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Optimization of culture conditions for antimicrobial metabolites production by *Streptomyces* sp. against bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae*

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Abstract
An investigation was conducted to explore the antimicrobial potential of *Streptomyces* sp. TC1 against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) which causes bacterial leaf blight disease in rice. The TC1 strain was found to produce maximum antimicrobial metabolites upto 7 days in starch casein broth. The starch and yeast extract positively influences the antimicrobial metabolites production. The produced antimicrobial metabolites in modified starch casein broth were extracted using ethyl acetate and the pH of broth adjusted to 3 prior to extraction process for the better recovery of antimicrobial metabolites. The ethyl acetate crude extract possess an MIC of 300 µg/mL and IC_{50} value of 125 µg/mL against the test pathogen *Xoo*. The culture filtrate and crude extract of TC1 strain also showed antimicrobial activity against various isolates of *Xoo*.

Keywords: Antimicrobial, *Streptomyces*, MIC, *Xoo*

Introduction
Actinomycetes are gram positive organisms known for producing several bioactive metabolites with diverse biological activities. The genus *Streptomyces* constitutes around half of the total soil actinomycete population (Xu *et al.,* 1996) [21] and are widely recognized as industrially important microorganisms because of their ability to produce novel secondary metabolites having utilization in agricultural, pharmaceutical and industrial applications (Miyadoh, 1993) [13]. Over 55% of antibiotics have been isolated from genus *Streptomyces* and therefore this genus is one of the several biological control agents which are widely studied and used to control various plant pathogens (Embley and Stackebrandt, 1994) [4]. Many of the seed, soil-borne and foliar diseases were efficiently managed by biocontrol means using *Bacillus* sp. *Pseudomonas* sp. *Streptomyces* sp. and *Trichoderma* sp. A numbers of antimicrobial metabolites were produced by these strains which have different mode of actions in suppression of plant pathogens.

BLB caused by *Xoo* is one of the major foliage diseases that limit annual rice production in India. Yield losses due to BLB have been reported to vary from 2% to 74% depending on location, season, crop growth stage and cultivar (Rao and Kauffman, 1977) [15]. Bacterial leaf blight is the most serious disease of rice in South-East Asia, particularly since the widespread cultivation of dwarf high-yielding cultivars (Feakin, 1971) [5]. BLB is characterized by yellow lesions with wavy margins on leaf blades that may extend to the sheath (Gnanamanickam *et al.,* 1999) [7]. The details of BLB causing pathogen and their physiology in disease development are greatly discussed by Liu *et al.* (2006) [12].

Production of secondary metabolites by microorganisms differs qualitatively and quantitatively depending on the strains and species of microorganisms used as well as on their nutritional and cultural conditions (Lam *et al.,* 1989) [10]. Designing a suitable fermentation medium is of critical importance in the production of secondary metabolites from microbial origin (Gao *et al.,* 2009) [6]. Fermentation for the production of secondary metabolites is influenced by various environmental factors including nutrients, growth rate, feedback control, enzyme inactivation and variable conditions including oxygen supply, temperature, light & pH (Lin *et al.,* 2010) [11].

Antimicrobial metabolite production from *Streptomyces* sp. RUPA-08PR was found to be higher in fermentation medium with glucose as carbon source and yeast extract as nitrogen source. The fermentation carried out for ten days yielded higher amount of antimicrobial metabolites. Maximum production of bioactive metabolites was obtained when NaCl concentration was 1% and among different minerals tested, K_2HPO_4 and NaCl showed positive influence on antibiotic production by the strain (Ripa *et al.*, 2009) [16].

Oskay (2011) [14] reported the culture filtrate of *Streptomyces* sp. KGG32 having antimicrobial activity against human pathogens *S. aureus*, *B. cereus*, *B. subtilis*, *E. coli*, *Enterococcus faecalis* and *C. albicans* under *in-vitro* growth conditions. He also reported that increase in antimicrobial metabolites were produced in the production medium containing sucrose and bacteriological peptone as carbon and nitrogen source respectively. With this background the study was conducted to optimize the media for production of antimicrobial metabolites by *Streptomyces* sp. TC1 isolate.

Materials and methods

Microbial culture

The *Streptomyces* sp. TC1 (GenBank accession number: KC954629) was grown in Ken knight agar slants for five days at 28ºC and maintained under refrigerated conditions. The test culture *Xanthomonas oryzae* pv. *oryzae* (Xoo) from various sources of rice varieties *viz.*, TN1, TNRH180, CO47, CO50, ADT39, ADT43 were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India.

Chemicals

The chemicals used in the present study were of analytical reagent grade. It was purchased from Himedia, Sigma, Qualigens, and SD Fine Chem., India.

Fermentation

A seed culture of *Streptomyces* sp. TC1 was prepared by inoculating a loop of biomass into a 200 ml Erlenmeyer flask containing 100 ml of Ken knight broth and then incubating at 28 ºC for 3 days. A 10 % level of this inoculum was transferred into 1000 ml of production medium contained in 3L Erlenmeyer flasks (15 in number). The production medium having the composition of soluble starch 1.0%, casein 0.03%, KNO_3 0.2%, NaCl 0.2%, K_2HPO_4 0.2%, $CaCO_3$ 0.002%, $MgSO_4.7H_2O$ 0.005% and $Fe_3SO_4.7H_2O$ 0.001% with pH 8.0. The inoculated production flasks were incubated for 7 days at 28 ºC.

Optimization of fermentation parameters

The effect of various carbon and nitrogen sources on the growth and antimicrobial activity of *Streptomyces* sp. TC1 isolate were carried out. The effect of different pH in the range between 3.0 to 10.0 was also evaluated for antimicrobial metabolite production. The carbon sources used were maltose, glucose, fructose, sucrose, mannitol, galactose, glycerol, starch and lactose. The nitrogen sources used were ammonium chloride, ammonium nitrate, ammonium sulphate, soybean meal, sodium nitrate, monosodium glutamate, potassium nitrate, yeast extract, peptone and urea. Hundred mL of the culture medium amended with different carbon and nitrogen sources were taken in 250 mL Erlenmeyer flasks and steam sterilized. The flasks were inoculated with 10 percent inoculum and incubated at 35 ºC for seven days. The biomass production and antimicrobial activity of the culture filtrate were analyzed after the incubation period. The optimized carbon and nitrogen sources *viz.*, soluble starch, yeast extract were supplemented separately to the starch casein broth at different percentages *viz.*, 0.2, 0.4, 0.6, 0.8, 1.0 percent of carbon and nitrogen and antimicrobial activity of the culture supernatant was measured after seven days by agar well diffusion assay.

Preparation of crude extract

The fermentation broth of strain TC1 was centrifuged at 10000 rpm and the supernatant was collected. The crude extract from the liquid culture medium was extracted by shaking with equal volume of ethyl acetate in a separating funnel. The solvent extract was then evaporated to dryness by concentrating under rotary vacuum evaporator. The obtained dried residue were considered as a crude extract and kept in tightly stoppered bottle in a refrigerator until used for the antimicrobial testing.

Preparation of test sample and antimicrobial activity assay

A quantity of 10 mg of crude extract obtained from solvent extraction of TC1 culture filtrate were dissolved in 1 mL of 100 percent ethanol and used for antibacterial assays against the *Xoo* isolates by agar well diffusion method. The Minimum Inhibitory Concentration (MIC) assay was also performed separately to find out the antimicrobial activity of crude extract. The inhibitory concentration 50 (IC_{50}) was performed to test the antibacterial activity of the crude extract using broth dilution technique (Hughes *et al*., 1987) [9]. IC_{50} is defined as a concentration at which 50 percent mortality of organisms is observed.

Results and Discussion

The present study was carried out to evaluate the antimicrobial potential of *Streptomyces* sp. TC1 against *Xanthomonas oryzae* pv. *oryzae*. Medium optimizations were attempted for increased production of antimicrobial metabolites.

Medium optimization

Antimicrobial metabolites production usually occurs during the late growth phase of the producing microorganism. The nature of their formation is genetic but the expression can be influenced greatly by change in culture conditions which might be regarded as an important criterion. So improvement and optimization of culture conditions are a prerequisite in expression of the antibiotics producing genes. In the present study the effect of pH, carbon and nitrogen sources were evaluated for increased antimicrobial metabolites production.

Experiments conducted to find out the optimum pH for antimicrobial activity showed that, pH 7.0 was the optimum pH for antimicrobial metabolites production. Maximum antimicrobial activity of 1.5 cm was noticed in pH 7.0 and the antimicrobial activity observed at other pH levels were lesser comparatively (Table 1). There was a positive correlation observed between biomass production and antimicrobial activity of *Streptomyces* sp. TC1. The present result is in accordance with Thakur *et al*. (2009) [20] who also reported the importance of inoculum density in increasing mycelial growth and metabolite production by *Streptomyces* sp.

Among the different carbon sources tested, starch significantly enhanced antimicrobial activity of TC1 isolate (1.5 cm) followed by sucrose (1.2 cm). The other carbon sources recorded comparatively lower antimicrobial activity

(Table 2). Among the various nitrogen sources evaluated, yeast extract enhanced the antimicrobial activity (1.6 cm) followed by peptone (1.4 cm). In general, organic sources of nitrogen supported better growth of the *Streptomyces* than inorganic nitrogen sources (Table 3). In the present study a number of carbon sources were investigated for their effect on growth and antibiotic production of *Streptomyces* sp. TC1. Starch proved to be an excellent source for increase antibiotic metabolites production. The other tested carbon sources are resulted in poor antibiotic production. It is possible that these carbon sources are utilized rapidly for the synthesis of cellular materials. So that little would be available as carbon and energy source for antibiotic synthesis. Starch may be utilized less rapidly and this was available during the phase of antibiotic production. This was supported by Augustine *et al.* (2005) [2] where starch was the best carbon source in the fermentation carried out by using *Streptomyces rochei* AK 39 for increased antimicrobial metabolites production. Slow metabolising nitrogen sources like amino acid proline showed increased titre values of chloramphenicol in fermentation carried out by *Streptomyces venezuelae* (Shapiro and Vining, 1983) [18].

Al-Zahrani (2007) [1] reported the *Streptomyces* sp. J12 grown in oat meal, starch casein and Sabouraud media exhibited higher antimicrobial activity against both gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and gram negative (*Escherichia coli* and *Salmonella typhi*) bacteria. The highest antimicrobial activity was observed with starch casein broth after 6 days of growth at pH 7.2.

The optimized carbon and nitrogen sources *viz.*, starch and yeast extract respectively were evaluated in different percentages in growth media for increased antimicrobial activity. The influence of different percentages of starch on the antimicrobial activity was presented in Table 4. The results revealed that maximum antimicrobial activity (1.4 cm) coupled with biomass production by TC1 isolate was observed at 0.8 percentage of starch, whereas maximum antimicrobial activity of 1.7 cm was observed at 1 percentage of yeast extract (Table 5). The antimicrobial activity produced by 0.4 to 1 percentage of yeast extract was statistically on par with each other. Hence 0.8 percentage of starch and 0.4 percentage of yeast extract were found to be the level for maximum antimicrobial activity. The antibiotic production of the TC1 strain was obtained optimally in the presence of starch as carbon source and yeast extract as a nitrogen source. Optimization of carbon and nitrogen sources will be useful for the development of the cultivation process for efficient antibiotic production on a large scale.

Table 1: Effect of pH of the medium on antimicrobial activity

pH of the medium	DIZ (in cm)	Biomass (g/100 mL on dry wt. basis)
3	0.7 ± (0.10)	1.17 ± (0.22)
4	0.9 ± (0.10)	1.31 ± (0.13)
5	1.1 ± (0.06)	1.41 ± (0.35)
6	1.4 ± (0.21)	1.47 ± (0.23)
7	1.5 ± (0.06)	1.54 ± (0.10)
8	1.4 ± (0.15)	1.53 ± (0.16)
9	1.4 ± (0.06)	1.49± (0.19)
10	1.3 ± (0.06)	1.45 ± (0.27)
SEd	0.091	0.179
CD(.05)	0.194	0.378

Values are Mean ± SD of three replicates

Table 2: Effect of different carbon sources on antimicrobial activity

Carbon source	DIZ (in cm)	Biomass (g/100 mL on dry wt. basis)
Glucose	0.8 ± (0.21)	1.23 ± (0.31)
Fructose	0.8 ± (0.06)	1.09 ± (0.34)
Starch	1.5 ± (0.06)	1.55 ± (0.18)
Lactose	0.5 ± (0.12)	0.81 ± (0.33)
Mannitol	1.1 ± (0.17)	1.44 ± (0.08)
Sucrose	1.2 ± (0.10)	1.38 ± (0.18)
Galactose	0.8 ± (0.12)	1.27 ± (0.36)
Maltose	0.7 ± (0.10)	0.95 ± (0.22)
Glycerol	0.6 ± (0.06)	0.75 ± (0.14)
SEd	0.098	0.209
CD(.05)	0.206	0.439

Values are Mean ± SD of three replicates

Table 3: Effect of different nitrogen sources on antimicrobial activity

Nitrogen source	DIZ (in cm)	Biomass (g/100 mL on dry wt. basis)
Ammonium chloride	0.4 ± (0.12)	0.50 ± (0.24)
Ammonium nitrate	0.4 ± (0.15)	0.56 ± (0.16)
Ammonium sulphate	0.5 ± (0.10)	0.76 ± (0.31)
Soybean meal	1.2 ± (0.06)	1.22 ± (0.10)
Sodium nitrate	0.8 ± (0.09)	0.98 ± (0.25)
Monosodium glutamate	0.8 ± (0.15)	0.89 ± (0.14)
Peptone	1.4 ± (0.06)	1.51 ± (0.38)
Yeast extract	1.6 ± (0.10)	1.85 ± (0.20)
Potassium nitrate	1.3 ± (0.06)	1.08 ± (0.13)
Urea	0.3 ± (0.06)	0.70 ± (0.37)
SEd	0.080	0.202
CD(.05)	0.168	0.421

Values are Mean ± SD of three replicates

Table 4: Effect of different percentage of starch on antimicrobial activity

Carbon source (%)	DIZ (in cm)	Biomass (g/100 mL on dry wt. basis)
0.2	0.8 ± (0.06)	1.06 ± (0.31)
0.4	1.1 ± (0.06)	1.18 ± (0.27)
0.6	1.2 ± (0.10)	1.30 ± (0.19)
0.8	1.4 ± (0.06)	1.49 ± (0.12)
1.0	1.4 ± (0.10)	1.53 ± (0.33)
SEd	0.063	0.209
CD(.05)	0.1401	0.465

Values are Mean ± SD of three replicates

Table 5: Effect of different percentage of yeast extract on antimicrobial activity

Nitrogen source (%)	DIZ (in cm)	Biomass (g/100 mL on dry wt. basis)
0.2	1.3 ± (0.15)	1.43 ± (0.27)
0.4	1.6 ± (0.10)	1.72 ± (0.34)
0.6	1.6 ± (0.15)	1.78 ± (0.26)
0.8	1.6 ± (0.06)	1.81 ± (0.08)
1.0	1.7 ± (0.06)	1.84 ± (0.25)
SEd	0.092	0.207
CD(.05)	0.205	0.462

Values are Mean ± SD of three replicates

Antimicrobial activity of culture filtrate and crude extract against various isolates of *Xoo*

The antimicrobial activity in terms of zone of inhibition against various isolates of *Xoo* was carried out for the TC1 culture filtrate as well as crude extract. The crude extract produced a zone of inhibition of 1.5-2.2 cm against the tested *Xoo* isolates (Table 6). The highest activity of 2.2 cm

inhibition zone was found against the *Xoo* isolate obtained from TN1 variety, whereas the lowest activity of 1.5 cm zone of inhibition was observed for *Xoo* isolate obtained from CO50 variety. The ethyl acetate crude extract exhibited higher antimicrobial activity compared to culture filtrate which produced an inhibition zone of 1.1-1.6 cm against the tested isolates. The crude extract contains higher amount of antimicrobial fractions compared to culture filtrate, this might be the reason for the increased antimicrobial activity of crude extract against *Xoo*. The variations observed in antimicrobial activity may be due to the different levels of susceptibility to crude extract among tested *Xoo* isolates. The variation observed in the antimicrobial activity against the *Xoo* pathogen is due to the existence of different resistance genes among the races of *Xoo* isolates. Goel *et al.* (1998) [8] reported the existence of variability in the pathogen and the emergence of new pathotypes might be the reason for resistance development among *Xoo* population.

Table 6: Antimicrobial activity of culture filtrate and crude extract of *Streptomyces* sp. TC1 against *Xoo* isolates

Source of *Xoo* isolate	DIZ (in cm)	
	Culture filtrate	Crude extract
ASD 16	1.6 ± (0.06)	2.1 ± (0.15)
ADT 39	1.5 ± (0.20)	1.8 ± (0.06)
ADT 43	1.2 ± (0.10)	1.7 ± (0.12)
CO 43	1.5 ± (0.12)	2.0 ± (0.06)
CO 47	1.2 ± (0.06)	1.6 ± (0.00)
CO 50	1.1 ± (0.12)	1.5 ± (0.06)
TN 1	1.6 ± (0.12)	2.2 ± (0.06)
TNRH 180	1.2 ± (0.06)	1.7 ± (0.15)
Oryza grandiglumis	1.4 ± (0.10)	1.9 ± (0.06)
Oryza merdionalis	1.5 ± (0.06)	2.0 ± (0.06)
SEd	0.079	0.073
CD(.05)	0.164	0.151

Values are mean ± SD of three replicates

Minimum Inhibitory Concentration (MIC) and Inhibitory concentration 50 (IC_{50}) of crude extract against *Xoo*

The MIC and IC_{50} are the basic laboratory assessments for testing the activity of antimicrobial agents. The MIC (minimum inhibitory concentration) was defined as the lowest concentration of antibiotics or crude extracts that did not show any growth of the tested pathogens at a minimum concentration. Whereas the IC_{50} is the concentration in which fifty percent of the target pathogens get killed.

The antimicrobial potential of the ethyl acetate crude extract was assessed quantitatively by determining the MIC. The MIC range of ethyl acetate crude extract against *Xoo* was evaluated and found to be between 0.1 to 1.0 mg/mL. The negative control (ethanol) produced microbial growth in all the dilutions. The positive control streptomycin exhibited an MIC range between 0.01 to 0.1 mg/mL. The MIC experiments carried out for an ethyl acetate crude extract to narrow down the range by NCCLS protocol resulted in the MIC value of 300 µg/mL against the test pathogen *Xoo*. The crude extract with concentrations above 300 µg/mL showed nil growth in MIC experiments. Whereas, the growth of the test culture *Xoo* was observed in concentration below 250 µg/mL. IC_{50} was carried out for ethyl acetate crude extract of *Streptomyces* sp. TC1 against *Xanthomonas oryzae* pv. *oryzae*. The IC_{50} was evaluated at the concentration between 25 to 300 µg/mL. Fifty percent of inhibition (IC_{50}) was observed at a concentration of 125 µg/mL against *Xoo*. An inhibition of 52.5 percentage of colonies was observed in that particular concentration.

The ethyl acetate crude extract obtained from the *Streptomyces* strain ACTN2 exhibited an MIC value of 156 µg/mL and 312 µg/mL against *Bacillus subtilis* and *Candida albicans* respectively (Sosovele *et al.*, 2012) [19]. The antimicrobial compound aloesaponarin II isolated from the culture filtrate of *Streptomyces termitum* strain ATC-2 by silica gel column chromatography possessed strong antimicrobial activity against *Xoo* with IC_{50} of 19.2 µg/mL (Donghua *et al.*, 2013) [3].

Saha *et al.* (2010) [17] reported the MIC of ethyl acetate crude extract obtained from *Streptomyces* sp. MNK7 were found to be in the range of 32-64 mg/mL against selected pathogenic bacteria including *Bacillus, Pseudomonas, Shigella* and *Streptococcus*. In the present study, the crude extract obtained from the TC1 culture filtrate possessed 300, 125 µg/mL of MIC and IC_{50} value respectively against the test pathogen *Xoo*.

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