Pak. J. Bot., 49(SI): 363-368, 2017.

ENHANCED LINCOMYCIN PRODUCTION BY *STREPTOMYCES LINCOLNENSIS* THROUGH OPTIMIZATION OF CULTURE CONDITIONS

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Abstract

In the present study, production of lincomycin by *Streptomyces lincolnensis* through submerged fermentation has been reported. Culture medium M6 consisting of glucose (15 g/L), starch (40 g/L), molasses (20 g/L), corn steep liquor (20 g/L), peptone (10 g/L) and $CaCO_3$ (8.0 g/L) was found best for growth of *Streptomyces lincolnensis* and production of lincomycin. The increased production of lincomycin by *Streptomyces lincolnensis* was obtained at 30˚C (64.6 mg/mL of dry cell mass with 3.12 mm diameter of inhibition zone), 7.5 pH (78.3 mg/mL of dry cell mass with 3.19 mm diameter of inhibition zone) and 144 hours incubation (80.8 mg/mL of dry cell mass with 3.30 mm diameter of inhibition zone), respectively. For maximum production of lincomycin by *Streptomyces lincolnesis*, different carbon sources, organic and inorganic nitrogen sources were also studied. 4% of lactose (82 mg/mL of dry cell mass with 3.30 mm diameter of inhibition zone) as a carbon source, 8% of meat extract (81.1 mg/mL of dry cell mass with 4.10 mm diameter of inhibition zone) as an organic nitrogen source and ammonium nitrate (82.4 mg/mL of dry cell mass with 5.00 mm diameter of inhibition zone) as an inorganic nitrogen source were optimized. The inoculum quality and quantity were also optimized for high yield of lincomycin. 2% of 72 hours old vegetative inoculum of *Streptomyces lincolnensis* in the form of pellet was found best for the growth of *Streptomyces lincolnensis* for subsequent lincomycin production using shake flask method.

Keywords: Antibiotic, Sensitivity, Inhibition zone, submerged fermentation

Introduction

The chemical substances produced by different microorganisms having ability to kill other microorganisms are very vital for unchecked control of microbes known as "Antibiotics". There are two classes of antibiotics mainly; one is effective against wide range of microbes and called as broad spectrum antibiotics while on other hand narrow spectrum antibiotics act against specific microbes. Lincomycin presented a very good action against gram positive microbes in the initial reports (Spizek & Rezanka, 2004). Lincomycin is a member of broad spectrum group and characterized by methyl-6,8-dideoxy-6-[(2S,4R)-1-methyl-4-propylpyrrolidine-2-carboxamide]-1-thio-D-erythro-α-D-galacto octopyranoside hydrochloride joined with proline moiety by an amide linkage (Shirling & Gottlieb, 1972). The mode of action of lincomycin may be bacteriostatic or bactericidal. Bacteriostatic lincomycin shows *in vitro* and *in vivo* activity which immediately inhibits the protein synthesis in sensitive bacteria. However, it may be bactericidal in higher concentration that can be reached *in vivo* (Spizek & Rezanka, 2003).

Actinomycetes are often referred to as antibiotic producers as they produce approximately 85% of the antibiotics (Sultan *et al.*, 2002). They are ubiquitous in nature and are excellent source of biologically active secondary metabolites especially antibiotics, anti-tumor, anti-fungal, anti-viral and immunosuppressant agents. The majority of actinomycetes are free living gram-positive bacteria and the most important one in this regard are *Streptomyces*. These are aerobic, Gram-positive, filamentous, saprophytic soil bacteria widely distributed as one of the major groups of soil population which may vary with the soil type, water and colonizing plants (Slavica *et al.*, 2005; Hayakawa *et al.*, 2004; Mizuno & Yoshida, 1993).

The growth of *Streptomyces* cells for antibiotic production has been found better in submerged culture than on solid media due to a better supply of nutrients (Reichl *et al.*,). Submerged fermentation has several advantages over solid-state fermentation, such as easy monitoring of pH and temperature, good homogenization of medium, easy inoculation of medium with microorganisms and easy supplementation of oxygen required for the growth of aerobic organisms. In submerged culture, *Streptomyces* leads to better yield of antibiotic than in the case of growth as free filaments.

The relationship between antibiotic production and its surroundings is very important to understand in order to achieve a greater production of drug. Cell growth and lincomycin production have been reported using batch cultures of *Streptomyces* sub sp. in chemically defined culture media under submerged aerobic conditions (Young *et al.*, 1985; Pandey *et al.*, 2008). Production of lincomycin from *Streptomyces* can be affected at different temperatures. The requirement of suitable temperature for spore production is strain dependent. The production of lincomycin has been reported at a temperature ranging from 18˚C to 45˚C (Shirling & Gottlieb, 1972; Benimeli *et al.*, 2007).

The production of antibiotic is controlled by genetic make-up that imparts highest expression and is influenced by the kind and quality of nutritional elements and environmental factor (Bhattacharyya *et al.*, 1998). The best production of antibiotic can be obtained by using such complex mediums that have ability to slowly metabolize its carbon as well as nitrogen source. The preferred carbon sources for the production of lincomycin by *Streptomyces* include glucose, glycerol, sucrose, starch, lactose, molasses, maltose and fructose (Bergy *et al.*, 1967; Bhattacharyya *et al.*, 1998). Similarly, corn steep liquor, yeast extract, beef extract, meat extract, malt extract, casein, peptone and urea are among the preferred nitrogen sources for lincomycin production (Bergy *et al.*, 1967; Bhattacharyya *et al.*, 1998).

pH is also reported to have a significant effect on the production of lincomycin and growth of *Streptomyces* (Bhattacharyya *et al.*, 1998). *Streptomyces* grows over a broad range of pH between 4.0-11.5, but most types prefer to grow on neutral to alkaline pH, the optimum being 6.5-8.0 (Kontro *et al.*, 2005). Besides the aforementioned factors, the quality and quantity of inoculum plays a crucial role in bioprocess results, i.e. the growth of *Streptomyces spp.* in medium and the production of lincomycin (Ettler, 1992).

Traditionally, lincomycin is determined by microbiological assay. However, because of the difficulty in differentiating lincomycin from other constituents and the presence of pharmaceutically active compounds in *Streptomyces* culture supernatants, thin layer chromatography (TLC) has been developed for the identification and quantification of lincomycin (Charles *et al.*, 2002). Lately, high performance liquid chromatography (HPLC) is replacing the microbiological and TLC methods because the later being less sensitive, less accurate and less selective of antibiotics. On the contrary, HPLC is a rapid, simple and sensitive method and is used to assess identity, strength, quality and quantity of the antibiotic. Lincomycin detection is carried out by UV detector at wavelength ranging from 208-210 nm (Dousa*et al.*, 2006).

Materials and Methods

Microorganism and maintenance: Lincomycin-producing *Streptomyces* subsp. were isolated from different soil samples using serial dilution method (Slavica *et al.*, 2005). These dilutions were spread on agar plates containing medium comprising of glucose (0.1%), yeast extract (0.01%), meat extract (0.04%), peptone (0.04%) and incubated at 30˚C for 72 hours. Individual colonies were then transferred to the slants containing the same medium and after incubation for growth, they were stored at 4˚C. Same procedure was repeated for the strain of *Streptomyces lincolnensis* (NRRL ISP 53SS) taken from the culture bank of the Institute of Industrial Biotechnology (IIB), Government College University, Lahore.

Inoculum development and fermentation technique: The medium for seed inoculum comprised of glucose (4g/L), yeast extract (10 g/L) and malt extract (pH 7.5). While the production medium (pH 7.2) consisted of starch (40 g/L), glucose (15 g/L), molasses (20 g/L), corn steep (20 g/L), peptone (10 g/L), $CaCO_3$ (8 g/L). Each of the media was prepared in 50 mL amount in 250-mL Erlenmeyer flasks followed by autoclaving. The production medium was inoculated with 1 mL of seed inoculum and incubated at 30˚C and 200 rpm rotation for 120 hours. All experiments were conducted in triplicates. This was followed by centrifugation of the contents at 10,000 rpm for 10 minutes and subjecting the supernatant for further analyses.

Analytical procedures: Dry cell mass of *Streptomyces lincolnensis* was determined by the simple procedure of weighing filter paper before and after filtering the fermented broth through it and was expressed in mg/mL. The activity of the antibiotic present in the supernatant was determined by agar well method, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) methods. Briefly, agar well method includes boring of wells in nutrient agar medium (8g/L nutrient broth and 18 g/L agar) solidified in petri plates and filling these with 0.2 mL of the supernatant obtained after centrifugation. Clear zones of inhibition are looked for and their diameters measured after incubation for growth at 37˚C for 48 hours.

TLC was be performed by having 10% citric acid, n-hexane and ethanol (80:1:1) as the 1st mobile phase, n-butanol, ethanol, chloroform and 25% ammonia (4:5:2:5) as the 2nd mobile phase. Two hundred and fifty mg of orcinol was dissolved in 44 mL of absolute ethanol and 6 mL H_2SO_4 as the locating agent. Alternatively, HPLC was also used for the determination and quantification of lincomycin in the fermented broth. The column chosen was reversed phase C-18 of size 250 x 4.6 mm with pore size 5 µm, while the mobile phase comprised of 0.2% phosphoric acid in water and acetonitrile mixed in the ratio of 875:125 (v/v). It was conducted at ambient temperature, with flow rate of 1.50 mL/min and injection volume of 20 µL. The detection was conducted at 208 nm.

Optimization of parameters: Different culture media were screened for the growth of *Streptomyces lincolnensis* and production of lincomycin. Six media were used for this purpose whose compositions are under-mentioned.

M 1: (g/L); yeast extract,1.0: Glucose, 10; peptone, 4.0: meat extract, 4.0; NaCl, 2.0 (pH 7.2).
M 2: (g/L); Glucose 15,; starch, 40; molasses, 20; $CaCO_3$, 8.0 (pH 7.2).
M 3: (g/L); Dextrose, 10; yeast extract, 4.7; beef extract, 2.4; peptone, 9.4; NaCl, 10 (pH 7.5).
M 4: (g/L); Dextrose, 1.0; yeast extract, 3.0; beef extract, 1.5; peptone, 6.0; casein, 4.0 (pH 7.5).
M 5: (g/L); Dextrose, 2.5; casein, 17; di-potassium phosphate, 2.5; soybean peptone, 3.0; NaCl, 2.0 (pH 7.2).
M 6: (g/L); Glucose, 15; starch, 40; molasses, 20; corn steep liquor, 20; peptone, 10; $CaCO_3$, 8.0 (pH 7.2).

The effect of initial pH of the fermentation medium on the growth of *Streptomyces lincolnensis* and, in turn, the production of lincomycin was studied by adjusting the pH of the medium in the range 6-8.5 with 0.1N HCl and 0.1N NaOH. Similarly, the incubation time was investigated by incubating the flasks in shaking incubator at 30˚C and 200 rpm rotation at time intervals of 24, 48, 72, 96, 120, 144 and 168 hours. The effect of incubation temperature on growth was studied at different temperatures of 25, 30, 35, 40 and 45˚C.

Also, different carbon sources, viz. molasses, lactose, glucose, fructose, starch, maltose and sucrose were also added in the fermented medium in concentrations ranging from 2 to 10 % (w/v) in order to optimize the concentration of carbon source in the culture medium.

Different nitrogen sources, including peptone, yeast extract, urea, corn steep liquor, meat extract, casein and malt extract were also added in the fermented medium in concentrations ranging from 2-10% (w/v).

The effect of addition of inorganic nitrogen sources, such as sodium nitrate, ammonium sulfate, ammonium acetate, ammonium chloride, ammonium nitrate, and potassium nitrate, on the production of lincomycin was also studied. Size of inoculum was also varied ranging from 1-6% (v/v) along with the type of inoculum, i.e. spore or vegetative inoculum, and ages of inoculum ranging from 24 to 96 hours to see their effects on the production of the antibiotic.

Results and Discussion

The strain provided by IIB gave maximum growth and production of lincomycin as compared to the four cultures of Streptomyces that were isolated from soil samples as shown by the dry cell mass of 51.3 mg/mL and inhibition zone of 2.8 mm against the test organism (Table I). Therefore this strain was selected for optimization of the cultural conditions for the production of lincomycin by submerged fermentation.

The presence of lincomycin was confirmed by TLC (Fig. 1) followed by HPLC (Fig. 2). The other peaks obtained in the latter showed the presence of some other antimicrobial agents in the fermentation broth.

Table 1. Screening of isolates for production of lincomycin by *Streptomyces lincolnensis*.

Sr. No.	Strain Code	Dry Cell Mass (mg/mL)	Inhibition Zone (mm)
1	IIB1	23.1 ±1.3	1.53
2	IIB2	39.4 ± 2.1	1.83
3	IIB3	25.6 ± 1.6	1.18
4	IIB4	41.6 ± 1.9	1.19
5	NRRL ISP 53SS	51.3 ± 1.4	2.80

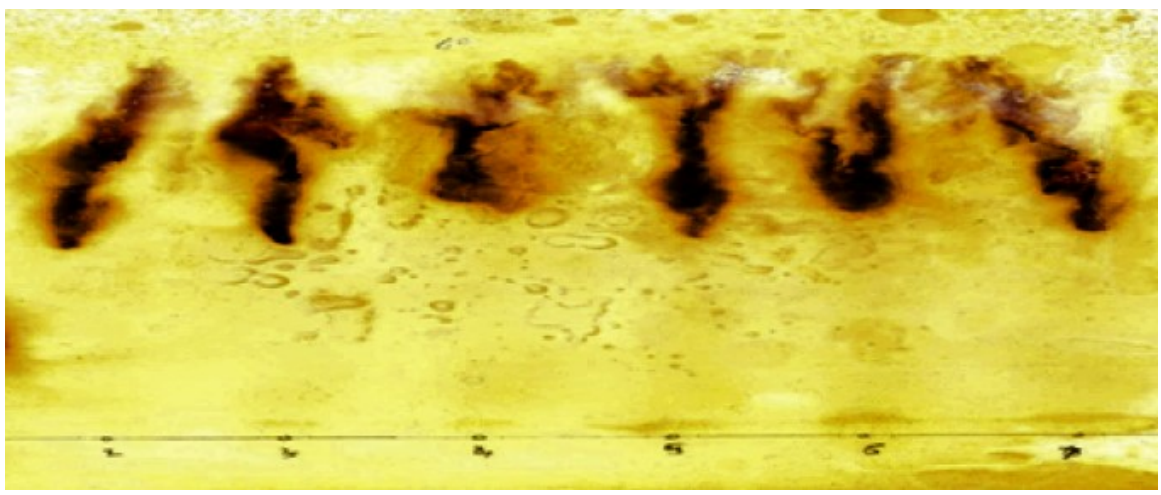

Fig.1. Thin layer chromatography (TLC) for the detection of lincomycin in fermented broths.

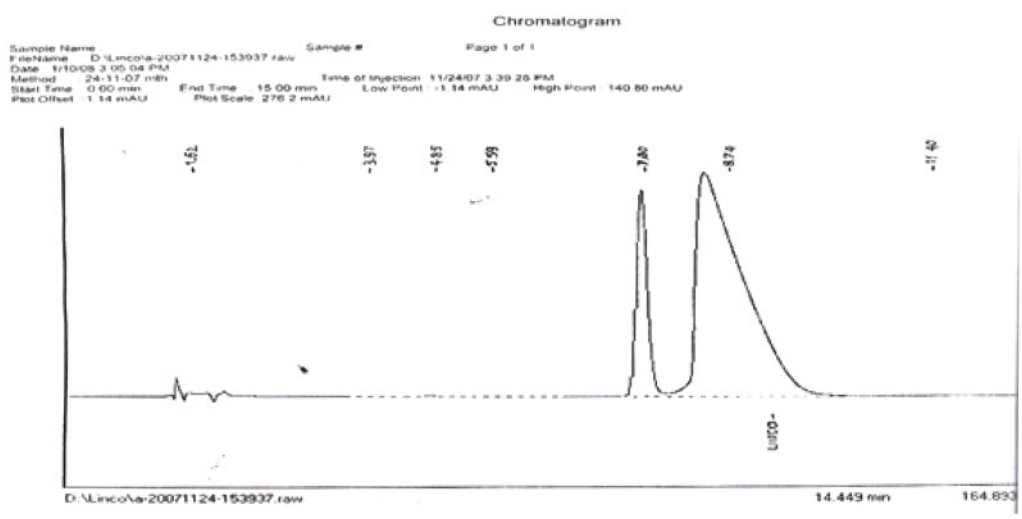

Fig. 2. High performance liquid chromatography (HPLC) for the detection of lincomycin in the fermentation broth.

Different parameters were investigated and optimized by one-factor-at-a-time strategy. The results obtained have been tabulated (Table 2) after graphical analysis (Fig. 3).

In thin layer chromatography analysis, the mobility of the sample spots was more due to the presence of other compounds in the fermented broth, while in HPLC analysis, the other two peaks have been obtained due to the presence of some other antimicrobial agents in fermentation broth. While selecting the culture medium, it was observed that the medium composition has a great influence on the growth of *Streptomyces lincolnensis* and lincomycin production. It commonly grows at remarkably variable nutritional environment and its growth in simple media was considerably slower, hence the minor modifications of ingredients. M6 was found to be the best suitable growth medium because this complex medium could provide all the necessary components in sufficient quantity, while the converse of the same reason could be true for M5 thus accounting for the minimum growth (Fig. 3a).

The amount of secondary metabolite synthesis is highly dependent on growth rate and temperature. In this study 30˚C was optimized to be the temperature of maximum growth as shown in Fig. 3c, as it also affects the lipid composition, fluidity, and permeability of organelle which help the antibiotic to diffuse out from the cell. High temperature retards the metabolic process by denaturing the enzymes and proteins, hence less growth and antibiotic production at temperatures above the optimized (Shapiro, 1989).

Production of lincomycin by *Streptomyces lincolnensis* is much dependent on pH of the fermentation medium. Changes in pH affect both the timing and extent of antibiotic production, and the present study has found the optimum pH as 7.5 indicated in Fig. 3d (Sultan *et al.*, 2002). The growth is also directly related to the incubation time which was found to be 144 hours (Fig. 3b) because of the fact that the organism had entered the stationary phase of growth and antibiotic production takes place in the stationary phase (Gamajo *et al.*, 1993). The decrease in the production of lincomycin might be attributed to the fact that after 144 hours of incubation nutrients started to deplete with accumulation of toxins (Sultan *et al.*, 2002).

Carbon source, another important determinant of the growth of *Streptomyces lincolnensis*, is required as an energy source and is known to greatly influence the lincomycin production by the fungus. Lactose (4 %) has been found to be the suitable carbon source because of its ability to serve the needs of both cellular growth and antibiotic production (Figs. 3e & 3f). Furthermore, nitrogen source is known to greatly influence the lincomycin production by constituting the nucleotides and amino acids. Meat extract (0.8%) was found as optimum organic source of nitrogen (Figs. 3g & 3h). It might be due to the reason that meat extract is easily assimilated, inexpensive and distinct from conventional sources of nitrogen (Schimana *et al.*, 2001). The inorganic nitrogen source is also considered to be necessary for the growth of the fungus and antibiotic production and the most suitable source was found to be ammonium nitrate (Fig. 3i).

The quantity of inoculum material plays an important role in the bioprocess results (Ettler, 1992). 2% incoulum size of 72 hours age was found best for the production of lincoycin as shown in Fig. 3j and 3k. Inoculum size less than 2% often results in pelleting (James & Turner, 1990). Also, the cells are not enough in number at such concentrations to utilize the essential amount of nutrients for rapid growth and antibiotic production. At higher concentrations, the anaerobic condition of fermentation medium due to tremendous growth of microorganism led to nutritional imbalance in the medium and in turn less production. The cells utilize all the nutrients to grow only and leave none to be utilized for the production of antibiotic (Deklava *et al.*, 1985) therefore an inoculum of intermediate size was found to be the best.

Table 2. Values of optimized parameters along with the maximum dry cell mass and diameter of inhibition zone obtained for each factor.

Parameter	Optimized result	Maximum dry cell mass (mg/mL)	Maximum diameter of inhibition zone (mm)
Culture medium	M 6	55.7	3.0
Incubation temperature	30˚C	64.6	3.12
Initial pH of culture medium	7.5	78.3	3.19
Incubation time for growth	144 hours	80.8	3.3
Carbon source	Lactose	81,7	3.4
Lactose concentration (w/v)	4%	82	3.3
Organic nitrogen source	Meat extract	80.3	3.63
Meat extract concentration (w/v)	8%	81.1	4.1
Inorganic nitrogen source	Ammonium nitrate	82.4	5.0
Inoculum quantity (v/v)	2%	85	7.2
Inoculum quality (age of inoculum; v/v)	72 hours	96.1	8.1
Type of inoculum	Vegetative	103	8.4
Type of vegetative inoculum	Pellets	111	9.6

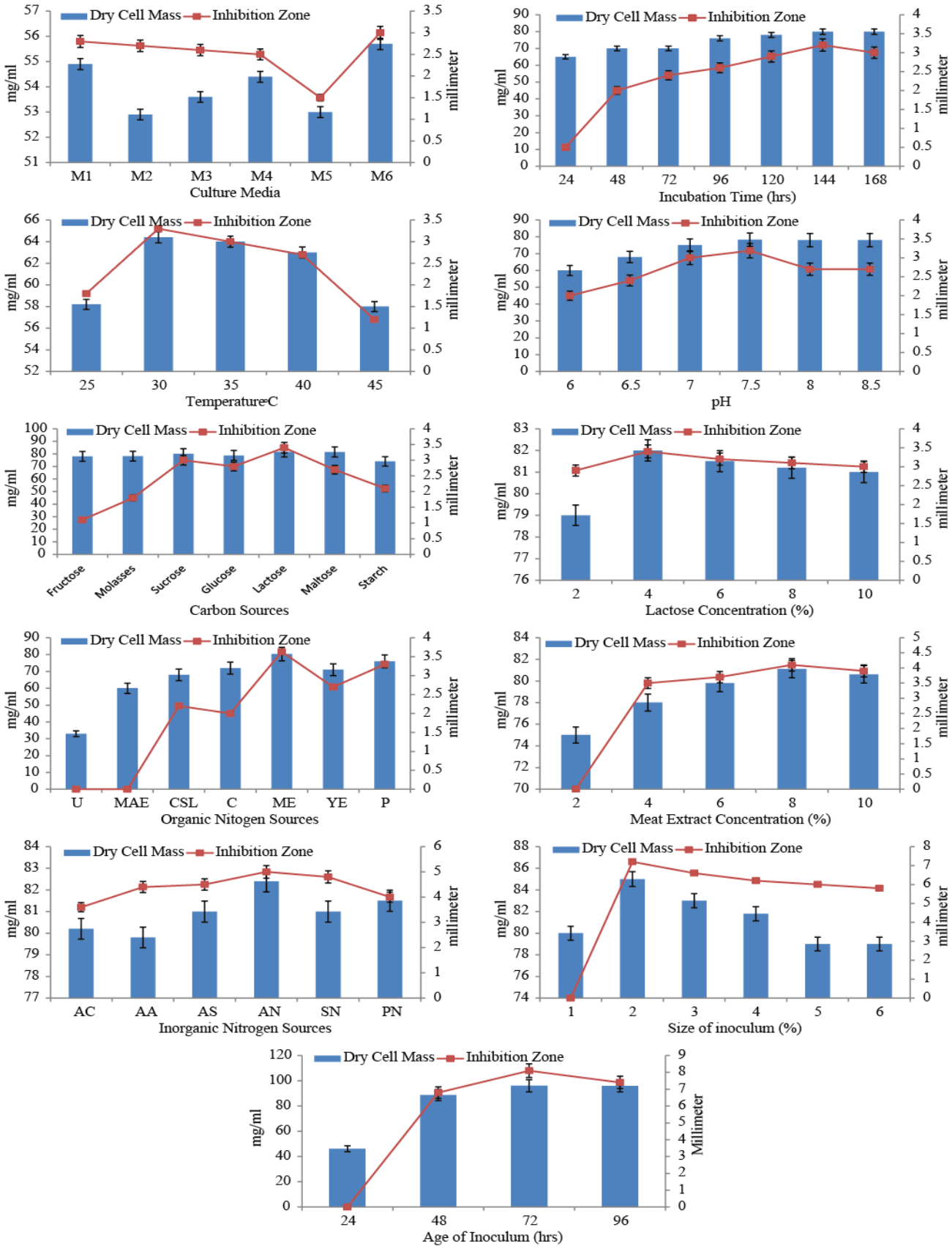

Fig. 3: a) Effect of medium composition; b) Effect of incubation time; c) Effect of incubation temperature; d) Effect of initial pH; e) Effect of carbon sources; f) Effect of different concentrations of lactose; g) Effect of nitrogen sources (U = urea, MAE = malt extract, CSL = corn steep liquor, C = casein, ME = meat extract, YE = yeast extract, P = peptone); h) Effect of different concentrations of meat extract; i) Effect of inorganic nitrogen sources (A.C = ammonium chloride, A.A = ammonium acetate, A.S = ammonium sulfate, A.N = ammonium nitrate, S.N = sodium nitrate, P.N = potassium nitrate); j) Effect of inoculum quantity; and k) Effect of inoculums age on lincomycin production by *Streptomyces lincolnensis*.

Conclusion

It is concluded from present study that a strain of *Streptomyces lincolnensis* was found to produce a considerable amount of lincomycin in shake flasks during batch fermentation. The production of lincomycin and growth of *Streptomyces lincolnensis* were substantially increased by optimization of fermentation media and cultural conditions during fermentation. Large-scale production by optimized cultural parameters can be used for application in poultry, veterinary, medicinal, agricultural, forest and horticultural industries.

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(Received for publication 15 January 2016)