

Original Research Article

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Optimization of Cultural Conditions for Production of Antifungal Bioactive Metabolites by *Streptomyces* spp. Isolated from Soil

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ABSTRACT

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The present research was undertaken with an aim to isolate and characterize antifungal compounds from actinomycetes and optimization of cultural characteristics for production of bioactive metabolite by actinomycetes. Actinomycetes isolate ACITM-1 was further cultivated in different conditions fermentation in order to optimize biosynthetic process of antibiotic production including: different pH values, different temperatures, different carbon and nitrogen sources were fully investigates. Actinomycetes isolate ACITM-1 was isolated from soil of Gwalior Chambal region, MP, (India) was found to show significant antifungal activity against phytopathogens of soybean crop. Based on physiological, biochemical characteristics the isolate was identified as *Streptomyces chilikensis* ACITM-1. This strain was further cultivated in different conditions in order to optimize the growth of secondary metabolite production. The antimicrobial activity and biomass production was highest when isolates were cultivated in media having starch as a carbon source. Peptone and potassium nitrate were found best nitrogen source for the highest antifungal activity and biomass production. Form the experiment it was observed that antifungal activity was found maximum with starch, peptone and potassium nitrate in 96 hours at 30° C.

Introduction

Actinomycetes are a group of Gram-positive bacteria. They are best source for bioactive compound production. Actinomycetes produce large number of important bioactive metabolites such as antibiotic compounds including Streptomycin, actinomycin, and tetracycline (Lo *et al.*, 2002; Gonzalez *et al.*, 2005; Kavitha *et al.*, 2009; Raja *et al.*, 2011). *Streptomyces* occur in a multiplicity of natural and man-made environments and a unique group having different morphological, cultural, biochemical and physiological characters. Among natural sources, several antifungal and antibacterial compounds are

reported from the genus *Streptomyces* (Singh *et al.*, 2012). The production of secondary metabolites from the genus *Streptomyces* can be influenced by optimization of the nutritional requirements and cultural conditions. These conditions play an important role in the production of these secondary metabolites (Khattab *et al.*, 2016).

The ability of *Streptomyces* to form these bioactive compounds is not a fixed property but can be greatly increased or decreased under different conditions of nutrition and cultivation media. Hence media composition

plays a vital role in the efficiency and economics of the ultimate process. Therefore, designing an appropriate fermentation medium is of critical importance in the production of secondary metabolites. Changes in the nature and type of carbon and nitrogen sources have been reported to affect antibiotic biosynthesis in *Streptomyces*. Also several cultivation parameters like pH, incubation period and temperature play a major role in the production of bioactive metabolites (Bundale *et al.*, 2015).

Materials and Methods

Collection of soil samples

Different soil samples were collected from different sites of Gwalior, (MP), India, such as garden, agricultural field, playground, and poultry farms. Soil samples were carefully taken with spatula down to a 10 cm depth into the soil. The samples were stored in sterile plastic bags then labeled properly then brought to lab and stored at 4°C until use.

Isolation of Actinomycetes

Soil samples were collected from different sites such as garden, agricultural field, playground, and poultry farms and actinomycetes were isolated by serial dilution technique on different media such as starch casein agar, soil extract agar, ISP medium, Nutrient agar, glycerol asparagine agar, starch agar, Yeast extract malt extract agar, actinomycetes isolation agar media and incubated for 6-7 days at 30°C. The isolated actinomycetes culture were further purified on respective fresh media and stored in BOD incubator for further use.

Screening of Actinomycetes for antifungal activity

After isolation of 80 actinomycetes, screening was done for the antifungal activity of

actinomycetes. All isolates were screened for their *in vitro* antifungal activity against pathogenic fungi of plants. Screening was done on the basis of primary and secondary method. Primary screening was done by the cross streak plate method (Saxena *et al.*, 2013).

Secondary screening was done by the well agar diffusion method. In this method two different types of media were prepared i.e. starch casein broth and potato dextrose agar then selected actinomycetes (ACITM-1) on the basis of primary screening, was inoculated into two flask of starch casein broth.

Flasks were incubated for 5 days in orbital shaker then prepared the potato dextrose agar media and made two wells in each plate using well cutter and each well was loaded with 150 µl of starch casein broth in the wells and put all tested fungi in each plate. Plates were incubated for 6-7 days at 25 °C (Mohanraj *et al.*, 2013).

Physiological, biochemical and molecular characterization

Most active antimicrobial producing isolate designated as ACITM-1 which was characterized on the basis of morphological, physiological, cultural and biochemical characters. The morphology of the spore bearing hyphae with entire spore chain along with the substrate and aerial mycelium was examined under scanning electron microscope (SEM).

Cultural characteristics (growth pattern, color of aerial and substrate mycelia, formation of pigment) was studied on different media yeast extract-malt extract agar (ISP-2), Oat meal agar (ISP-3), Starch inorganic salt agar (ISP-4), Asparagine glycerol agar (ISP-5), Starch casein agar, Actinomycetes isolation agar and Nutrient agar. Ability of isolate producing

different type of secondary metabolite was examined by using standard methods.

Optimization of the actinomycetes growth and secondary metabolite production in different conditions

Effect of different media

The selected isolate ACITM-1 was inoculated in different broth medium to test the maximum bioactive compound production in broth medium. The media used Yeast extract-Malt extract broth, Oatmeal broth, Inorganic Starch-Salt broth, Glycerol asparagines broth, Starch casein broth, Starch broth and Peptone yeast extract iron broth. Afterwards 5 ml of the culture filtrate from each medium were taken aseptically and the antifungal activity was measured using the inhibition zone method.

Effect of different incubation period

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch casein medium each were inoculated with the selected isolate (ACITM-1) and incubated on rotary shaker (150 rpm) at $30 \pm 1^\circ\text{C}$ for various incubation periods (e.g 1, 2, 3, 4, 5, 6 and 7 days). At each incubation period, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured using the inhibition zone method. Three plates were used within each incubation period for each fungus.

Effect of different temperature

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch casein medium each were inoculated with the selected isolate and incubated on rotary shaker (150 rpm) for the optimum incubation period (7 days), at different temperatures (20,25, 30, 35 and 40°C). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured using the inhibition

zone method described earlier. Three plates were used for each temperature for each fungus.

Effect of different pH

Erlenmeyer flasks (250 ml) containing 100 ml sterile starch casein medium each were adjusted at various levels of pH (2, 4, 6, 7, 7.5, 8 and 10) using a phosphate buffer before the sterilization and then inoculated with the selected isolate and incubated for the optimum incubation period (7 days) at the optimum temperature (31°C). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured using the inhibition zone method described earlier. Three plates were used for each pH level for each fungus.

Effect of different carbon sources

In this experiment, glucose, fructose, sucrose, lactose, xylose, dextrose, starch and maltose were tested as substitute carbon sources. Carbon source of SCA medium was substituted with one of the tested sources (containing the same quantity of carbon). Erlenmeyer flasks containing starch substituted starch-nitrate medium were inoculated with the selected isolate. The initial pH of the various media was adjusted at 7.5, before sterilization and the flasks were incubated for 3 days at 30°C on a rotary shaker (150 rpm). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured by the inhibition zone method described earlier. Three plates were used for each carbon source for each fungus.

Effect of different nitrogen sources

In this experiment, potassium nitrate, ammonium nitrate, soya peptone, tryptone, peptone, beef extract, malt extract and yeast

extract were tested as substitute nitrogenous sources. Nitrogenous source of SCA medium was substituted with one of the tested sources (containing the same quantity of nitrogen) Erlenmeyer flasks containing SCA medium (sodium nitrate substituted with one of the tested sources) were inoculated with the selected isolate. The initial pH of the various media was adjusted at 7.5, before sterilization and the flasks were incubated for 3 days at 31°C on a rotary shaker (160 rpm). For each, 5 ml of the culture filtrate were then taken aseptically and the antifungal activity was measured by the inhibition zone method described earlier. Three plates were used for each nitrogenous source for each fungus.

Results and Discussion

Sample collection and isolation of actinomycetes

Different soil samples were collected from different areas of Gwalior Chambal region as ITMU garden, ITMU medicinal plant area, ITMU playground, Poultry farm, Agriculture field, Chambal revines and agriculture field. Actinomycetes were isolated as 10 from ITMU garden soil, 9 from medicinal plant soil, 12 from ITMU playground soil, 7 from poultry farm soil, 16 from agriculture field soil, 11 from Chambal soil, and 5 from ITM sitholi campus soil by serial dilution method.

Total 80 actinomycetes were isolated from different sites of Gwalior Chambal region (Fig. 1). Maximum actinomycetes were found from Agriculture field soil area. Different ISP media were used for isolation of actinomycetes but maximum isolates were grown well on starch casein agar media.

Primary screening- Cross streak plate method

All 80 isolated actinomycetes were tested against each isolated fungus. Purified colonies of actinomycetes were subjected to antifungal

analysis against five sensitive strains of fungi viz. *Macrophomina phaseolina*, *Fusarium oxysporum*, *Colletotrichum truncatum*, *Rhizoctonia solani* and *Sclerotium rolfsii*. Primary screening of isolates was done to check their antifungal activity by cross streak method. Among 80 isolated actinomycetes 6 isolates were found to give antifungal activity against different fungus. ACITM-1) was selected on the basis of screening because ACITM-1 gave zone of inhibition against all tested fungi named *Macrophomina phaseolina*, *Fusarium oxysporum*, *Colletotrichum truncatum*, *Rhizoctonia solani* and *Sclerotium rolfsii*.

ACITM-1 showed activity against all fungi, ACITM-16 showed activity against 1 fungus, ACITM-25 showed activity against 3 fungi, ACITM-36 has abtifungal activity against 1 fungus, and ACITM-53 has antifungal activity against 2 fungi and ACITM-78 isolate showed activity against 2 fungi. On the basis of primary screening one isolate (ACITM-1) was selected for secondary screening in which *Macrophomina phaseolina*, *Fusarium oxysporum*, *Colletotrichum truncatum*, *Rhizoctonia solani* gave zone of inhibition.

Secondary screening- Agar well diffusion method

Secondary screening was done by agar well method. In agar well diffusion method, starch casein broth culture of selected ACITM-1 was used in the agar wells. The result indicates that isolate ACITM-1 has antagonistic activity against four fungi *M.phaseolina*, *C. truncatum*, *F. oxysporum*, *R. solani* but *S.rolfsii* had no dead zone of inhibition.

Culture Characteristics of actinomycetes strain on different agar media

The colour of mycelium above the petri dishes and colour of media were shown. various media were used for growth studies

like Yeast extract malt extract agar, Starch agar, Starch casein agar, Oat meal agar, Actinomycetes isolation agar, Glycerol asparagines agar, Peptone yeast iron agar, Inorganic salt starch agar, Tryptone yeast extract agar, Nutrient agar, Potato dextrose agar. The selection of growth medium was done on the basis of comparative incubation time and growth of actinomycetes in solid agar media. The result indicates that the maximum and fast growth of selected actinomycetes was found on Starch Casein Agar (SCA) media therefore the starch casein agar was selected as a medium for selected isolate.

Identification of Actinomyceytes

On the basis of microscopic, biochemical and molecular characterization the screened ACITM-1 actinomycetes was identified as *Streptomyces chilikensis*.

Optimization of different conditions for the growth & bioactive component production of actinomycetes

Effect of different media

Results showed that isolate ACITM-1 had highest antimicrobial activity and biomass production in starch casein broth followed by Starch Broth (SB), Yeast Extract Malt Extract broth (YEMEB), Inorganic Salt Starch Broth (ISSB), and Nutrient Broth (NB). Among the media tested, starch casein broth showed more zone of inhibition due to more antibiotic production. Hence, starch casein broth was selected as suitable medium for shake flask fermentation of ACITM-1 (Fig 2).

SCB= Starch casein broth, SB= Starch broth, NB= Nutrient broth, YEMB= Yeast extract malt extract broth, ISSB= Inorganic salt starch broth, GAB= Glycerol asparagine broth, TB= Tryptone broth, OB= Oatmeal broth.

Effect of different incubation period

The antifungal compound production and growth of *Streptomyces chilikensis* ACITM-1 was increasing continuously from 72 hrs to 96 hrs. Further increase in incubation time showed a gradual decrease in the production of antifungal compound and the growth of actinomycetes. Therefore optimum incubation time for the maximum production of secondary metabolite was at 96 hrs. The zone of inhibition was found to be maximum after 96 h of fermentation in seeded starch casein broths. This indicated that the maximum production of antibiotic was occurring at the end of 4th day of fermentation (Fig 3).

Effect of different temperature

The amount of secondary metabolite production *Streptomyces* highly depend on the temperature and growth rate. Optimum temperature for maximum growth and productivity of *Streptomyces chilikensis* ACITM-1 was determined zone of inhibition (Fig. 4). From the temperature optimization experiments, it was observed that the temperature adequate for growth is the same as that for antibiotic production. ACITM-1 strain has proved to show maximal growth and antibiotic production at 30 °C followed by 35 °C, 40 °C, 25 °C, 20 °C, 45 °C according to the measurement of zone of inhibition. The optimum growth and antimicrobial compound production was observed at 30 °C and beyond optimum temperature the growth and antimicrobial metabolite production was decreased. However higher temperature showed adverse effect on both growth and bioactive compound production.

Effect of different pH

pH of the culture medium affects not only growth but also production of the antibiotic. Optimum pH for production of antimicrobial

compound was found 7.5 and 7 (Fig.5). It has been observed that maximum antibiotic production was obtained at pH 7.5 by *Streptomyces chilikensis* AITM-1 and beyond optimum pH the growth and antimicrobial metabolite production was decreased. However higher pH showed adverse effect on both growth and bioactive compound. Fermentation of ACITM-1 was carried out using starch-casein broth.

Effect of different carbon source

It was observed that no significant difference in antimicrobial activity and biomass production when isolates were cultivated in media having starch as a carbon source followed by the dextrose, glucose, maltose, sucrose and fructose. In contrast no activity was observed with xylose, lactose (Fig.6). Starch is reported as an important medium component for the production of antifungal compounds from microorganisms. Maximum growth and antibiotic production is found to occur when starch used as the sole source of carbon.

Effect of different nitrogen source

Peptone and potassium nitrate were found

best nitrogen source for the highest antifungal activity and biomass production followed by the yeast extract, malt extract in contrast no activity was observed with ammonium nitrate, tryptone (Fig.7), Peptone considered as suitable medium component for the production of antifungal from the *Streptomyces chilikensis* ACITM-1.

Screening and isolation of actinomycetes from the soils of Gwalior- Chambal region was identified as *Streptomyces chilikensis* ACITM-1. *Streptomyces chilikensis* ACITM-1 has been reported as producer of the strong antifungal activity against the various fungal pathogens.

In this study, we focused on the optimization of culture conditions for production of antifungal secondary metabolite by a new isolate *Streptomyces chilikensis* ACITM-1. The optimization of fermentation medium is as important as selection of an organism to obtain antibiotic production. The source of carbon and nitrogen in the fermentation media plays an important role. It has been reported in literature that Glycerol was reported as an important medium component for the production of antifungal compounds from microorganisms.

Table.1 Culture Characteristics of actinomycetes strain on different agar media

Media used	Growth	Aerial mycelium	Substrate mycelium
Yeast extract malt extract agar	Good	Grey	White
Starch agar	Good	Grey	White
Starch casein agar	Excellent	Grey	White
Oat meal agar	Good	Grey	White
Actinomycetes isolation agar	Good	Grey	White
Glycerol asparagines agar	Good	Grey	White
Peptone yeast iron agar	Good	Grey	White
Inorganic salt starch agar	Mild	Grey	White
Tryptone yeast extract agar	Mild	Grey	White
Nutrient agar	Mild	Grey	White
Potato dextrose agar	Good	Grey	White

Fig.1 Screening of actinomycetes for antifungal activity

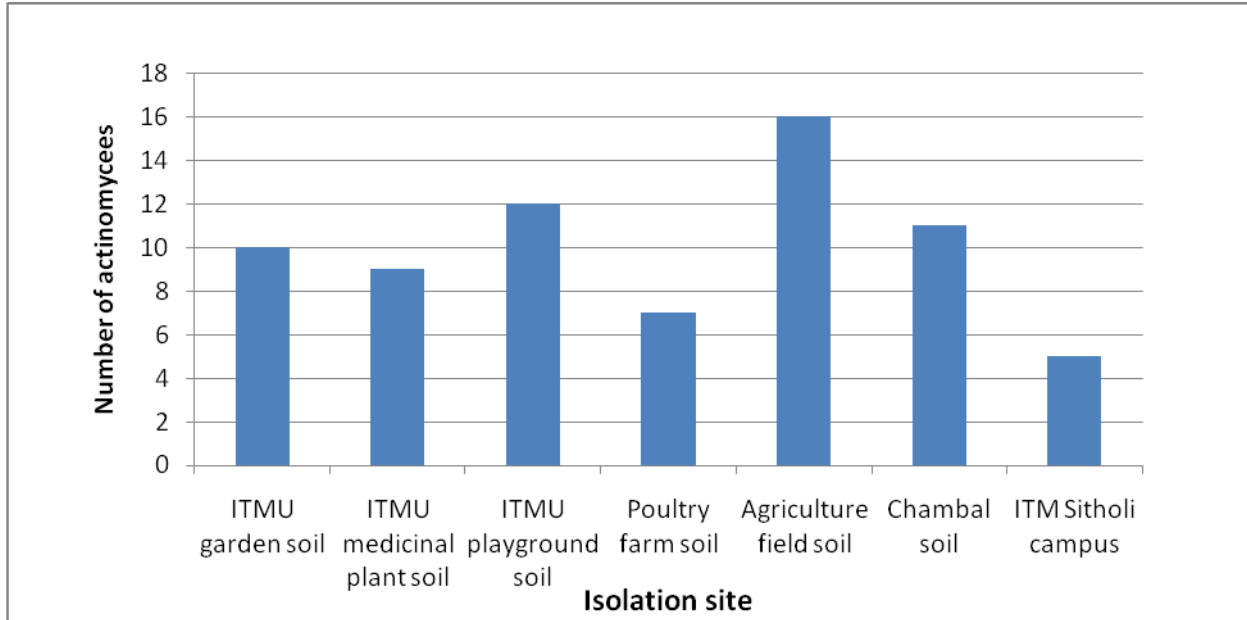


Fig.2 Effect of different media on growth of *Streptomyces chilikensis* ACITM-1

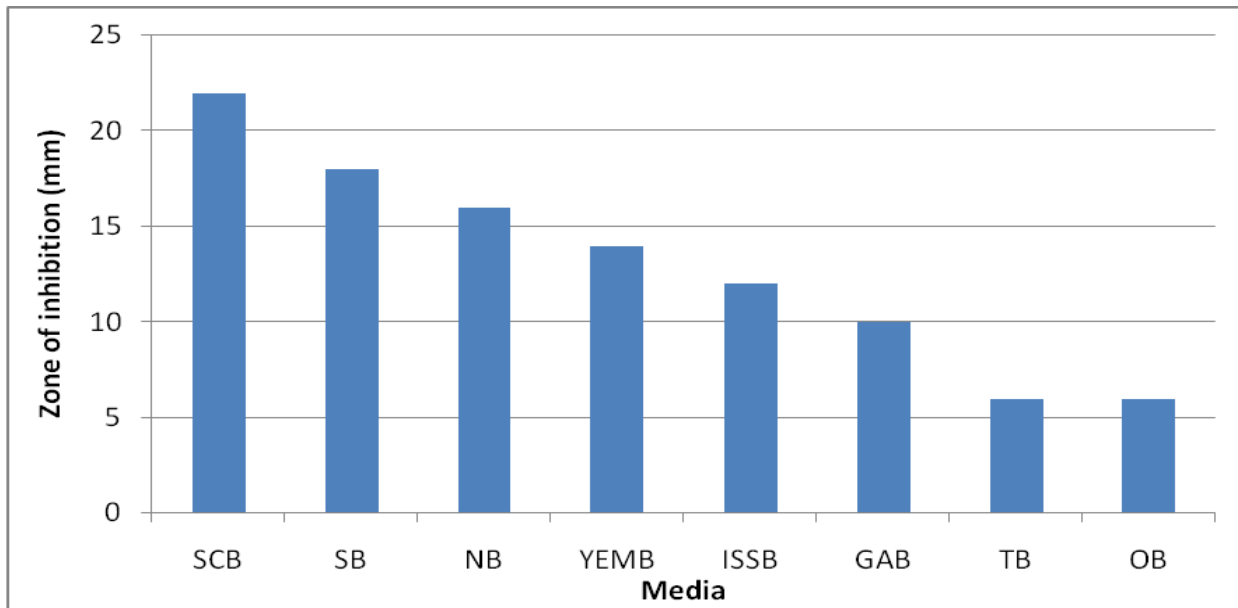


Fig.3 Effect of incubation period on growth of *Streptomyces chilikensis* ACITM-1

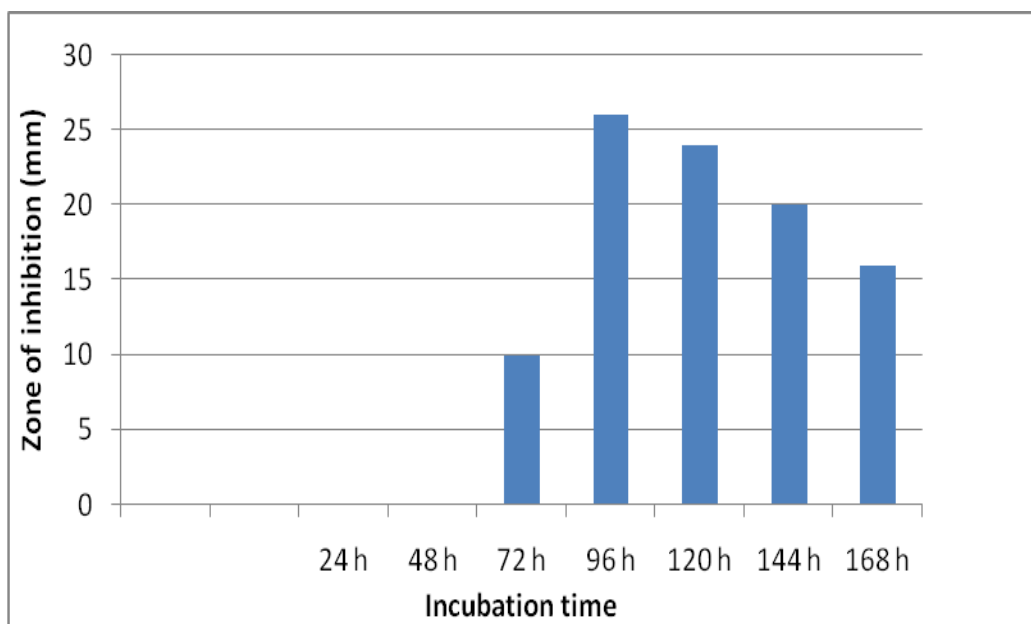


Fig.4 Effect of different temperature on growth of *Streptomyces chilikensis* ACITM-1

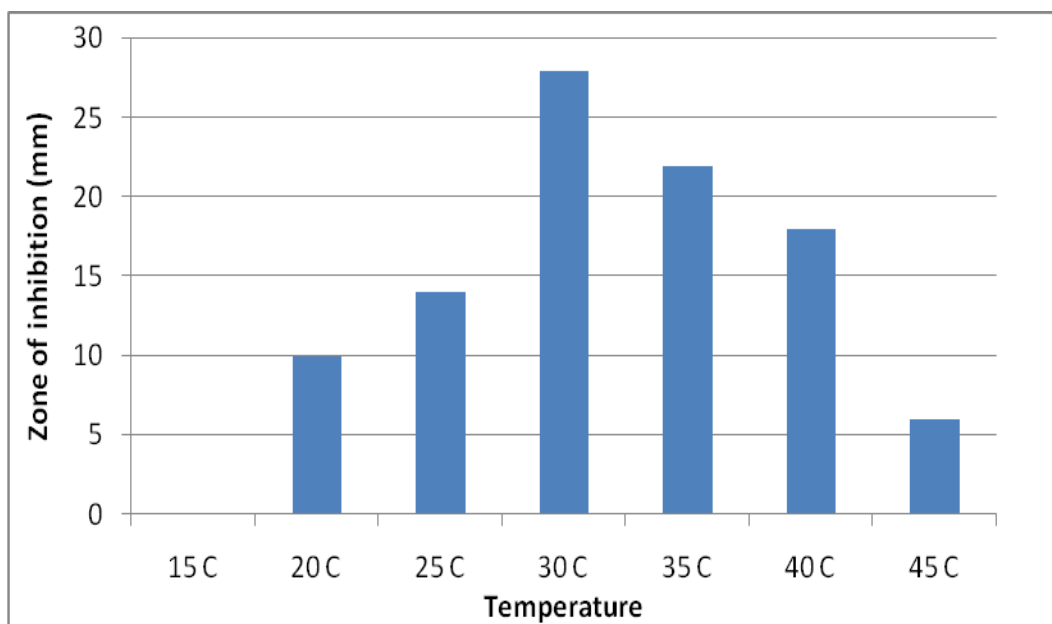


Fig.5 Effect of different pH on growth of *Streptomyces chilikensis* ACITM-1

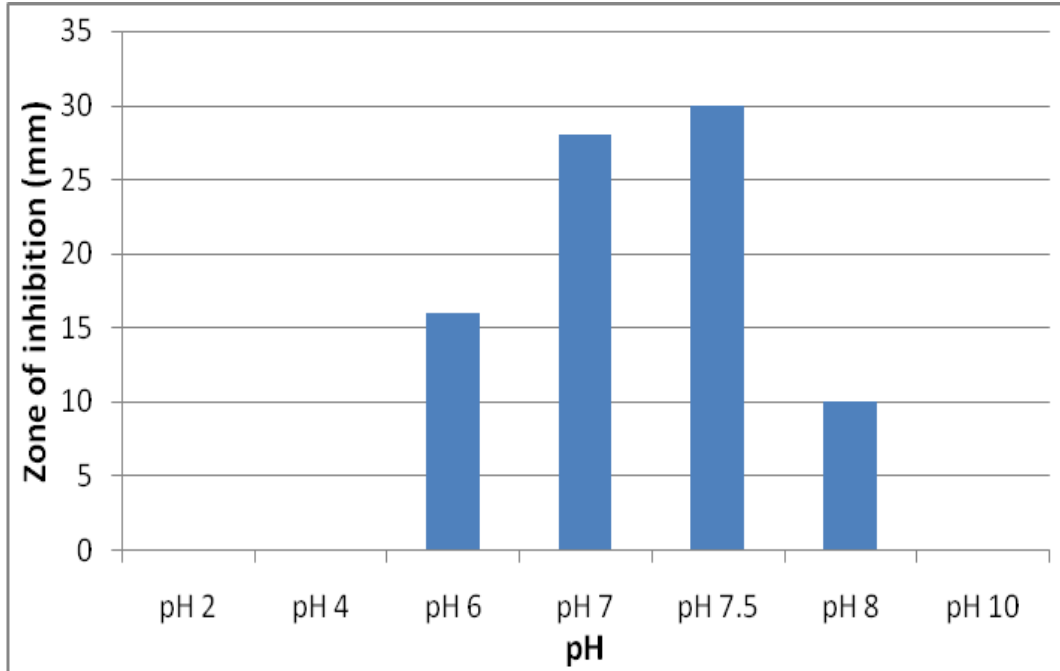


Fig.6 Effect of different carbon sources on growth of *Streptomyces chilikensis* ACITM-1

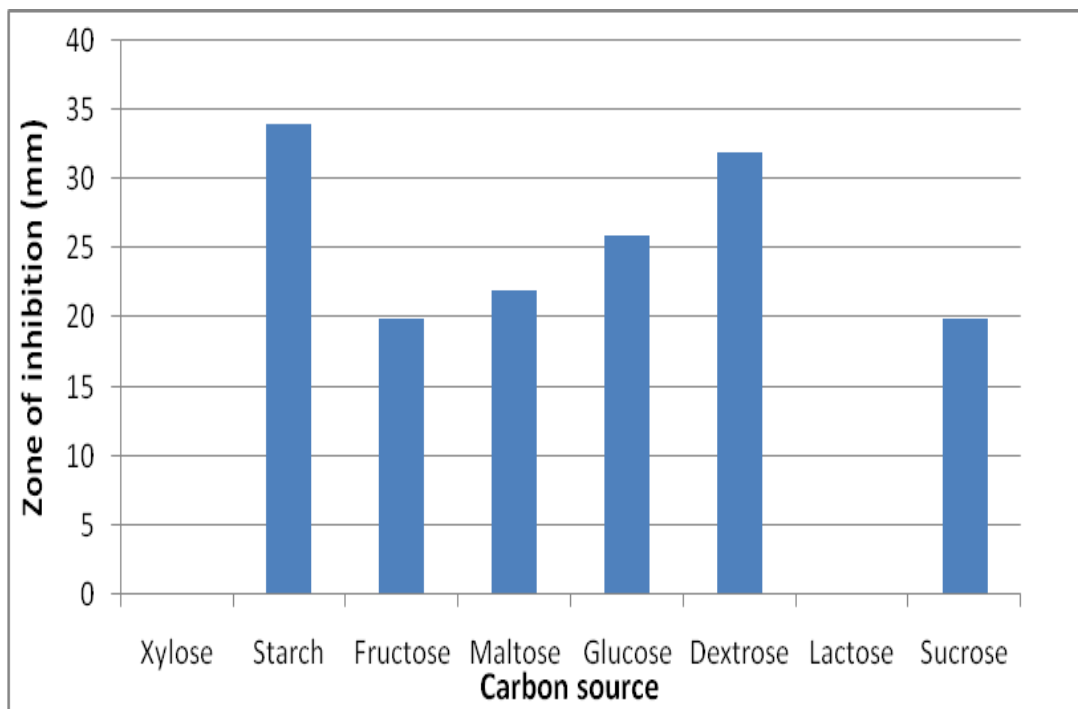
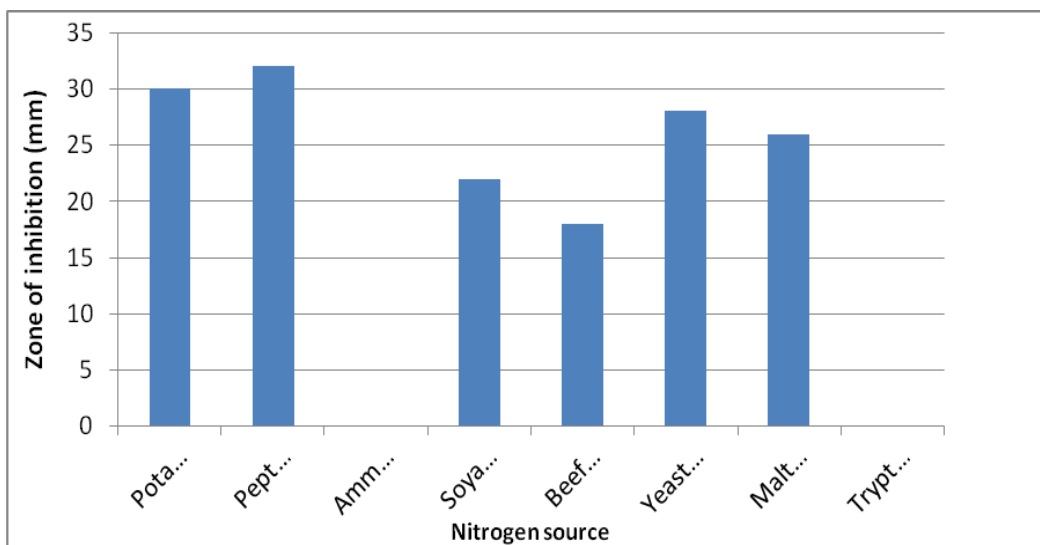


Fig.7 Effect of different nitrogen sources on growth of *Streptomyces chilikensis* ACITM-1



Soybean meal and peptone were found best nitrogen source for the highest antimicrobial activity and biomass production. They reported optimum pH for production of antimicrobial compound was 7.5 by *Streptomyces* strains. The optimum temperature for maximum antimicrobial activity and biomass production was found to be 28°C (Pavani *et al.*, 2014).

Investigation was made that the Factors affecting the production of the antifungal compounds. The results showed that incubation of *S. spororaveus* RDS28 for 72 h at 31°C and initial pH 7.5 on a medium containing glucose as a carbon source and proline as a nitrogen source gave the best antifungal antibiotic production (Askar *et al.*, 2011).

In the present study results showed that isolate ACITM-1 had highest antimicrobial activity and biomass production in starch casein broth followed by Starch Broth (SB), Yeast Extract Malt Extract broth (YEMEB), Inorganic Salt Starch Broth (ISSB), and Nutrient Broth (NB) and the zone of inhibition was found to be maximum after 96 h of fermentation in seeded starch casein

broths as well as it was observed that the temperature adequate for ACITM-1 strain which showed maximum growth and antibiotic production at 30°C.

It has been observed that maximum antibiotic production was obtained at pH 7.5 by *Streptomyces chilikensis* ACITM-1. From the experiment it was observed that antimicrobial activity and biomass production was highest when isolates were cultivated in media having starch as a carbon source. Peptone and potassium nitrate were found best nitrogen source for the highest antifungal activity and biomass production.

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