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RESEARCH ARTICLE

Bioactive metabolite production by *Streptomyces albolongus* in favourable environment

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ABSTRACT

Objectives: Demand for new antibiotic is rising up due to continuous resistance risk against conventional antibiotic. This attempt was taken to find out a novel antimicrobial metabolite.

Methods: Chili field antagonistic actinomycetes *Streptomyces albolongus* was isolated and tested for optimum antimicrobial metabolite production. Primary screening was done by selective media and antibiotic assay was done by agar cup plate method. Fermented product was recovered by separating funnel using suitable solvent.

Results: Maximum antimicrobial metabolite production was found at temperature 35°C and pH 9.0 and on 6th day of incubation. The medium consisting of corn steep liquor (0.2%), glucose (1.0%), NaCl (0.5%), K_2HPO4 (0.1%) was screened out as suitable medium for maximum antimicrobial production. Sucrose was found as the best carbon source among four sources. The antimicrobial metabolite was found to be stable at pH and temperature up to 11.0 and 100°C respectively. The active agent was best extracted with chloroform. The antimicrobial spectrum of the metabolite was wide and shows activity against *Shigella dysenteriae* (AE14612), *Shigella sonnei* (CRL, ICDDR, B), *Salmonella typhi* (AE14296), *Vibrio cholerae* (AE14748), *Pseudomonas aeruginosa* (CRL, ICDDR, B), *Bacillus cereus* (BTCC19), *Staphylococcus aureus* (ATCC6538), *Bacillus subtilis* (BTTC17) and *Bacillus megaterium* (BTTC18).

Conclusions: The findings of antibacterial activity of *S. albolongus* against several species of human pathogens including both Gram-positive and Gram-negative bacteria indicated that our produced material might be an alternative antimicrobial substance to control human diseases. *J Microbiol Infect Dis 2013; 3(2): 75-82*

Key words: *Streptomyces albolongus*, antimicrobial metabolite, optimum production, antimicrobial spectrum

Uygun çevre koşullarında Streptomyces albolongus ile oluşan biyoaktif metabolit üretimi

ÖZET

Amaç: Günümüzde konvansiyonel antibiyotiklere karşı artan direnç riski nedeniyle yeni antibiyotiklere gereksinim vardır. Bu çalışmada yeni bir antimikrobiyal bileşiğin elde edilmesi denenmektedir.

Yöntemler: Bir aktinomiçes türü olan *Streptomyces albolongus* izole edilerek optimal antimikrobiyal metabolit üretimi açısından değerlendirildi. İlk tarama seçici besiyeri ve antibiyotik assay ise "agar cup-plate" metodu ile yapıldı. Fermente ürün uygun bir çözücü yardımıyla ayırma hunisi ile elde edildi.

Bulgular: Maksimum antimikrobiyal metabolit üretimi 35°C sıcaklık ve pH 9.0'da inkübasyonun altıncı gününde gözlendi. Denen besiyerleri arasında mısır ıslatma suyu (%0,2), glukoz (%1), NaCl (%0,5) ve K_2HPO4 (%0,1) içeren besiyeri maksimum metabolit üretimi için en uygun besiyeri idi. Sukroz, diğer dört kaynak arasında en iyi karbon kaynağı idi. Bulunan antimikrobiyal metabolit sırasıyla pH 11,0 ve 100°C sıcaklığa kadar dayanıklı idi. Aktif ajan en iyi kloroform ile ekstrakte edildi. Bulunan metabolitin antimikrobiyal spektrumu geniş idi ve *Shigella dysenteriae* (AE 14612), *Shigella sonnei* (CRL, ICDDR, B), *Salmonella typhi* (AE14296), *Vibrio cholerae* (AE14748), *Pseudomonas aeruginosa* (CRL, ICDDR, B), *Bacillus cereus* (BTCC19), *Staphylococcus aureus* (ATCC6538), *Bacillus subtilis* (BTTC17) ve *Bacillus megaterium* (BTTC18) gibi bakterileri kapsıyordu.

Sonuç: Araştırmada *S. albolongus*'un Gram-positif ve Gram-negatif bakterileri içeren değişik insan patojenlerine karşı etkisi hakkında elde edilen bulgular ürettiğimiz materyalin insanlardaki hastalıkların kontrolünde alternatif antimikrobiyal bir madde olabileceğini göstermektedir.

Anahtar kelimeler: *Streptomyces albolongus*, antimikrobiyal metabolit, optimum üretim, antimikrobiyal spektrum

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INTRODUCTION

There is great opportunity for discovering new groups of microorganisms with industrial and clinical importance in soil.[1] The top cultivable antibiotic producers present in soil are the actinomycetes.[2] These bacteria comprise about 10-33% of the total bacterial community present in soil.[3] The genus Streptomyces is responsible for the synthesis of the majority of antimicrobial agents with clinical importance.[4] On the whole, the last 55 years have seen the discovery of more than 12.000 antibiotics and actinomycetes yielded about 70% of these.[5] Most of the bioactive compounds from actinomycetes sort into several major structural classes such as aminoglycosides (e.g. streptomycin and kanamycin), ansamycins (e.g. rifampin), anthracyclines (e.g. doxorubicin), β-lactams (e.g. cephalosporins), macrolides (e.g. erythromycin) and tetracycline.[6-11] Most of the antibiotics are extracellular-secondary metabolites which are normally secreted in culture media and serve as intermediates from primary metabolisms as precursors for their biosynthetic process and they have been used as herbicides, anticancer agents, drugs, immunoregulators and antiparasitic agents.[12] Despite critical need for new antibiotics to treat drug-resistant infections and other infectious diseases, very few new antibiotics are being developed.[13] The nutritional sources like carbon, nitrogen, time, and minerals, the environmental factors such as temperature and pH are found to have profound influence on antibiotic production by actinomycetes.[14] Optimization of the culture conditions is essential to get high yields of the metabolites. Considering above-mentioned facts the present study was undertaken to find out and optimize the growth of antibiotic producing microbial isolates.

METHODS

Isolation and purification of the antibiotic producing microorganisms

The media used for the isolation of antibiotic producing microorganisms were nutrient agar medium, oat meal agar medium and starch casein medium.[15] One gram of soil sample was mixed with 100 ml of sterile water in a conical flask, shaken well with magnetic stirrer and then allowed to stand for 30 minutes for sedimentation. Necessary dilution (up to 10^{-5}) was made with this mother solution. The samples (0.1 ml) were used for inoculating 15 ml molten medium at 45-50°C in Petri plates and the plates were incubated at 37°C for about 4 to 5 days. Observation was made at 24 hours intervals to detect any colony surrounded by a clear zone of inhibition. Both the organisms (inhibiting and inhibited) involved in antagonistic reaction were isolated aseptically and transferred to slant of the same medium. The inhibiting and the inhibited colonies were marked as X-organisms and Y-organisms respectively. The isolated organisms were purified through repeated plating. Streaking on nutrient agar and oat meal agar was done for these purposes. The purified isolates were then transferred to starch casein agar.

Primary screening by sensitivity spectrum analysis

Primary screening was made by sensitivity spectrum analyses test against 10 test organisms. Perpendicular streak plate method was used for this purpose.[16] In the present study, to screen the antimicrobial activity of microbial secondary metabolites 10 human standard pathogenic bacteria strains were used as test organisms. Among them, 6 were Gram negative including *Shigella dysenteriae* (AE 14612), *Shigella sonnei* (CRL, ICDDR, B), *Salmonella typhi* (AE 14296), *Vibrio cholerae* (AE 14748), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (CRL, ICDDR, B) and 4 were Gram positive including *Bacillus cereus* (BTCC 19), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (BTTC 17) and *Bacillus megaterium* (BTTC 18). Three different media namely nutrient agar (NA) medium, starch casein agar (SCA) medium and Czapek Dox Agar (CDA) medium were used for primary screening. In each case, the melted medium was poured into Petri plate and allowed to solidify. The isolate was streaked across the surface of the agar medium at the middle position of the plate and incubated at 37°C for growth. After growth, the test organisms were streaked perpendicularly. A space of 2-3 mm was kept between two streaks. Then the plates were incubated at 37°C for the growth of the test organisms. The plates were then observed for organisms sensitive to the metabolites produced by the isolates. A clear zone of inhibition appeared against those organisms.

Identification and optimization of culture conditions for isolates

The selected isolate MU 37 was identified on the basis of morphological, cultural and physiological behaviors. These characteristics were compared with standard description of Bergey's Manual of Determinative Bacteriology.[17] The organisms which showed a considerable inhibition to the test organisms in primary screening were selected for mass culture in liquid media. In solid media an organism

may produce an antimicrobial metabolite but it may not do so in liquid media. The production of an antibiotic may be influenced by different types of factors such as composition and pH of media, incubation period, temperature and condition (Shake or stationary). So the maximum yield of an antibiotic depends on the optimization of these factors.

Antibiotic assay by agar cup plate method

In performing the sensitivity spectrum analyses by agar cup plate method, nutrient agar plates were heavily seeded uniformly (2.7 x 10^3 cells/ml) with the test organisms. Then a hole was made in media by gel cutter in sterile condition. Then one drop of melted agar was poured into hole and allowed to solidify to make a base layer. After that specific amount of culture filtrate (0.1 ml) was poured into the hole. Then plates were kept at low temperature (4°C) for 2-4 hours to allow maximum diffusion. The plates were then incubated at 37°C for 24 hours at inverted position to allow maximum growth of the organisms. The antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter.[18] The experiment was carried out more than once and mean of reading was recorded.

Establishment of suitable culture medium

Four different media were prepared for antibiotic production: Medium A: Beef extract (3 g), Peptone (5 g), NaCl (5 g), Distilled Water (1000 ml); Medium B: Yeast extract (0.2%), Glucose (1.0%), K_2HPO_4 (0.1%), NaCl (0.5%); Medium C: Beef extract (0.75%), Peptone (0.25%), NaCl (1.5%), KCl (1.0%), $MgCl_2$ (1.0%), $FeSO_4$ (1.0%); Medium D: Corn steep liquor (0.2%), Glucose (1.0%), K_2HPO_4 (0.1%), NaCl (0.5%). The pH of media was adjusted at 7.0. 150 cc of the medium was taken in each 500 cc conical flask. Each type of the medium was prepared in duplicate. Then the sterile media were inoculated with the specific organisms at 10% inoculums potential (15 cc) and incubated at 37°C. The product was assayed by agar cup method at an interval of three days and continued up to 6 days.

Optimization of incubation period, temperature, media pH, carbon sources and stationary and shaking incubation condition

The effect of incubation periods on the antibiotic production by selected isolates was also studied. For this reason 50 ml of selected medium was taken in each 100 ml conical flask and autoclaved. After cooling the broth medium was inoculated with equal quantity of inoculums, incubated at 37°C for 2, 4, 6 and 8 days. Then the culture filtrate was assayed for antibiotic production. After inoculation, the culture medium with optimum pH was incubated at different temperatures such as 27°C, 30°C, 35°C, 40°C, and 45°C for optimum incubation period. The culture filtrate was assayed for maximum antibiotic production by agar cup plate method. To observe the effect of pH on antibiotic production, the selected medium was prepared at different pH (6.0, 7.0, 8.0, 9.0, 10.0 and 11.0), dispensed 50 ml per 100 ml conical flask and autoclaved. Then the medium was inoculated and incubated at 37°C for optimum days. After incubation the culture filtrate was assayed for antibiotic production. The production of antibiotic using different carbon source was studied in the selected culture medium. Four carbon sources (glucose, fructose, sucrose, starch) were added to the selected medium and after specific period of incubation the culture media were filtered and assayed for antibiotic production. To determine the effect of stationary and shaking conditions broth medium was inoculated and incubated in both stationary and shaking conditions keeping all other experimental conditions at optimum. After specific period the culture media were filtered and assayed for antibiotic production of antimicrobial metabolite.

Production and recovery of antimicrobial metabolite

i) Fermentation: Selected media were prepared, sterilized and then inoculated with 5-10% (v/v) of inoculums of selected strain MU 37. All optimum conditions were maintained for maximum production of antimicrobial metabolite.

ii) Isolation of antimicrobial agent: The isolation of the antibiotic from the culture broth was achieved by extraction with an organic solvent not completely miscible with water. In this case the solubility of the antibiotic in different organic solvent was first investigated in order to find out a suitable solvent. The solvents used were petroleum ether, n-butanol, chloroform and benzene. For solvent- solvent partitioning of product the fermented product was gently shaken in a separating funnel with almost equal volume of pure petroleum ether, which is immiscible with aqueous alcohol. The mixture was then kept for several minutes for separating of the organic layer from the aqueous phase. The materials of the crude extract were partitioned between the two phases depending on their affinity towards their respective solvents. The organic layer was separated and collected in a conical flask and the process was repeated thrice. After collection of organic phase, the aqueous phase thus obtained was further extracted with other organic solvents usually of increasing

polarity like n-butanol, chloroform and benzene in the same way. Finally all the fractions were collected separately and dried in an oven (50°C). Finally gummy substances were collected and mixed with distilled water with concentration of 0.5 mg/ml. For the test of antimicrobial sensitivity of the extracts disc diffusion method was used and 50 µg of concentration was tested against pathogens and distilled water was used as a blank.

Studies for the antimicrobial metabolite stability at different pH and temperatures

Twenty-five ml of potent culture filtrate was taken in 50 ml conical flask in duplicates and the pH of the filtrate was adjusted at pH 3, 5, 7, 9, 11 by adding buffer solution. Then the flasks were kept inside the refrigerator for two hours and then the antibiotic was solvent extracted and assayed by agar cup plate method. Twenty-five ml of potent culture filtrate was taken in a 50 ml conical flask in duplicates and heated in water bath at different temperature (60°C, 70°C, 80°C, 90°C, 100°C and autoclave temperature 121°C) for half an hour. Then the antibiotic was extracted with the best solvent (chloroform) and assayed by agar cup plate method.

RESULTS

Primarily, 15 soil samples were collected from different chili fields in Chittagong, Bangladesh. Then the soil samples were examined by pour plating technique on three different media namely- nutrient agar medium, oat meal agar medium and starch casein medium to find out any antagonistic microorganisms present. Following incubation, 14 antagonistic microorganisms (actinomycetes and bacteria) surrounded by a zone of inhibition and the inhibited ones were isolated. These were purified and preserved and the inhibiting isolates were tested for their antimicrobial activity.

The previous sensitivity spectrum analysis tests on NA medium revealed that the antimicrobial agent produced by the isolate MU 37 was active strongly against *B. subtilis, V. cholerae, E. coli, B. cereus, P. aeruginosa, S. dysenteriae*, and weakly active against *S. aureus, S. sonnei, B. megaterium* and *S. typhi* and wide spectrum antimicrobial agent production by actinomycetes isolate was also reported.[19] On the basis of their better antimicrobial activity against 10 pathogenic test organisms in three different media, the actinomycetes isolate MU 37 was finally selected for detailed study (Table 1). On the basis of their morphological, cultural and biochemical characteristics, the actinomycetes isolate MU 37 was found to belong to the genus Streptomyces. It was provisionally identified as Streptomyces albolongus.[17]

Optimization of culture conditions for maximum production of antimicrobial metabolite

Among the four media used the medium D was found suitable for antimicrobial metabolite production (Figure 1). Following medium selection other tests were done on it. *S. albolongus* exhibited a wide range of antimicrobial activity (zone of inhibition) against different test organisms. The highest activity was found against *S. sonnei, B. subtilis* and *S. aureus* after 6 days of incubation which is very close to a report describing the maximum of antimicrobial activity at 5th day of incubation by *Streptomyces spp.*[20]

Effect of incubation temperature and pH on antimicrobial metabolite production

The actinomycetes isolate *S. albolongus* exhibited maximum antimicrobial metabolite production at 35°C (Table 2). It also showed maximum antimicrobial activity against *S. aureus* (zone of inhibition 33 mm) at pH 9.0.

Effect of carbon sources on the production of antimicrobial metabolite

Among the four carbon sources sucrose has significant effect on the production of antimicrobial metabolite which results in maximum activity against *B. subtilis* (zone of inhibition 31.5 mm).

Effect of incubation condition (stationary/ shaking) on antimicrobial metabolite production

Shaking incubation condition was found optimum for the production of antimicrobial metabolite as compared to stationary culture (Figure 2). This result complies with the report which described better bioactive metabolite production at shaking condition by *Streptomyces* spp. 201.[21]

Antimicrobial sensitivity assay of extracted metabolites

The activity of different extracted fraction of the metabolites was tested by agar cup plate method. Chloroform was found best for the solvent extraction of the metabolites produce by *S. albolongus.*

Determination of pH and temperature stability of the antimicrobial metabolite

The activity of the antimicrobial metabolite was found to be stable up to pH 11.0 (Figure 3) and temperature at 100°C which corresponds to the report on heat stable active component production by *Streptomyces*.[20]

Table 1. Morphological, cultural, physiological and biochemical characteristics of the MU 37 actinomycetes isolate

Character	Observations
Vegetative mycelium	Filamentous, non-septated, branched
Spore	Smooth chain
Gram staining	Gram positive
Acid fast staining	Non-acid fast
Colonies on starch casein agar	Form: circular; Margin: erose; Elevation: umbonate, Surface: concentric; Mycelium color: whitish; Spore color: grey; Diffusible pigment: light brown
Starch casein agar slant	+++, filiform, gray sporulation, yellow diffusible colour
Nutrient agar slant	++, scanty white sporulation, yellow diffusible color
Nutrient broth	Ring formation, low turbid and sediment present
Modified nutrient broth	Pellicle, turbid, sediment present
Starch casein broth	+++, Ring formation, turbid, sediment present
Catalase test	Positive
Deep glucose agar test	Heavy growth on surface, few colonies just below the surface
Casein hydrolysis	Strongly positive
Starch hydrolysis	Weakly positive
Egg albumin test	Negative
Gelatin hydrolysis	Positive
Nitrate reduction test	Positive
Fermentation test	Acid (without gas): Glucose Glycerol Strong alkali: Sucrose, Lactose, xylose, arabinose, Raffinose, Maltose, cellulose Moderate alkali: Starch, galactose, rhamnose, inulin and week from fructose No fermentation: Mannitol

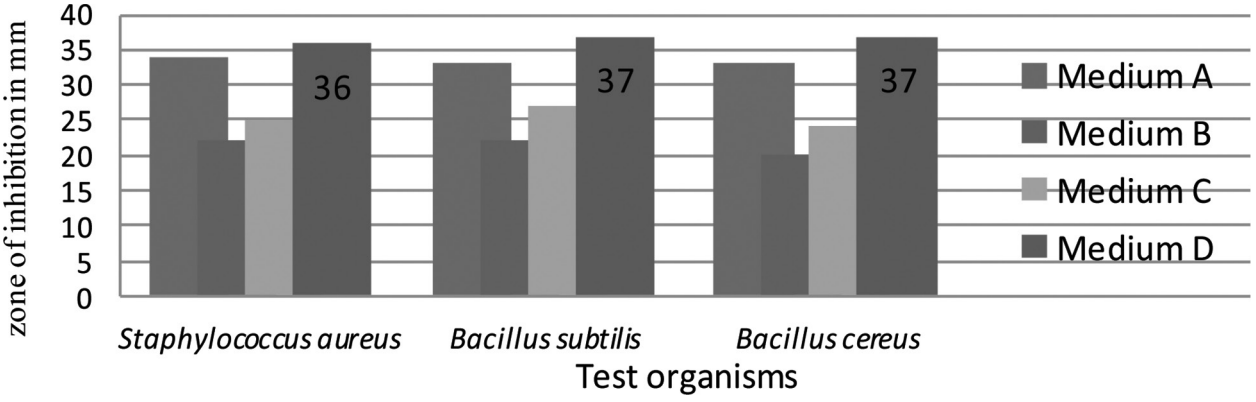

Figure 1. Production of active agents in different culture media by *Streptomyces albolongus*

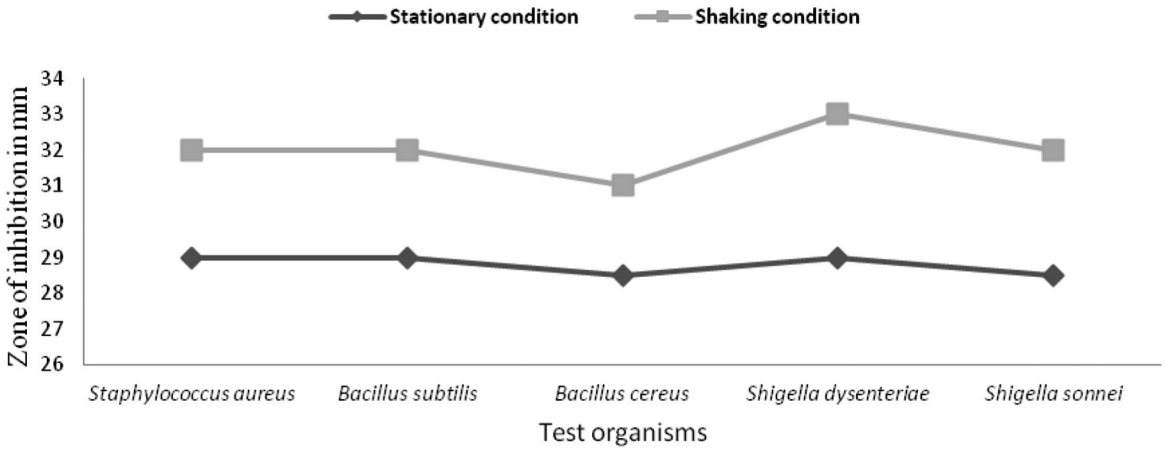

Figure 2. Production of active agents at stationary and shaking condition by *Streptomyces albolongus*

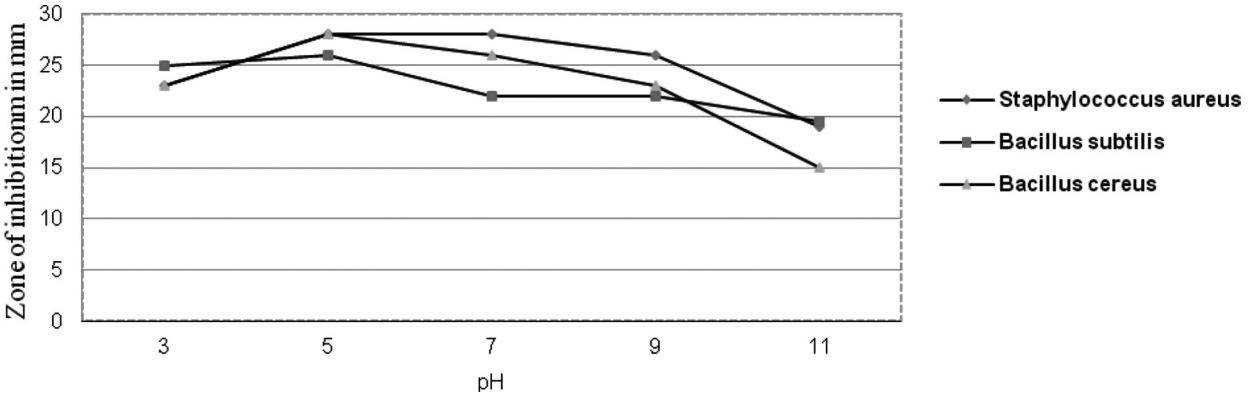

Figure 3. Stability of the active metabolite produced by *Streptomyces albolongus* at different pH values.

Table 2. Obtained inhibition zones against test microorganisms by MU 37 isolate in different incubation temperatures*

Test organisms	27°C	30°C	35°C	40°C	45°C
Staphylococcus aureus	26	25.5	30.5	23.5	-
Bacillus subtilis	25.5	26	29.5	24	-
Bacillus cereus	24.5	26	27.5	24	-
Shigella dysenteriae	26.5	29	31.5	29	-
Shigella sonnei	28	28	35	25.5	-

*Data presented as mm

DISCUSSION

Antibiotic production by microorganisms is greatly dependent on the selective environmental pressure prevailing at its source of isolation and manipulation of growth and nutritional conditions during fermentation exerts substantial influence on the level of metabolite production.[22] The composition and concentrations of the constituents of the media are closely linked with the metabolic capacities of the producing organism and greatly influence the biosynthesis of the bioactive molecules. Equally, the effect of other factors including medium volume, oxygen transfer rate, temperature, initial pH of the medium, and incubation time on secondary metabolites production were frequently reported. These factors have great motivation on increase of metabolite production by different kinds of actinomycetes. All these parameters were considered during production of antimicrobial metabolites by MU 37 and during recovery by solvent chloroform optimized conditions were followed.

In our study, use of medium D (Figure 1) provides maximum antimicrobial metabolite production. Other parameters were also optimized. Sanchez and Demain[14] have listed several carbon sources which interfere with the antibiotic production. Sugar can affect the metabolism directly by decreasing the

time for switching over to stationary phase.[23] We found during production 1% glucose concentration in medium D has greatly induces antibiotic production. Abbanat et al.[24] have found that glucose concentration influence metabolite production. Moreover, when medium D have supplemented with 1% sucrose (data not shown) production of metabolite has increased in a significant way.

The level of antibiotic production may be greatly influenced by the nature; type and concentration of the nitrogen source supplied in the culture medium.[25] Mohamed et al.[26] showed that nitrogen source (inorganic and organic) exhibited a significant effect on the natamycin production. Our results are in agreement with the results of the study. The medium D used for metabolites production contains corn steep liquor which is a good source for all amino acids, and nitrogen compounds. The mineral K_2HPO_4 (in medium D) also have good contribution on bioactive metabolite production by the investigated strain MU 37. Other environmental factors like pH, incubation temperature, incubation period, and air relation were also found to have profound influence on antibiotic production.[27] The maximum antibiotic activity was obtained at a pH of 9.0 suggesting its inclusion in the alkaliphilic actinomycetes group. This is in agreement with the results obtained by Sanjay et al.[28] They found for good antibiotic production by *S. griseus* at a pH value of 9.0 was suitable. The influence of temperature on the bioactive metabolite production of the strain is presented in Table 2. Highest growth, as well as antimicrobial compound production, was obtained at 35°C. In terms of its optimum temperature for growth, the organism appeared to be mesophilic. These results are in complete accordance with those reported by Narayana and Vijayalakshmi.[29]

In this study, the stability of antibiotics at different temperatures and pH values were examined. The metabolites from isolate MU 37 were stable at temperature ranges from 60 to 100°C however unstable after autoclaving. Stability was also examined at different pH varies from 5 to 11. Maximum activity was found at pH 7 and activity was decreased with increase or decrease of pH value. Mustafa et al.[30] also reported about study of antimicrobial activity of antibiotic metabolites on subjection to different temperatures and pH levels. Using all optimum conditions metabolites were trying to recover from broth culture using different solvents like n-butanol, petroleum ether, chloroform and benzene. Among them, chloroform (Table 3) was found best for recovery of the metabolites.

Table 3. Obtained inhibition zones by different extracted fractions (1) against test microorganisms*

Test microorganisms	n-butanol	Petroleum ether	Chloroform	Benzene	Control
Staphylococcus aureus	21		42	27	0
Bacillus subtilis	22.2	-	42.5	28.5	0
Bacillus cereus	22	11.5	42	29	0

*Data presented as mm

In conclusion, it is clear that based on morphological, cultural, physiological and biochemical characteristics the new *Actinomycetes* MU 37 has been assigned as *S. albolongus*. Optimal conditions for the production of bioactive metabolites and recovery using solvent were determined, and metabolites showed good antimicrobial activity against both Gram positive and Gram negative bacteria. Further studies on their purification, characterization and identification of bioactive metabolites are in progress.

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Conflict of interest: The authors report no conflict of interest.

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