphate, 1 g; (0.1 g FeSO4. 7H2O, 0.1 g MnCl2.4H2O and ZnSO4.7H2O per 100 ml distilled water) glucose, 7.5; and lysine, 2.0 (Cruz *et al.*, 1999).

M2 (g/L): yeast extract, 1.0: beef extract, 1.0g; casein enzymic hydrolysate, 2.0; dextrose, 10 (Murray *et al.*, 2003).

M3 (g/L):17.0; soybean, 3.0; dextrose, 2.50; sodium chloride, 5.0 and K2HPO4, 2.50 (Forbes *et al.*, 1998).

M4 (g/L): yeast extract, 4.0; malt extract, 4.0; glucose, 1.0. (Downes and Ito, 2001).

M5 (g/L): casein, 1.0; starch, 10.0; potassium nitrate, 2.0; dipotassium hydrogen phosphate, 2.0; sodium chloride, 2.0; magnesium sulphate, 0.50; calcium carbonate, 0.20; and ferrous sulphate, 0.01(Jensen *et al.*, 2000).

The maximum amount of dry cell mass and maximum production of antibiotic was observed when M1 medium was used for fermentation. The production of APHE antibiotics is also affected by the constituents of the medium. (Theobald *et al.*, 2000; Schimana *et al.*, 2001). The maximum growth of *Streptomyces griseocarneus* NRRL B1068 and highest production of APHE antibiotics in modified minimal media was due to the reason that this composite medium supplied all the essential constituents e.g. carbon and nitrogen sources, salts, minerals and growth factors that is vital for the growth of the organism in the correct amounts (Maria *et al.*, 2001). Langlykke (2001) revealed that K_2HPO_4 helps in the elution of the bound antibiotics from the mycelia. The minimum growth in tryptic soy broth (TSB) medium may be due to the reason that it contains much smaller amounts of nutrients.

Effect of incubation time: The time of incubation is an important factor to affect the growth of microorganism and production of APHE antibiotics. The incubation time of the fermentation technique has a strong association with the production of APHE antibiotics. Therefore, the effect of incubation time on the production of APHE antibiotics was studied by incubating the fermentation flasks for different intervals of time ranging from 1-10 days. It was observed that the maximum growth of *Streptomyces griseocarneus* NRRL B1068 (4.2 mg/ml) and highest zones of inhibition of 30mm, 22mm and 21 mm were produced at 7th day of incubation against *A. niger, E. coli and B. subtilis* respectively. It can be seen from the Fig 4.2 that the APHE antibiotics production started to decline after 7th day of incubation. At 6th day of incubation, there was a reduced growth of microorganism (3.8 mg/ml) and inhibition zones of 14mm, 9mm and 12mm were observed against *A. niger, E. coli and B. subtilis* respectively. While at the 8th day of incubation, zones of 25mm, 23mm and 22mm were produced against *A. niger, E. coli and B. subtilis* respectively, with biomass of 4.0 mg/ml. Although, the growth of the microorganism was obtained at 9th day of incubation (3.1 mg/ml) but there was no antimicrobial activity. So, the time course of 7 days was

selected as the best time of incubation for the production of APHE antibiotics by *S. griseocarneus* NRRL B1068.

The growth of microorganism began to increase steadily as the fermentation process started. The highest production of APHE antibiotics after 7 days of incubation might be due to the fact that microorganism has entered the stationary phase of growth and it has been reported that the antibiotic production by *Streptomyces* takes place in stationary phase of the growth (Gramajo *et al.*, 2003). The decrease in the APHE antibiotics production after 7 days of incubation may be due to the decrease in the nutrients availability to the microorganism or accumulation of noxious byproducts and metabolites. So, the increase in time does not mean to synthesize more antibiotics, but it may produce more secondary metabolites. It may produce even more toxins that slow down the production of antimicrobial compounds.

Effect of Incubation temperature: The effect of different incubation temperature on APHE antibiotics production by *Streptomyces griseocarneus* NRRL B1068 was observed by changing the temperatures of incubation from 25-40 ºC. It was observed that the maximum growth of the microorganism (3.2 mg/ml) and highest production of APHE antibiotics was obtained at 30ºC with the inhibition zones of 35mm, 25mm and 24 mm against *A. niger, E. coli and B. subtilis* respectively as shown in fig 4.3. At 25 ºC, there was minimum growth of microorganism (2.0 mg/ml) and smaller zones of inhibition of 12mm, 14mm and 11mm were produced against *A. niger, E. coli and B. subtilis* respectively. There was no production of APHE antibiotics but the strain was capable to grow at 40 ºC (1.8 mg/ml). As a result, the best temperature for the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotics production was found as 30 ºC.

The optimum temperature for the growth of microorganism and antibiotic production was found as 30 ºC (Fig. 3). Gradual increase in the amount of dry cell mass and diameter of inhibition zone was observed when the incubation temperature was increased from 25-30º C. Above 30 ºC; there was a rapid decline in both the growth and antibiotic production with no antibiotic production at 40ºC. Different workers have reported that optimum temperature for the growth of *Streptomyces* is 25-35 ºC (Buchanan & Gibbons, 1974). Lesser growth of microorganism at higher temperature is due to the fact that high temperature retards the metabolic processes of the microorganism by denaturing enzymes, transport carriers and other proteins.

Effect of Initial pH: The pH of the fermentation broth is the foremost parameter in most studies on the microbial fermentations. In order to study the effect of different media pH on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotics production, pH of the fermentation medium was varied from 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. The optimum pH of the fermentation broth for the growth of *Streptomyces griseocarneus* NRRL B1068 (4.75 mg/ml) and subsequent production of APHE antibiotics were produced at the neutral pH of 7.2 with the mean zones of inhibition of 35mm, 25mm and 24mm against A. *niger, E. coli and B. subtilis* respectively. At pH 7.5, there was considerable growth of microorganism (4.6 mg/ml) and inhibiton zones of 13mm, 16mm and 15mm were produced against *A. niger, E. coli and B. subtilis* respectively. All the other values of the medium pH (5.5-8.5) supported reduced growth of microorganism (2.5-3.72 mg/ml) and exhibited low production of APHE antibiotics as shown in Fig 4.4.

The pH plays an important physiological and morphological role in the growth and metabolism of the microorganism. The growth of *Streptomyces griseocarneus* NRRL B1068 in the acidic side of pH was much less with no production of APHE antibiotics. But, when the pH value was raised above 7.0, there was an increase in the biomass and APHE production. The change in the pH values affects both the time and amount of APHE antibiotics production (James and Edwards, 2000). So, it was concluded that the microorganism was neutrophilic and capable to produce APHE antibiotics at the neutral pH ranges between 7.0-7.2.

Effect of carbon sources: Different carbon sources such as glucose, fructose, sucrose, maltose, galactose and xylose were screened to study their effectiveness on APHE antibiotics production by *S.griseocarneus* NRRL B1068 in submerged fermentation. The growth of the microorganism and production of APHE antibiotics was substantial in the entire sources of carbon tested, but the optimum results were achieved by using glucose. It is obvious from the Fig. 4.5a that the glucose gave highest growth of the microorganism (3.6 mg/ml) and produce maximum yield of APHE antibiotics with mean zones of inhibition of 24mm, 21mm and 22mm against *A. niger, E. coli and B. subtilis* respectively after 7 days incubation at 30◦C.

Different amounts (1-10 %) of glucose were supplemented in the fermentation broth to find the optimum level of glucose for enhanced production of APHE antibiotics by *S.griseocarneus* NRRL B1068 (Fig. 4.5b). The production of APHE antibiotics was raised by increasing the amount of glucose from 1 %, and the highest production of APHE antibiotics (3.6 mg/ml) was obtained at 8.0 %glucose level, but above this level there was a slow decrease. So, 8.0 % glucose level was chosen for the preparation of the media in further fermentation experiments.

The amount of carbon source has an obvious effect on the metabolic processes of microorganisms. The low production of APHE antibiotics in small amount of carbon source may be because of the fact that less amount might not accomplish the requirements of microorganism to grow and synthesize maximum APHE antibiotics. The amount of carbon source has a marked effect on the metabolism of *Streptomyces griseocarneus* NRRL B1068. The production of APHE antibiotics by *S. griseocarneus* NRRL B1068 stopped when glucose in the medium was finished (Soliveri *et al.*, 1988).

All living organisms need carbon as a major element for the growth of the cell and to carry out balanced metabolism of the body. However, requirements of carbon sources vary in every organism. Carbon source which had been fully utilized during growth would prove unsatisfactory for antimicrobial production of metabolites. On the other hand, a compound which is only partially utilized during cell growth might be more appropriate for the subsequent antibiotics production (Dekleva *et al.*, 2005).

Effect of nitrogen source: In order to screen out the best nitrogen source for maximum APHE antibiotics production, different amino acids such as lysine, glycine, cysteine, asparagin, leucine and isoleucine were tested to study their effectiveness on APHE antibiotics production from *S.griseocarneus* NRRL B1068 in submerged fermentation. It was found that lysine produced highest growth of microorganism (4.3 mg/ml) and maximum yield of APHE antibiotics with mean zones of inhibition i.e. 24mm, 21mm and 23mm against *A. niger, E. coli and B. subtilis* respectively, after 7 days of incubation at 30◦ C. Cysteine produced inhibition zones of 18, 13 and 14mm against *A. niger, E. coli and B. subtilis* respectively with biomass of 3.2 mg/ml as shown in Fig 4.6a.

Different amounts 1-3 % (v/v) of lysine was used to observe their effects on the growth of microorganism and APHE antibiotics production. It was analyzed that maximum growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotics production were obtained from 1.5 to 2.5 % (v/v) lysine level. At 2.0 % lysine, the maximum biomass (3.3 mg/ml) and high APHE production of antibiotics was obtained but above that there was a decline in both the growth and APHE production (Fig. 4.6 b). Therefore, 2.0 % (v/v) lysine level was used in the further fermentation experiments.

The sources of nitrogen given to microorganism have obvious effect on the nature of the APHE antibiotics produced (Theobald *et al.*,

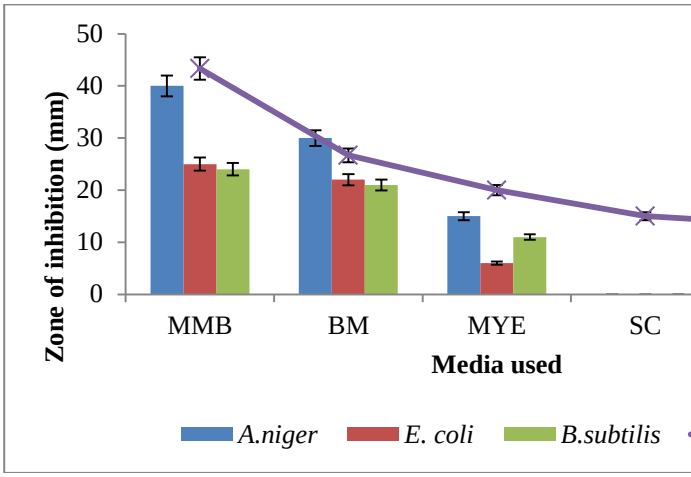
2000) and the requirements of nitrogen sources also fluctuate among different organisms. In the current study, the medium containing lysine as nitrogen source supported maximum production of APHE antibiotics. For this reason, lysine was selected as the best source of nitrogen and was used in the further studies. The same source of nitrogen was used by Motkova *et al*., 1981 during tobramycin production from *S. ceremeus* and cephamycin from *S. griseus*. Furthermore, it was observed that APHE antibiotics production is very sensitive to even small amounts of cysteine in the culture medium, promoting the moderate rate of growth but no synthesis of APHE antibiotics. However, L-cysteine is good nitrogen source for many other different *Streptomyces* strains (Pen-Chaur *et al.*, 1992) but synthesis of cysteine is a complex process and expensive too.

Effect of inorganic nitrogen sources: Different inorganic nitrogen sources such as ammonium sulphate, sodium nitrate, peptone and ammonium chloride were evaluated for maximum growth of *Streptomyces griseocarneus* NRRL B1068 and highest production of APHE antibiotics in submerged fermentation. It was found that the maximum growth of the microorganism (3.8 mg/ml) and highest production of APHE antibiotics was shown by ammonium sulphate with inhibition zones of 15, 12 and 9mm against *A. niger, E. coli and B. subtilis* respectively. The growth of the microorganism was moderately low (3.2 mg/ml) when sodium nitrate was used and showed antimicrobial activity with the inhibition zones of 13, 12 and 11mm against *A. niger, E. coli and B. subtilis* respectively. Whereas, peptone showed reduced growth (2.9 mg/ml) with inhibition zones of 10, 9 and 6mm against *A. niger, E. coli and B. subtilis* respectively. There was no inhibition activity exhibited by ammonium chloride whereas biomass of 1.9 mg/ml was obtained from it as shown in Fig 4.7.

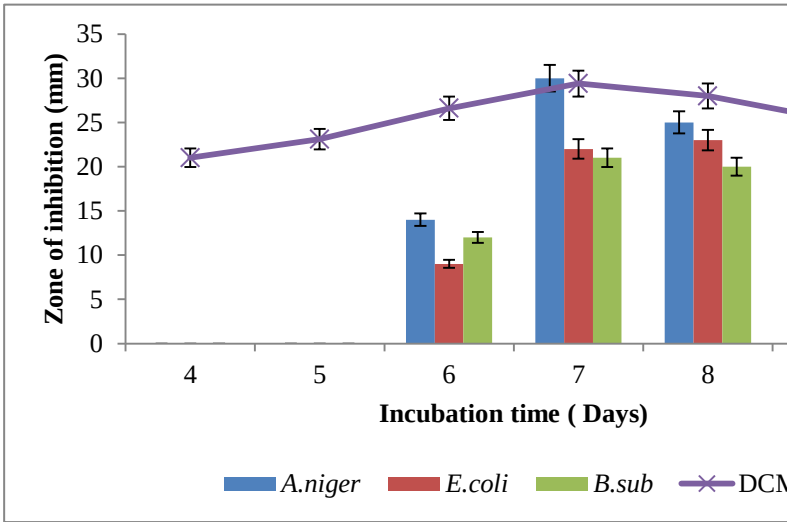
The fermentation broth was supplemented with each of these inorganic sources of nitrogen at a level of 0.8 %. Ammoniun sulphate was selected as the best inorganic nitrogen source for the production of APHE antibiotics from *Streptomyces griseocarneus* NRRL B1068. It might be due to the fact that it provided both the ammonium and sulfate ions that is required for the filamentous cell growth of cells and APHE antibiotics production (Mekala *et al*., 2008). Mangat & Mandahr (1998) reported that nitrogen sources significantly affect the APHE production so they must be used in appropriate quantities. The elevated amounts of inorganic nitrogen sources can cause vitrification of the medium that is generally unusable by microorganisms.

Effect of inoculum size: The inoculum volume of the fermentation broth was varied from 1-3% for the highest growth of *Streptomyces griseocarneus* NRRL B1068 and maximum production of APHE antibiotics. 50 µl of the fermentation broth was poured in each well for the determination of antimicrobial activity. It was observed that the optimum growth of the microorganism (5.2 mg/ml) and maximum production of APHE antibiotics was achieved at the inoculum volume of 3 % with mean zones of inhibition of 14, 12 and 13against *A. niger, E. coli and B. subtilis* respectively.

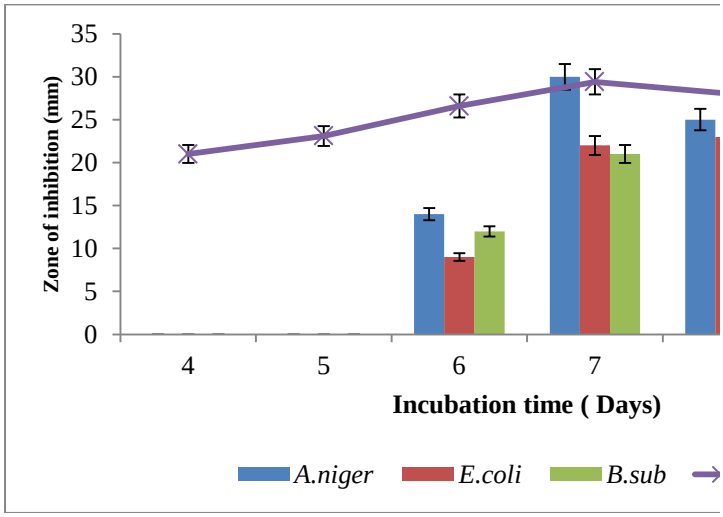
The amount and quality of inoculums play an important role in the bioprocessing of APHE antibiotics (Ettler, 2002). It has been found that cell inoculums at stationary phase produced the best growth of microorganism and APHE production which is very much similar to the results reported by Dekleva *et al.*, 2005.



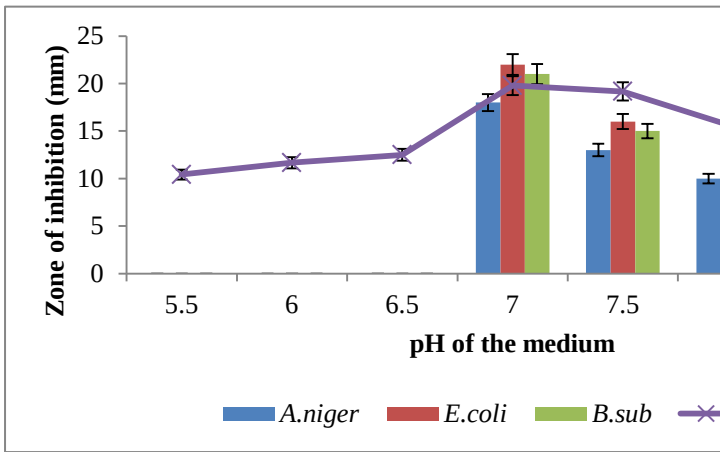
Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.
Submerged fermentation conditions: pH 7.0, Incubation temperature 30° C, Incubation time 7 days.
Abbreviations: MMB= Modified Mineral Basal Medium, BM= Bennet Medium, TSB= Tryptic soy broth medium, SC= Starch casein medium, MYE= Malt yeast extract medium.
Fig.1: Effect of fermentation media on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotics production.



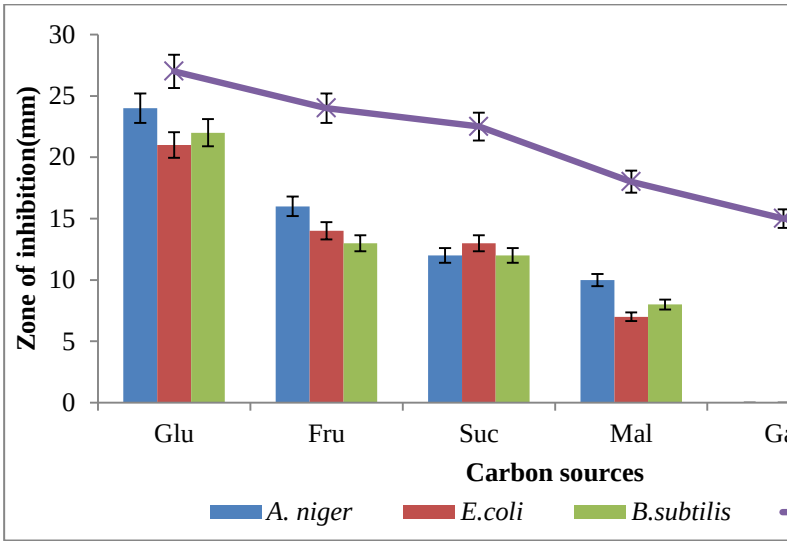
Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.
Submerged fermentation conditions: pH 7.0, Incubation temperature 30° C, Modified minimal basal medium.
Fig.2.Effect of incubation time on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotics production.



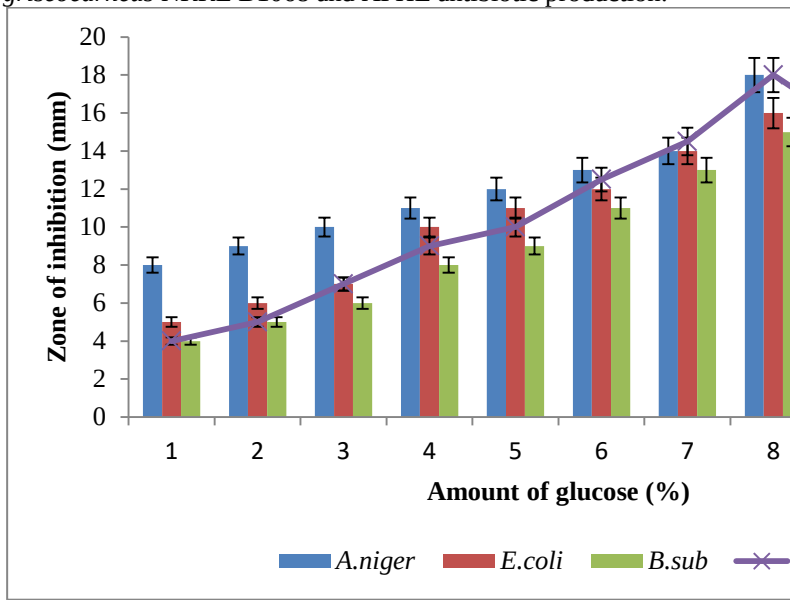
Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.
Submerged fermentation conditions: pH 7.2, Incubation time 7 days, Inoculum size 2% in Minimal basal fermentation medium.
Fig.3. Effect of incubation temperature on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotics production.



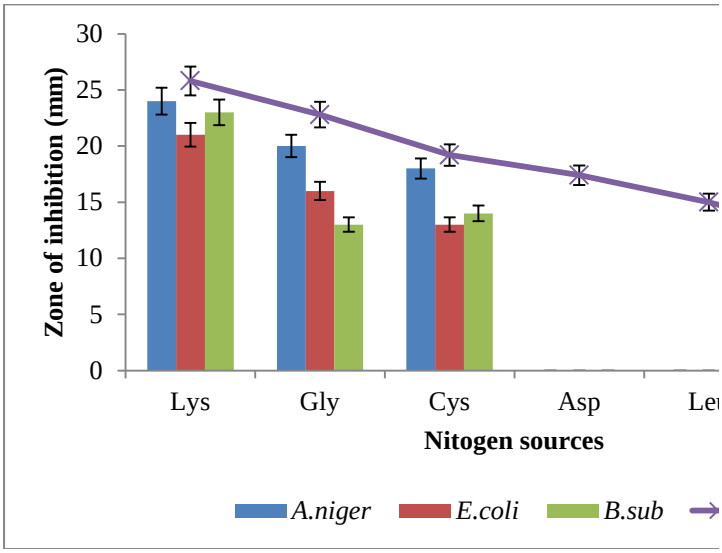
Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.
Submerged fermentation conditions: Incubation temperature 30° C, Incubation time 7 days, Modified minimal basal media.
Fig. 4.Effect of initial pH for on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotics production



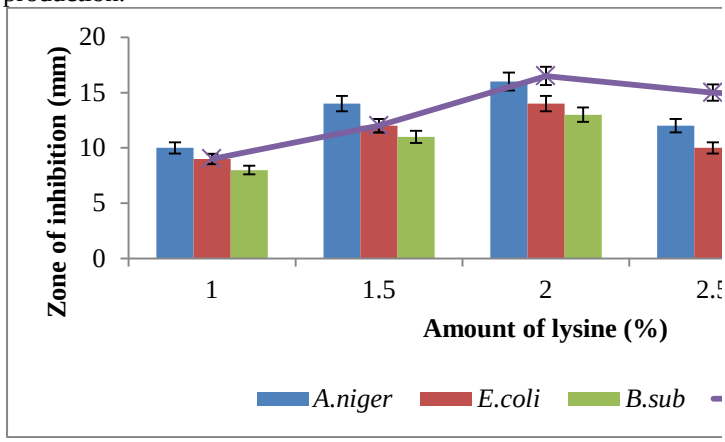
Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.
Submerged fermentation conditions: Incubation temperature 30° C, pH 7.2, Incubation time 7 days, Modified minimal basal media.
Fig. 5: Effect of type of carbon source on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotic production.



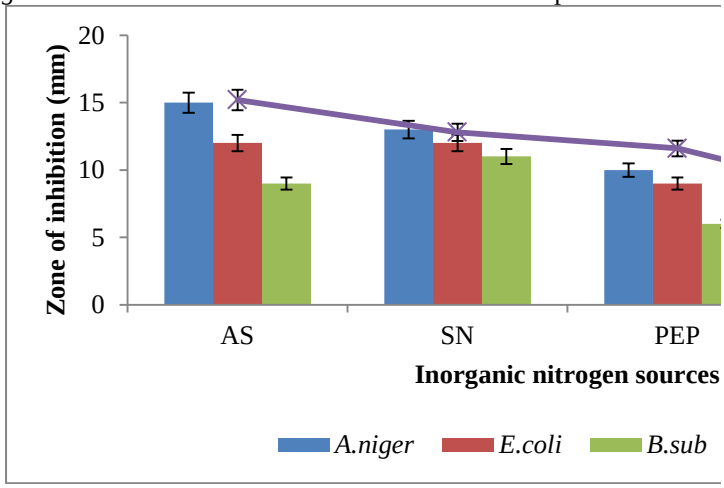
Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.
Submerged fermentation conditions: Incubation temperature 30° C, pH 7.2, Incubation time 7 days, Modified minimal basal media.
Fig. 6: Effect of amount of carbon source on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotic production



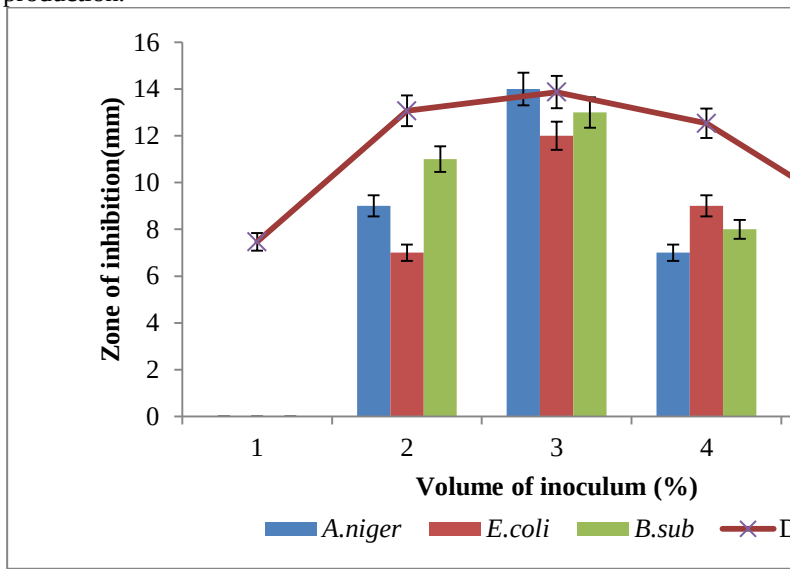
Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.
Submerged fermentation conditions: Incubation temperature 30° C, pH 7.2, Incubation time 7 days, Modified minimal basal media.
Abbreviations: Lys = Lysine, Gly = Glycine, Cys = Cysteine, Asp = Asparagin, Leu = Leucine, Iso=Isoleucine.
Fig.7. Effect of type of nitrogen source on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotic production.



Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.
Submerged fermentation conditions: Incubation temperature 30° C, pH 7.2, Incubation time 7 days, Modified minimal basal media.
Fig. 8. Effect of amount of lysine on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotics production.



Each value is a mean of three replicates. Y-error bars indicate the standard error from the mean.
Submerged fermentation conditions: pH 7.0, Incubation temperature 30° C, Incubation time 7 days, Inoculum size 2% in MMB medium.
Abbreviations: AS= Ammonium sulphate, SN= Sodium nitrate, PEP=Peptone, AC= Ammonium chloride.
Fig. 9. Effect of inorganic nitrogen sources on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotics production.



Each value s a mean of three replicates. Y-error bars indicate the standard error from the mean.
Submerged fermentation conditions: pH 7.0, Incubation temperature 30° C, Incubation time 7 days, Inoculum size 2% in MMB medium.
Fig.10: Effect of volume of inoculums on the growth of *Streptomyces griseocarneus* NRRL B1068 and APHE antibiotics production.

Conclusion: The purpose of the study was to optimize the fermentation parameters for the APHE antibiotics production from *Streptomyces griseocarneus* NRRL B1068. It is concluded by the present study that the microorganism showed maximum titers of APHE antibiotics under the optimized conditions of submerged fermentation. The optimization of the process significantly enhanced the yield of APHE antibiotics. The microorganism showed antimicrobial activity against all the three microorganisms tested and it can be accomplished that *Streptomyces griseocarneus* NRRL B1068 acts as a promising source of antimicrobial agent in future.

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