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Growth and production kinetics of antimicrobial compound from *Streptomyces albidoflavus* 321.2

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In this study, an antimicrobial compound was isolated from the fermented broth of *Streptomyces albidoflavus* 321.2 (MTCC 3662), a new soil isolate. Arginine glycerol salt medium was found best for the production and was maximum after the 6th day of fermentation at 32°C using 6% (v/v) inoculum at static condition to yield 250 µg/ml. The compound exhibited less toxicity against experimental plant and animal systems.

Key words: *Streptomyces*, antimicrobial, fermentation, optimization.

INTRODUCTION

Indiscriminate use of antimicrobials enhances the probability of microbial resistance. Resistance is also with the use of continuous and developed antimicrobial compounds. Commonly used synthetic chemicals (Sosa and Cventic, 2005; Pepelnjak et al., 2005; Mohamed et al., 2005) are sharply losing its acceptability mainly due to cause by environmental problems (Wang et al., 1999). Thus, it necessitates searching for new natural antimicrobial compounds to support the medical practitioners and also to the pharmaceutical industries (Mendo et al., 2004). The genus *Streptomyces* is the largest producer of commercially important antimicrobials. A model suggested that the number of bioactive compounds from *Streptomyces* would be very close to a lakh and a fraction of which had been unearthed so far (Watve et al., 2001). It further emphasized the need to continue the search programme.

The efficiency of a producer largely depends upon the nutritional and environmental conditions (Ueki et al., 1997). There is no common methodology to ascertain the growth and production ability of microorganisms. It is recommended that by varying the conditions of cultivation, one can stimulate the production ability. Such conditions emphasize to pay special attention to the

selection of suitable medium to enhance the rate of physicochemical reactions to ease production. To ensure good screening practice for antibiotic, it is important to explore new areas from where organisms with potential usefulness can be identified (Umezawa, 1982). Enzymatic potential of *Streptomyces albidoflavus* has been reported (Bressollier et al., 1999), however, the isolate *S. albidoflavus* 321.2, showed notable antimicrobial activity. The pH - and thermo - tolerant, active compound (Roy and Sen, 2006) was identified as dibutylphthalate (DBP), an antimetabolite of proline (Roy et al., 2006). This report describes physical growth kinetics for the optimal production of antimicrobial compound by *S. albidoflavus* 321.2 (MTCC 3662), a new soil isolate and evaluation of its toxicity.

MATERIALS AND METHODS

Microorganism used

The producer organism, *S. albidoflavus* 321.2 [MTCC 3662], a soil isolate (Roy and Sen, 2002), was maintained in glucose-asparagine agar and test organism *Escherichia coli* ATCC 25922 in nutrient agar. Organisms were stored at 4°C.

Media and inoculum

Several recommended media for streptomycete (mentioned elsewhere) were tested. For inoculum, suspension of 1.2×10^7

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spores/ml of well-sporulated (10 days old) slant was made.

Fermentation condition

Fermentation was carried out for 7 days at initial pH of 7.0 and at 30°C using 4% inoculums, if not stated otherwise. Fermentation parameter was optimized by varying one condition and keeping the rest constant.

Growth measurement

The cell mass was separated from fermented broth by centrifugation (5000 rpm, 15 min), washed properly with distilled water and dried at 70°C for 16 h. The growth was estimated in terms of dry weight (mg/25 ml).

Antimicrobial assay

After cell separation, clear and faint yellow supernatant was used as crude to test for its antimicrobial activity by agar cup assay method (Higashida et al., 1971). In petridish, glucose-asparagine agar medium was surface seeded with 1 ml of test organism (1.2×10^5 CFU). After solidification, cups (9 mm, diameter) were made and filled with crude active compound (0.1 ml) and incubated at 30°C for 24 h. The inhibition zone diameter was recorded.

Yield

Yield ($\mu\text{g/ml}$) was determined using a standard calibration curve of purified sample against the same test organism (*E. coli*).

Toxicity test

Both plant and animal toxicity tests were done. To measure the rate of photosynthesis, fresh twig of *Hydrilla* plant (~3 g) was used against active compound (50, 100, 200, 300, 400, and 500 $\mu\text{g/ml}$) with NaHCO_3 (trace) as inducer. Keeping it for 30 min, in normal sunlight, the amount of oxygen liberated in each set was recorded from the graduated tube. To test the cell membrane permeability, test was conducted with plant cells. For this purpose, thoroughly washed fresh beet roots were scooped out with cork borer to produce $7 \times 2 \text{ mm}^3$ cylinders. After proper washing, the slices (~4 g) were placed into solution of active compound (~10 ml of 50, 100, 200, 400, and 500 $\mu\text{g/ml}$) in test tubes. Keeping the set for 6 h at $30 \pm 2^\circ\text{C}$, the leached out pigment was recorded at 520 nm using spectrophotometer (JASCO, 7800, Japan). Animal toxicity test was performed following the method of Ezaki et al. (1995). Sterile solution of active compound was injected intraperitoneally (100 mg/Kg body weight) into male mice (Swiss albino strain) having average body weight of 22 g. They were observed for 60 days under standard condition. The optimized parameter of an experiment was considered for designing of subsequent experiments. All the experiments were made in triplicate in all treatments and each test case.

RESULTS AND DISCUSSION

Cell metabolism depends upon adequate supply of energy, thus, quality of medium. Hence, formulation of

medium and designing of growth and production conditions are essential part of successful fermentation experiments, as there is no common recommended medium for fermentation of antimicrobial compound. The production ability of the isolate 321.2 was tested with commonly used media for *Streptomyces*. It was found that the isolate 321.2 could produce maximally in arginine glycerol salt (AGS) medium, followed by Gause's mineral salt medium and ISP-5 (Table 1). Though soybean meal broth supported growth maximally but considering the yield, AGS medium was selected for further optimization.

Likewise medium suitability, physical parameters also influence the physiological performance of organisms. To any optimization process, the period of fermentation is very crucial. Therefore, to optimize the fermentation period for growth and production, experiment was continued up to 9 days. The results revealed that the growth and production increased with the extended fermentation period which was up to day 6, thereafter, the yield declined (Figure 1). The lysis of hyphae probably causes the lowering of pH value, in addition to the accumulation of organic acids that lead to alter the environmental growth condition. Optimum production at 6th day was also reported with *Streptomyces* sp. (Kojiri et al., 1992). Further, to optimize the temperature, experiment was conducted in a range of 25 to 40°C. Most favourable growth was found in between 30 to 34°C. But the highest yield was recorded at 32°C; above this temperature growth, production was declined, indicating its mesophilic nature (Figure 2). Temperature suitability of streptomycetes was also observed as 32°C (Boeck et al., 1985). For the successful fermentation experiment, inoculum size is an important factor. While standardizing the optimum inoculum size for growth and production, 2 to 10% (v/v) of inoculum was attempted. An inoculum size of 10 and 6% were found best for growth and production, respectively (Figure 3). Franco et al. (2000) used 8-10% vegetative inoculum for *Streptomyces* Y-86, 36923 and 10% inoculum was used for *S. hygroscopicus* (Bhattacharyya and Sen, 2002). The pH of medium is vital for biosynthesis of cell metabolites and their dissociation. The growth and production was found optimum at initial pH of 7.0 and 6.75, respectively (Figure 4). Neutral pH is most favoured by *Streptomyces* (Williams et al., 1971). Likewise, the isolate 321.2 is found to produce optimally at pH near neutrality (El-Gammal, 1986).

Acceptability of a new compound depends much on its toxicity value. Thus, toxicity test is an integral part of the research for antimicrobial compound and commonly subjected to both plant and animal systems. To study the phytotoxicity, the rate of photosynthesis of treated plants revealed that the photosynthetic rate gradually decreased with the increase in concentration of active compound (Figure 5a). However, only 14% loss of photosynthetic rate was observed at 500 $\mu\text{g/ml}$ concentration. The effect

Table 1. Suitability of medium for growth and antimicrobial production by isolate 321.2.

Medium	Growth (mg/25 ml)	Yield ($\mu\text{g/ml}$)
Glucose-asparagine (Krainsky, 1941)	040	-
Pridham and Gottlieb (Pridham and Gottlieb, 1948)	075	100
Lindenbein (Lindenbein, 1952)	060	135
Mineral salts (Gause et al., 1958)	060	145
Arginine glycerol salt (El-Nakeeb and Lechevalier, 1963)	050	175
ISP-2 (Pridham et al., 1956)	080	-
Starch-yeast extract (Agate and Bhat, 1963)	045	125
ISP-5 (Pridham and Lyons, 1961)	060	-
Sporulating medium (Hobbs et al., 1989)	100	145
Sucrose nitrate (Waksman, 1957)	125	-
Soybean meal (Tsao et al., 1960)	150	085
Glucose-peptone-yeast extract (Abou-Zeid et al., 1991)	075	050

Figure 1. Effect of time period for growth and antimicrobial production by isolate 321.2. **Figure 3.** Effect of inoculum size for growth and antimicrobial production by isolate 321.2. **Figure 2.** Effect of temperature for growth and antimicrobial production by isolate 321.2. **Figure 4.** Effect of pH for growth and antimicrobial production by isolate 321.2.

Figure 5. Toxicity test of the antimicrobial compound A) Phytotoxicity and B) Animal toxicity.

of cell permeability was studied with beet root. The experiment did not show much leaching of anthocyanine pigment even up to 6 h of treatment but the rate of leaching increased with increased concentration of active compound (Figure 5a). The compound did not influence much in photosynthesis rate and membrane permeability even in higher dose. Haque et al. (1996) noted insignificant inhibition in photosynthetic rate of the antimicrobial compound produced by *S. antibioticus* sr 15.4. In the animal toxicity experiment, there was no incidence of death of treated animals which was up to 60 days, while administered with active compound (100 mg/Kg). However, the loss of body weight was observed (Figure 5b). But the results of animal toxicity tests may vary with the chemical nature of the compound and the route of administration to the experimental animal. The lowest observed adverse effect level (LOAEL) of DBP was 66 mg/kg/day with diet in SD rats (Foster et al., 2000). Production of antimicrobials as secondary metabolite is controlled by the genetic make up of the organism. The fullest expression of this property is profoundly influenced by quality and quantity of medium, along with the environmental factors. The experimental data revealed that the isolate 321.2 under optimized physical parameters of selected medium could also be able to produce 250 µg/ml of antimicrobial and exhibited very low toxic effects.

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