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Optimization of Cultural Conditions for Production of Antibacterial Metabolites from *Streptomyces coelicoflavus* BC 01

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Abstract

The aim of the present study was to optimize various cultural conditions for the production of antibacterial metabolites by *Streptomyces coelicoflavus* BC 01 isolated from mangrove soil, Visakhapatnam, Andhra Pradesh, India. The effect of various factors such as carbon and nitrogen sources, different concentrations of NaCl and K_2HPO_4, different temperature, pH, incubation time and agitation on antibacterial metabolites production were studied. The production of antibacterial metabolites by the isolate *Streptomyces coelicoflavus* BC 01 was greatly influenced by the cultural conditions. Glucose (1.2%) and soya bean meal (1%) seemed to be the best carbon and nitrogen source respectively, followed by NaCl (1%) and K_2HPO_4 (0.25%). Maximum production of antibacterial metabolites was observed at a temperature of 30 °C, with pH 7.2, at 160 rpm for 96 hrs. These optimized parameters can be further useful to design a fermentation medium to achieve maximum yield of antibacterial metabolites from *Streptomyces coelicoflavus* BC 01.

Keywords: optimization, antibacterial metabolites, cultural conditions, *Streptomyces coelicoflavus,* fermentation medium

Introduction

Actinomycetes are the most widely distributed group of microorganisms in natural and manmade environment. They play an important role in producing secondary metabolites of novel structures, which includes antibacterial, antifungal, antitumor, antiprotozoic and antiviral properties, vitamins, enzymes etc. (Priya *et al.,* 2012). As on today, 70% of the known antibiotics are isolated from actinomycetes and among them, two thirds are produced from *Streptomyces* (Miyadoh, 1993). A number of clinically important antibiotics, as well as widely used drugs against common diseases, have been derived from *Streptomyces* (Bibb, 2005). *Streptomyces* are supposed to produce about 75% of commercially and medically useful antibiotics (Usha *et al*., 2013). *Streptomyces* species are widely recognized as industrially important microorganisms because of their ability to produce different kinds of novel secondary metabolites (Solanki *et al.,* 2005).

The production of antimicrobial metabolites depends on the nutritional and physiological conditions of the microorganisms. Hence, designing an appropriate culture medium is very important, as the medium composition can significantly affect the yield of the antimicrobial metabolites. Media components and their optimum levels are essential for the production of antimicrobial metabolites by microorganisms. The production of antibiotics through fermentation is influenced by the concentration and type of carbon, nitrogen, phosphorous sources, as well as trace elements and also variable conditions like temperature, pH and aeration (Lin *et al*., 2010; Ruiz *et al*., 2010; Sanchez *et al.,* 2010). In addition to nutrients, medium may also contain various inhibitors of microbial growth and biosynthesis, which may affect the antibiotic production. For improving the antibacterial metabolites from *Streptomyces* sp., medium components and environmental conditions play an initial and vital role.

The present study was undertaken to investigate the effect of different nutrients and cultural conditions on the production of antibacterial metabolites by the isolate *Streptomyces coelicoflavus* BC 01 and to determine the optimal conditions for maximum production.

Materials and methods

Isolation of actinomycetes

The actinomycetes strain BC 01 was isolated from mangrove soil Visakhapatnam, Andhra Pradesh, India by using serial dilution plating techniques on yeast extract, malt extract, glucose (ISP-2) agar slants at 28 °C to get good sporulation and stored under refrigeration at 4 °C until further use.

Morphological, cultural and physiological characteristics of the strain were studied by using International Streptomyces Project (ISP) media recommended by Shirling and Gottlieb (1966). The 16S rRNA gene partial sequence of the strain BC 01 was deposited in NCBI nucleotide database with Accession No JX126485 and the sequence was correlated with genus *Streptomyces* by using BLASTN. A phylogenetic tree was reconstructed by using neighbor-joining method. The results stipulate that the BC 01 strain closely resembles with the genus *S. coelicoflavus* NBRC

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15399T (AB184650). By comparing the phenotypic and phylogenetic data, it was confirmed that the strain BC 01 belongs to *Streptomyces coelicoflavus* (Rao and Rao, 2013).

The production of antibacterial metabolite yield by the *Streptomyces coelicoflavus* BC 01 strain was optimized by using different nutritional and environmental parameters such as carbon, nitrogen, NaCl, pH, temperature, agitation and aeration.

Test microorganisms

All test organisms employed in the present investigation were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The test organisms used for the determination of antibacterial activity are *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 441), *Bacillus cereus* (MTCC 430), *Pseudomonas aeruginosa* (MTCC 424), *Escherichia coli* (MTCC 443) and *Proteus vulgaris* (MTCC 426).

Inoculum preparation

Five ml of sterile 0.9% NaCl solution were added to a 7 day old, well sporulated, slant of the culture. The spores were scraped from the slant into sterile saline solution and the resulting spore suspension, at 10% level, was aseptically transferred into a 500 ml Erlenmeyer flask containing 200 ml of inoculum medium. The inoculum medium comprises (g/L): glucose 10.0; soya bean meal 10.0; NaCl 5.0; $CaCO_3$ 5.0, with pH 7.0. The inoculated flasks were kept in an orbital shaker (120 rpm) at 28 °C for 48 hrs. The contents of the flasks were centrifuged at 3,000 rpm for 10 minutes, followed by the supernatant removal. The cell pellet was washed thoroughly and suspended in 0.9% NaCl solution. This cell suspension was used as inoculum.

Submerged fermentation

Five ml of inoculum (5 mg/ml dry cell weight) were added to 200 ml of production medium in 500 ml Erlenmeyer flask. Pridham and Gottlieb's inorganic salts medium (Pridham and Gottlieb, 1948) was used as the production medium base. It was supplemented with different carbon and nitrogen sources to study their effect on growth and antibacterial metabolite production. The flasks were kept at 28 °C on an orbital shaker (120 rpm) for 96 hrs. At the end of the fermentation process, 5 ml broth were collected and centrifuged at 3,000 rpm for 15 minutes. The clear mycelia free culture supernatant was used for determination of antibiotic assay by using agar well diffusion method.

Effects of various carbon sources

Effect of various carbon sources on growth and antibiotic production was studied by incorporating them at 1% (w/v) level into the Pridham and Gottlieb's inorganic salts medium. The carbon sources used were glucose, fructose, galactose, sucrose, mannose, sorbitol, inositol, xylose, arabinose and glycerol. In order to optimize the concentration of the best carbon source for maximum growth and antibacterial metabolite production, different concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 g/100 ml were added to the production medium.

Effects of various nitrogen sources

The influence of various nitrogen sources on growth and antibiotic production was studied by adding inorganic nitrogen sources and organic nitrogen sources at 0.4% (w/v) level into the Pridham and Gottlieb's inorganic salts medium. The optimized carbon source was used for further investigations. The inorganic compounds used were ammonium citrate, ammonium nitrate, ammonium sulphate, potassium nitrate and sodium nitrate. The organic nitrogen compounds employed were soya bean meal, peptone, beef extract, yeast extract, tryptone, amino acids-leucine, histidine, methionine, asparagine and glutamate. The concentrations of optimized nitrogen source (soya bean meal) used to determine the optimum concentration for growth and antibiotic production was 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 g/100 ml. Each concentration of soya bean meal was incorporated into the Pridham and Gottlieb's inorganic salts medium.

Effects of different K_2HPO_4 concentrations

In order to optimize the concentration of K_2HPO_4 for antibiotic production, different concentrations of 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 g/100 ml were incorporated into the production medium.

Effects of different NaCl concentrations

Different NaCl concentrations 0.2,0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 g/100 ml were incorporated into the Pridham and Gottlieb's inorganic salts medium to determine the optimum concentration of NaCl for antibiotic production.

Effects of incubation temperature

Inoculated production medium was incubated at different temperatures of 15, 20, 25, 30, 35, 40, 45 and 50 °C for 96 hrs to determine the optimum incubation temperature for antibiotic production.

Effects of initial pH

The production media were adjusted to different initial pH values of 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 10.2 and 11.2 in order to study the effect of initial pH of the medium on growth and antibiotic production.

Effects of incubation period

In order to investigate the optimal incubation period for maximum antibacterial metabolite production, the flasks with the mediums were inoculated and incubated at 30 °C for every 12 hours up to 144 hrs.

Effects of agitation

The effects of agitation on growth and antibacterial metabolite production were investigated by conducting the fermentation at different agitation speeds of 60, 80, 100, 120, 140, 160, 180, 200, 220 and 240 rpm at 30 °C for 96 hrs consecutively.

Growth measurements

The growth of the organism was represented by the dry weight of the mycelium. The contents of the culture flask were filtered through a previously weighed dry Whatmann No. 1 filter paper, washed twice with distilled water. The filter paper together with the mycelial mass was dried in a hot air oven at 80 °C for 18-24 hrs. At the end, the filter paper was weighed.

Antibiotic assay

Culture samples (1 ml) were taken at specified time intervals, centrifuged at 3,000 rpm for 15 min at 4 °C and then separated into culture filtrate and mycelium. The antibiotic activities of mycelia free culture supernatant against test organisms were measured by agar well diffusion method.

Statistical analysis

All investigations were conducted in triplicates. The obtained data was exposed to standard deviation and bar diagrams were generated using Microsoft Excel 2010.

Results and discussions

Medium formulation is an essential stage for the production of specific bioactive compounds by pilot scale development and manufacturing processes. The constituents of a medium must satisfy the elemental requirements for cell biomass and metabolite production and there must be an adequate supply of energy for biosynthesis and cell maintenance. Numerous studies on the nutritional requirement for production of antibiotics and other non-essential metabolites have demonstrated that there is a relation between nutrient limitation and biosynthesis of secondary metabolite (Fisher and Sonnenshein, 1991; Vilches *et al*., 1990).

Effects of carbon source on antibiotic production

The optimization of the antibacterial metabolite production was carried out in batch cultures. The *Streptomyces coelicoflavus* BC 01 isolate was cultivated in Pridham and Gottlieb's inorganic salts medium, supplemented with different carbon sources 1% (w/v) and their effect on growth and antibacterial metabolite yield was studied.

The *Streptomyces coelicoflavus* BC 01 isolate have grown well on media supplemented with different carbon sources. However, maximum antibacterial activity was obtained when media was supplemented with 1% (w/v) glucose followed by glycerol and sorbitol. The growth of the isolate with various carbon sources was studied in terms of dry weight of the mycelium. The effect of various carbon sources on growth and antibacterial metabolite production was tabulated in Table 1. The highest biomass production was observed with glucose (3.2 mg/ml) followed by glycerol (2.2 mg/ml) and sorbitol (1.2 mg/ml). The scanty biomass production was observed with mannose (0.2 mg/ml) followed by sucrose (0.5 mg/ml), inositol (0.5 mg/ml) and arabinose (0.5 mg/ml).

The maximum zone of inhibition was observed at 1% (w/v) glucose; for *B. subtilis* and *P. vulgaris* it was 33 mm, whereas for *B. cereus, S. aureus, E. coli* and *P. aeruginosa* it was 29 mm. The results show that among all the carbon sources used in this study, glucose exhibited the maximum antibacterial activity. Therefore, further optimization process was carried out by using different concentrations of glucose as a carbon source.

The effects of different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 g/100 ml) of glucose on growth and antibacterial metabolite production were examined. Results are shown in Fig. 1. Among all the concentrations, the maximum zone of inhibition was observed at 1.2 g/100 ml concentration of glucose with a biomass of 3.5 mg/ml and the maximum zone of inhibition observed were *P. vulgaris* (38.33 ± 0.94), *E. coli* (35.66 ± 1.24), *B. subtilis* (35.33 ± 0.94), *P. aeruginosa* (34.66 ± 0.94), *S. aureus* (34.33 ± 1.69) and *B. cereus* (33.33 ± 1.24). The results suggested that 1.2 g/100 ml of glucose concentration was optimum for the antibacterial metabolite production. The increase of the glucose level of concentration to 1.4 g/100 ml lead to a reduced zone of inhibition, therefore further optimizing process was carried out by employing 1.2 g/100 ml of glucose as carbon source. The reduced zone of inhibition might be due to the uptake of glucose at higher concentrations than other carbon sources by the genus *Streptomyces* as was stated by Van Wezel *et al*. (2005) in case of *Streptomyces coelicolor*. Similarly Tarhan *et al*. (2011) found that antibiotic production of *Streptomyces* sp. M4018 was higher in glucose containing medium when compared with glycerol and starch. The same result was also obtained by Vasavada *et al*. (2006) who reported that the highest antibacterial activity of *Streptomyces sannanensis* strain RJT-1 was obtained when glucose at 1% (w/v) was used as a carbon source, followed by xylose and arabinose. Similar findings were also reported by Pandey *et al*. (2005) and Ripa *et al*. (2009) by which glucose was proved to be the best carbon source for antibiotic production by *Streptomyces kanamyceticus* M27 and *Streptomyces* sp. RUPA-08PR respectively. The study of Jakeman *et al*. (2006) indicated that the addition of carbon source to the media has a strong impact on the antibiotic production by *Streptomyces venezuelae*. However, the high concentration of glucose in the medium was observed to decrease the growth and also the antibiotic production by Gesheva *et al*. (2005) and Zhu *et al*.

Table 1. The effects of various carbon sources on growth and antibacterial metabolite production by *Streptomyces coelicoflavus* BC 01

Carbon Source 1% (w/v)	Growth in dry weight (mg/ml)	Zone of inhibition in mm					
		Gram Positive Bacteria			Gram Negative Bacteria		
		S. aureus	*B. subtilis*	*B. cereus*	*E. coli*	*P. vulgaris*	*P. aeruginosa*
Glucose	3.2	29	33	29	29	33	29
Fructose	0.7	15	13	11	11	14	13
Galactose	0.8	13	12	13	14	13	11
Sucrose	0.5	15	14	10	13	12	13
Mannose	0.2	10	12	11	12	10	11
Sorbitol	1.2	14	15	13	14	14	12
Inositol	0.5	13	15	12	12	11	13
Xylose	0.6	11	12	10	12	13	11
Arabinose	0.5	12	14	16	14	13	12
Glycerol	2.2	17	18	16	17	18	18

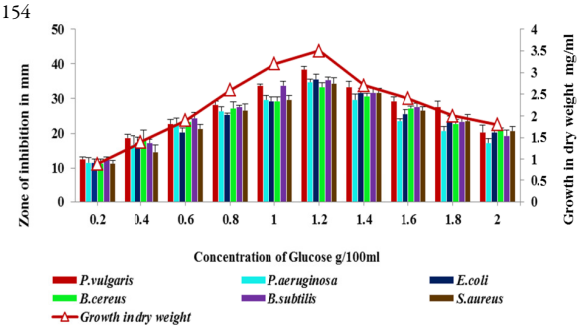

Fig. 1. Effects of glucose on growth and production of antibiotic activity against test organisms in *Streptomyces coelicoflavus* BC 01

(2007) in *Streptoverticillium* sp. 43/16 and *Streptomyces viridochromogenes* respectively.

Effects of nitrogen source on antibiotic production

Nitrogen source along with the carbon source play an important role in the production of antibacterial metabolites. The effects of various organic and inorganic nitrogen sources on the growth and antibacterial metabolites production were represented in Table 2. Among the various inorganic nitrogen sources, the maximum antibacterial activity was obtained by ammonium nitrate with a biomass of 2.6 mg/ml, followed by ammonium sulphate with a biomass of 1.8 mg/ml. The antibacterial activity of sodium nitrate, potassium nitrate and ammonium citrate was almost equal, but vary in biomass.

Among the organic nitrogen sources, the highest antibacterial activity was attained by soya bean meal with a maximum zone of inhibition observed in *P. aeruginosa*, *B. cereus* and *P. vulgaris* (18 mm), whereas *E. coli* and *B. subtilis* zone of inhibition was of 17 mm. In case of *S. aureus* the zone of inhibition was 15 mm. At 0.4% (w/v) of soya bean meal with a biomass of 3.2 mg/ml followed by yeast extract and beef extract with a biomass of 2.2 mg/ml and 1.8 mg/ml respectively. The lowest levels of antibacterial activity were obtained by peptone and leucine with a biomass of 1.3 mg/ml and 1.4 mg/ml respectively. Substitution of inorganic nitrogen sources with amino acids revealed that all the amino acids glutamate, asparagine, histidine tryptone and methonine showed worthy antibacterial activity, whereas leucine showed moderate antibacterial activity.

In the case of inorganic and organic nitrogen sources, soya bean meal was found to be the best for production of antibacterial metabolites. For this reason different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 g/100 ml) of soya bean meal were used for further optimization processes. Results are shown in Fig. 2. The highest antibacterial activity was attained at 1.0 g/100 ml of soya bean meal with a maximum zone of inhibition observed for *E. coli* (36.66 ± 1.24), *S. aureus* (35.33 ± 1.69), *B. subtilis* (33.66 ± 0.47) and *P. vulgaris* (33.33 ± 1.24), with a biomass of 3.4 mg/ml. The isolate *Streptomyces coelicoflavus* BC 01 was optimized at 1.0 g/100 ml of soya bean meal. Similar results where soya bean meal increases the production of antibiotic yield were reported by Singh *et al.* (2009) in *Streptomyces tanashiensis* strain A2D, Narayana and Vijayalakshmi (2008) in *Streptomyces albidoflavus,* Viana *et al.* (2010) in *Streptomyces* DAUFPE 3060 and Wu *et al.* (2008) in *Streptomyces padanus* PMS-702 respectively. All these results suggest that the level of antibiotic production may be greatly influenced by the nature and type of nitrogen source supplied in the culture medium. The use of complex nitrogen sources like soya bean meal, corn steep liquor and yeast extract, which increase the production of antibiotics by *Streptomycetes*, might be due to slow decomposition of these compounds in the medium. As a result, the use of inorganic nitrogen sources lead to high ammonium concentrations in the culture medium and suppress the antibiotic production in many microorganisms including *Streptomyces coelicoflavus* BC 01.

Effects of K_2HPO_4 on antibiotic production

The effects of different concentrations of K_2HPO_4 on growth and antibacterial metabolite production were studied. Results were shown in Fig. 3. The maximum antibacterial activity was obtained at 0.25 g/100 ml concentration of K_2HPO_4, with a biomass of 3.2 mg/ml. The maximum zone of inhibition was observed in *P. aeruginosa* (35.66 ± 0.47), *E. coli* (35.33 ± 0.47), *S. aureus* (35.0 ± 0.81), *P. vulgaris* (34.66 ± 0.47), *B. cereus* (34.33 ± 0.47) and *B. subtilis* (32.66 ± 0.94)

Table 2. Effects of different nitrogen sources on growth and antibacterial metabolites production by *Streptomyces coelicoflavus* BC 01

Inorganic Nitrogen sources (0.4% w/v)	Growth in dry weight (mg/ml)	Zone of inhibition in mm					
		Gram Positive Bacteria			Gram Negative Bacteria		
		S. aureus	*B. subtilis*	*B. cereus*	*E. coli*	*P. vulgaris*	*P. aeruginosa*
Ammonium citrate	1.4	12	11	13	10	11	10
Ammonium nitrate	2.6	14	16	16	15	17	16
Ammonium sulphate	1.8	13	13	12	14	13	15
Potassium nitrate	1.3	11	10	11	9	10	11
Sodium nitrate	1.6	12	11	10	11	10	12
Organic Nitrogen sources (0.4% w/v)							
Soya bean meal	3.2	15	17	18	17	18	18
Peptone	1.3	13	12	11	10	12	10
Beef extract	1.8	14	13	14	12	13	12
Yeast extract	2.2	14	16	15	14	17	16
Tryptone	1.6	13	12	13	15	14	13
Leucine	1.4	12	11	10	12	12	11
Histidine	1.9	12	15	16	15	14	16
Methionine	1.6	13	13	12	14	12	13
Asparagine	2.1	14	15	16	16	16	15
Glutamate	2.4	12	16	17	15	17	16

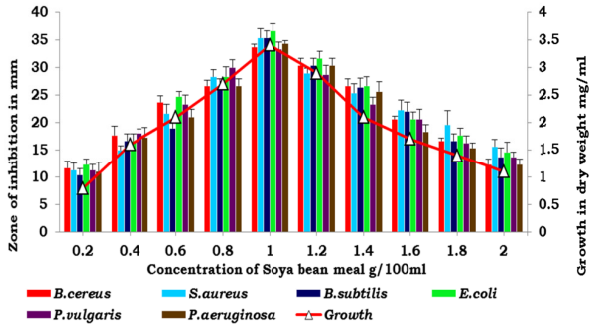

Fig. 2. Effects of soya bean meal on growth and production of antibacterial activity against test organisms in *Streptomyces coelicoflavus* BC 01

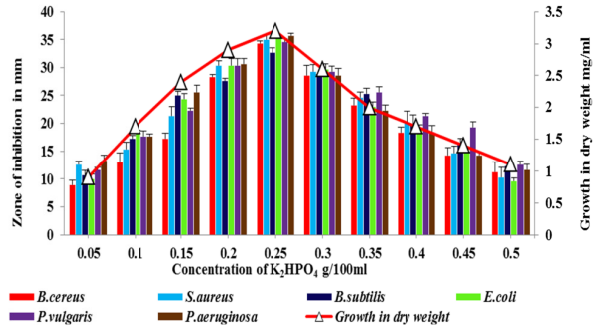

Fig. 3. Effects of K_2HPO_4 meal on growth and production of antibacterial activity against test organisms in *Streptomyces coelicoflavus* BC 01

respectively. From the obtained results K_2HPO_4 was optimized at a concentration of 0.25 g/100 ml, whereas further increase in the concentration of K_2HPO_4 showed negative impact and produced low levels of antibacterial activity and biomass growth. Similar results where low concentration of K_2HPO_4 had a positive effect on the antibiotic production was given by Bibb (2005), Martin (2004) and Majumdar and Majumdar (1965). This is because when concentration of inorganic phosphate in the culture media was high, the intracellular concentration of ATP increased and the primary metabolism was accelerated, inhibiting secondary metabolite production. In the present study increasing concentration of K_2HPO_4 tended to decrease antibiotic production. Similar reports where phosphate concentrations of more than 10 mM suppressed antibiotic synthesis in most microorganisms were given by Lounes (1996). Aharonowitz and Demain (1977) studied the relation between the concentration of inorganic phosphate in a medium and the production of antibiotics in the fermentation of cephalosporin by *Streptomyces clavuligerus*; the production was increased when phosphate concentration increased to 25 mM. Further addition of phosphate progressively decreased the production. Similar findings were reported by Kishimoto *et al.* (1996) for production of tetracycline, actinomycin and candicidi.

Effects of NaCl concentration on antibiotic production

Salt concentration has a thoughtful effect on the production of antibacterial metabolite from microorganisms due to its effect on the osmotic pressure to the medium (Pelczer *et al.,* 1993). To observe this effect NaCl was added to culture media at different concentrations such as 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 1.2%, 1.4%, 1.6% 1.8 and 2.0% respectively. The effect of different concentrations of NaCl on growth and antibacterial metabolite yield was investigated and the results were presented in Fig. 4. The results indicated that NaCl concentration greatly influenced the production of the antibacterial metabolite. The maximum antibacterial metabolite yield was obtained at 1.0 g/100 ml concentration of NaCl with a biomass of 3.2 mg/ml and the maximum zone of inhibitions observed were *B. cereus* (35.33 ± 0.94), *S. aureus* (33.66 ± 1.24), *P. vulgaris* (32.33 ± 0.47), *B. subtilis* (32.33 ± 1.24), *E. coli* (31.33 ± 0.47) and *P. aeruginosa* (30.66 ± 1.24) at 1.0 g/100 ml concentration of NaCl. The growth of the organism gradually decreased with the increase of NaCl concentration. Similar types of results were reported by Ripa *et al.* (2009) in *Streptomyces* sp. RUPA-08PR and by El-Refai *et al.* (2011) in *Nocardioides luteus* where maximum antibiotic production was at 1% of NaCl in culture medium. The requirement of NaCl for the production of bioactive metabolites seems to be different among *Streptomyces* strains. Saha *et al.* (2010) reported maximum antibiotic production

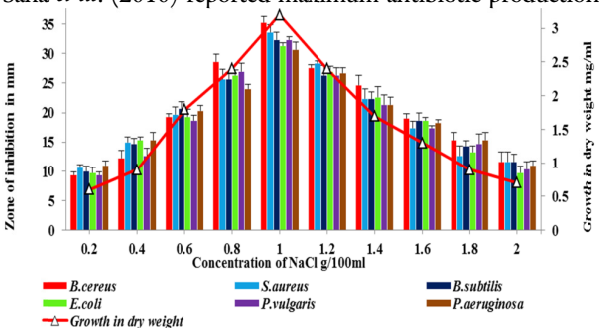

Fig. 4. Effects of NaCl on growth and production of antibacterial activity against test organisms in *Streptomyces coelicoflavus* BC 01

by *Streptomyces* sp. MNK 7 at 5% of NaCl and Singh *et al.* (2009) at 2% NaCl by *Streptomyces tanashiensis* A2D. Salinity levels must be optimized for different growth and production phases, by reducing the sea water levels in media composition, which increase the growth and production.

Effects of temperature on antibiotic production

Streptomyces coelicoflavus BC 01 showed a narrow range of incubation temperature for a relatively good growth and antibacterial metabolite production. The effect of temperature on growth and antibacterial metabolite production was shown in Fig. 5. The increase of the incubation temperature from 25 °C to 35 °C increased the growth of the biomass and the production of the antibacterial metabolite respectively. The maximum antibiotic yield was obtained at 30 °C with a biomass of 3.8 mg/ml and the maximum zone of inhibition observed were *P. aeruginosa* (36.66 ± 0.94), *B. subtilis* (36.33 ± 1.24), *S. aureus* (34.66 ± 0.94), *P. vulgaris* (34.33 ± 0.47), *E. coli* (35.66 ± 1.69) and *B. cereus* (35.33 ± 0.47) respectively. From the observed results the temperature was optimized at 30 °C for antibacterial metabolite production with a biomass of 3.8 mg/ml. The antibacterial activity was decreased consistently with the cell mass by increasing the growth temperature range between 35-

50 °C. Even though biomass was deposited at 45 °C and 50 °C, the antibacterial metabolite yield was negligible. Similar findings for production of antibiotics from *Streptomyces aureofacines* MY18 and *Streptomyces roseviolaceus* MR13 were reported by Tawfik *et al.* (1991). Comparable studies by Vastrad and Nelagund (2011), Abdel-Aal *et al.* (2011) confirmed the optimum temperature of 30 °C for production of neomycin, kanamycin and anicomycin production by *Streptomyces fradiae*, *Streptomyces kanamyceticus* and *Streptomyces griseolus* respectively. *Streptomyces* usually produces antibiotics at temperature near 27 °C. Generally, the range of a temperature supporting good growth is as wide as 25 °C, but the temperature range adequate for a good production of secondary metabolites is narrow, of 5~10 °C. Usually, cultivation for antibiotic production is performed under one constant temperature from the beginning to the end as stated by James and Edwards (1989).

Effects of initial pH on antibiotic production

The effects of pH on the growth and antibacterial metabolite production was shown in Fig. 6. The maximum antibacterial activity was obtained at pH 7.2, with a biomass of 3.8 mg/ml and the maximum zone of inhibition observed were *P. aeruginosa* (36.33 ± 1.24), *B. subtilis* (35.66 ± 1.24), *B. cereus* (35.33 ± 1.69), *P. vulgaris* (34.0 ± 1.69), *E. coli* (34.66 ± 2.05) and *S. aureus* (32.66 ± 0.47) respectively. The change in the pH of the production medium below and above the optimum conditions resulted in biomass growth, but decreased the antibacterial metabolite yield (Fig. 6). The results show that the isolate was capable to produce antibiotic within the optimum pH range

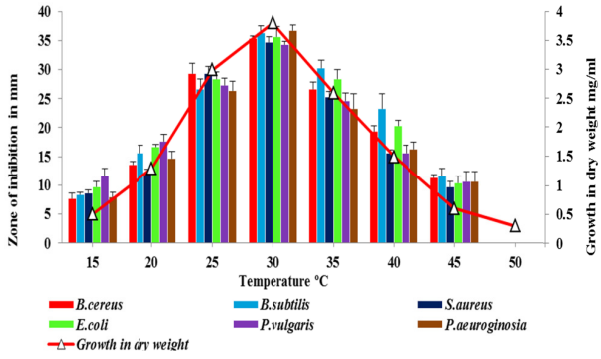

Fig. 5. Effects of temperature on growth and production of antibacterial activity against test organisms in *Streptomyces coelicoflavus* BC 01

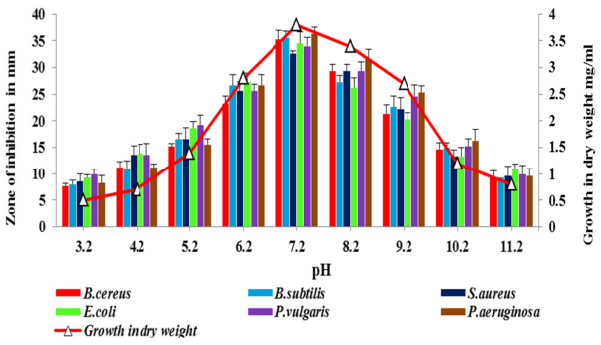

Fig. 6. Effects of pH on growth and production of antibacterial activity against test organisms in *Streptomyces coelicoflavus* BC 01

(7.2-8.2) although the strain withstands a broad range of pH (5.2-10.2). The isolate *Streptomyces coelicoflavus* BC 01 was optimized at pH 7.2 in the culture medium. The effect of pH is one of the vital physiological factors, which influence not only the growth, but also the production of antibiotics. The balanced use of the carbon and nitrogen sources will form a basis for pH control, as buffering capacity is provided by the proteins, peptides and amino acids in the medium. Most of the bacterial strains have their optimum growth on neutral environment. As a result, most of the antibiotics are optimally produced in pH close to 7.0. For instance, granaticin production was the highest when initial pH of cultural medium was adjusted to 6.5-7.0 (James *et al.*, 1991). A similar finding was reported by Bystrykh *et al.* (1996) in *Streptomyces coelicolor* for actinorhodin. The existence of high concentrations of glucose in the production medium makes it acidic in nature. As the fermentation progress, the cell mass increase by utilizing the nutrients, and thus depletion of nutrients make the medium alkaline in nature (Basak and Majumdar, 1973; Bhuyan, 1962). Hence, the pH of culture medium for the production of antibiotics must be neutral in *Streptomyces coelicoflavus* BC 01.

Effects of incubation period on antibiotic production

The growth of *Streptomyces coelicoflavus* BC 01 and the production of antibacterial metabolite were monitored over a period of 6 days. The fermentation samples were withdrawn periodically and assayed in order to study the optimal incubation time for production. The effects of different incubation periods on antibiotic yield were shown in Fig. 7. The antibacterial metabolite production by *Streptomyces coelicoflavus* BC 01 occurred in a growth-phase dependent manner and the highest antibacterial metabolite yield was obtained in the late exponential phase and the stationary phase. The results indicated that the maximum antibacterial activity was obtained at 96 hours incubation period with a biomass of 3.2 mg/ml and the maximum zone of inhibition observed were *B. cereus* (35.66 ± 0.94), *E. coli* (34.67 ± 2.49), *P. aeruginosa* (34.66 ± 0.94), *P. vulgaris* (34.33 ± 1.69), *B. subtilis* (33.67 ± 1.24) and *S. aureus* (33.0 ± 0.81) respectively. The results showed that incubation period of *Streptomyces coelicoflavus* BC 01 for antibacterial metabolite production was optimized at 96 hours. The incubation time for the production of antibacterial metabolite seems to be different among *Streptomyces* strains. From the present study incubation time of 96 hours was best suited for antibacterial metabolite production. Thakur *et al.* (2009) reported that antibacterial metabolites elaborated from 144

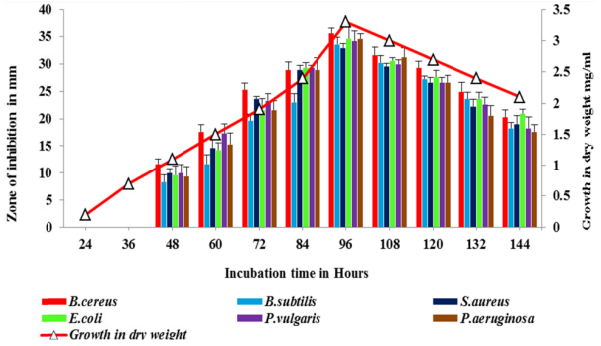

Fig. 7. Effects of incubation on growth and production of antibacterial activity against test organisms in *Streptomyces coelicoflavus* BC 01

hours old culture of *Streptomyces* sp. 201 showed worthy antimicrobial activity. Secondary metabolites collected from 168 hours old culture of *Streptomyces tanashiensis* strain A2D exhibited worthy antimicrobial activity as reported by Singh *et al.* (2009). Similar results were observed for streptomycin production in batch cultures of *Streptomyces griseus* ATCC 12475 when they were grown in a mineral medium by Fazeli *et al.* (1995) and for the production of candicidin in liquid grown cultures of *Streptomyces griseus* by Martin and McDaniel (1975).

Effects of agitation on antibiotic production

The agitation provides greater aeration to the culture and also creates conditions for greater availability of nutrients to the cells. Hence, the effect of agitation on growth and antibacterial metabolite production was determined and showed in Fig. 8. The highest antibacterial activity was observed at 160 rpm with the biomass of 3.2 mg/ml and the maximum zone of inhibition observed were *E. coli* (40 ± 0.81), *S. aureus* (40.6 ± 0.43), *P. aeruginosa* (41.23 ± 0.71), *P. vulgaris* (40.50 ± 0.40), *B. cereus* (41.0 ± 0.81) and *B. subtilis* (40.5.0 ± 0.40) respectively. At 160 rpm *Streptomyces coelicoflavus* BC 01 was optimized to get the best antibacterial yield, although at 180-200 rpm the antibiotic activity was moderate. Further increase in the agitation speed decreased the antibacterial activity along with biomass. Thus the results revealed that the antibacterial metabolite yield and the

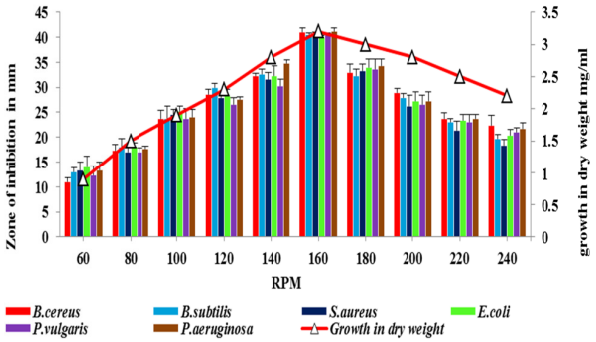

Fig. 8. Effect of agitation speed (rpm) on growth and production of antibacterial activity against test organisms in *Streptomyces coelicoflavus* BC01

growth rate were associated with agitation speed. Most of the antibiotic producing microorganisms are aerobic microorganism. Therefore oxygen supply has a great impact on their growth and antibiotic production. The decrease in antibiotic productivity was observed at both lower and higher agitation speeds. In the present study agitation speed of 160 rpm yielded biomass of 3.2 mg/ml. Although agitation is usually considered only from the viewpoint of oxygen, it may have other effects. The increase in agitation speed might affect the cellular damage and therefore decreases the product yield. The oxygen limitation acts in an analogous manner to substrate limitation imposed by dissolved nutrients, stimulating secondary metabolite production in some cases and inhibiting it in others as stated by Clark *et al.* (1995).

The optimized media after fermentation conditions

The medium optimized in the present investigation for the maximum production of antibacterial metabolite by *Streptomyces coelicoflavus* BC 01 has the following composition: 12.0 g/L glucose; 10.0 g/L soya bean meal; 2.5 g/L K_2HPO_4; 1.0 g/L NaCl; trace salt solution 1.0 ml {($CuSO_4.5H_2O$ (0.64 g/L); $FeSO_4.7H_2O$ (0.11 g/L); $MnCl_2.4H_2O$ (0.79 g/L); $ZnSO_4.7H_2O$ (0.15 g/L)} at 30 °C for 96 hours at 160 rpm. The present study results evidenced that the antibacterial productivity employing optimized fermentation conditions was higher than the initial fermentation conditions.

Conclusions

The present study focused mainly on the optimization of nutritional constituents and culture conditions for production of antibacterial metabolites in *Streptomyces coelicoflavus* BC 01 by altering one parameter at a time. It has been observed that antibacterial metabolite yield depends not only on the nature of the strain, but also on the composition of carbon and nitrogen sources, inorganic phosphorous, cell density, aeration and agitation conditions. Significant increase in the biomass and yield was attained by using newly formulated production medium under optimized cultural conditions. Therefore, large scale fermentation medium can be planned under these optimized parameters, to achieve a very good antibiotic yield.

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