Pak. J. Bot., 44(1): 445-452, 2012.

PRODUCTION OF ANTITUMOR ANTIBIOTIC BY *STREPTOMYCES CAPOAMUS*

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Abstract

The present study is concerned with the production of antitumor antibiotic by *Streptomyces capoamus* in batch fermentation. Antibiotic activity was tested against *Bacillus subtilis* by cylinder plate method. Different culture media were screened and M3 medium consisting of glucose (10.0 g/L), yeast extract (1.0 g/L), meat extract (4.0 g/L), peptone (4.0 g/L) and NaCl (2.0 g/L) was found to be the best. Optimum temperature, pH and incubation period for the production of antitumor antibiotic were found to be 30°C, 7.5 and 72 hrs, respectively. 2% maltose as carbon source, 2% corn steep liquor as nitrogen source and 48 hrs old inoculum at a concentration of 8% (v/v) were found to be the best for antitumor antibiotic production by *Streptomyces capoamus* NRRL B3632.

Introduction

Antitumor antibiotics (cytotoxic/anticancer antibiotics) are drugs that inhibit and combat the development of tumors. Anthracyclines are important group of antitumor antibiotics and seven members of this group have been shown to be clinically important in cancer treatment which include daunorubicin, doxorubicin, epirubicin, idarubicin, pirarubicin, zorubicin and aclarubicin (Kremer *et al.*, 2001; Fischer *et al.*, 2003). Anthracycline antibiotics were first isolated as red substances from microorganisms in 1939 and their antibiotic properties were studied in the 1950s. These antibiotics killed bacteria quite readily but were too toxic to be used against the infections in humans. It was after 1960s that anthracycline antibiotics were tested for antitumor properties and found to be active against cancer cells (Taatjes *et al.*, 1997). Among all groups of microorganisms, the antitumor antibiotics produced by *Streptomyces* are invaluable in the medical field (Mueller & Nicole 2002; Azambuja *et al.*, 2005). All the seven members of anthracyclines are produced by *Streptomyces* species (Martins & Souto- Maior, 2003).

Most of the antibiotics are produced by staged fermentations where the source microorganism is grown in large containers containing a liquid growth medium (Madigan & Martinko, 2005). Batch fermentation with complex medium containing slowly metabolizing carbon and/or nitrogen source is normally used for the production of anthracyclines (Ciclamycin) from *Streptomyces capoamus* (Martins and Souto- Maior, 2003). Fed-batch fermentation can also be used for anthracyclines production (Pamboukian & Facciotti, 2004). Industrial production of daunorubicin and doxorubicin has also been reported (White & Stroshane, 1984) but very little has been published on process and media development for maximal titre production of anthracyclines in commercial fermentations (Martins & Souto-Maior, 2003).

In submerged cultures, *Streptomyces* tends to form fluffy spherical pellets (Vecht-Lifshitz *et al.*, 1989). Cell growth in the form of pellets led to better yield of antibiotic than growth as free filaments. Increasing the shaking speed and decreasing the medium volume improves antitumor production. The use of high glucose concentration resulted in catabolite repression of the biosynthesis of the antitumor antibiotic. Also increasing antibiotic activity was observed both intra and extracellularly during growth under the carbon and nitrogen limiting conditions (Lilley *et. al.*, 1981). On an industrial scale, culture media for antibiotic production generally contain complex nitrogen sources such as soybean meal. These sources have been selected for their ability to sustain high antibiotic titres and this property is supposed to be linked to the slow release of nitrogenous components during the course of the fermentation. More generally, several studies have shown that nitrogen assimilation is crucial for regulation of antibiotic production but the mechanisms involved have not yet been unraveled. In addition, there is experimental evidence for repression of antibiotic production exerted by some nitrogen sources and especially ammonium (Martin & Demain, 1980).

The present study is aimed at the optimization of some critical parameters for the production of antitumor antibiotics from *Streptomyces capoamus* and having an insight into the production process for obtaining maximum titer of antibiotic for commercial productions.

Materials and Methods

Microorganism: A strain of *Streptomyces capoamus* was taken from the culture bank of the Institute of Industrial Biotechnology, GC University Lahore. The culture was provided by the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, IL and was designed as NRRL B3632. Stock culture was sub cultured in a 250ml flask containing 50ml of the basal medium consisting of (g/L): yeast extract, 0.3; casamino acids, 0.3; glucose, 0.3 and dipotassium hydrogen phosphate, 2.0 (pH 7.0) (Reddi & Rao, 1971) and maintained on solid medium in slants.

Inoculum preparation: Inoculum was prepared in 250ml Erlenmeyer flask containing 50ml of inoculum medium consisting of (g/L): glucose, 4; maltose, 10; yeast extract, 4 (pH 7.0). The flasks were incubated for 48 hrs at 30°C and 200rpm (Martins & Souto-Maior, 2003). The inoculum was used to inoculate the fermentation flasks.

Batch process: Batch process was carried out in 250 ml shake fermentation flasks containing (g/L): glucose, 10.0; yeast extract, 1.0; meat extract, 4.0; peptone, 4.0 and NaCl, 2.0 (pH 7.0) (Emerson *et al.,* 1946). The inoculated fermentation flasks were placed in shaking incubator for 48 hrs at 30°C and 200 rpm. After fermentation, the broth was filtered using Whatman filter paper No. 42 and filtrate was used for further analysis.

Determination of biomass: Biomass was recorded as dry cell mass (DCM) and was calculated according to the method of Martins & Souto-Maior (2003). Fermentation broth was filtered through a pre-weighed Whatman filter paper No. 44. Residue was dried in oven at 105°C for 24 hrs and was weighed again. Dry cell mass was calculated by subtracting pre-weight from after-weight.

Antibiotic activity: Antibiotic activity was determined by cylinder plate method. *Bacillus subtilis* was used as test organism. 0.2ml of the filtrate from batch flasks was poured into the cylinder made in the solidified nutrient agar plates already containing a lawn of inoculated test organism. These plates were incubated at 37°C for 24 hrs. Antibiotic activity was observed and expressed in millimeter of the inhibition zone size formed around the point of delivery of filtrate in the lawn of bacterial growth.

Results and Discussion

Screening of Fermentation media: Seven different media were tested for the growth of *Streptomyces capoamus* and production of antitumor antibiotic (Fig. 1). The composition of these media is as follows:

M1 (g/L): yeast extract, 0.25; K_2HPO_4, 0.5 (Reddi & Rao, 1971).

M2 (YCD medium) (g/L): yeast extract, 1; K_2HPO_4, 0.5: casamino acids, 1; D- glucose, 1 (Reddi & Rao, 1971).

M3 (g/L): glucose, 10; yeast extract, 1; meat extract, 4; peptone, 4; NaCl, 2 (Emerson *et al.,* 1946).

M4 (g/L): glucose, 5; malt extract, 10; yeast extract, 4 (Emerson *et al.,* 1946).

M5 (g/L): peptone, 3; meat extract, 2; yeast extract, 2; glucose, 2; $MnCl_2$, 0.2 (Reddi & Rao, 1971).

M6 (g/L): glucose, 4; malt extract, 10; yeast extract, 4 (Martins & Souto-Maior, 2003).

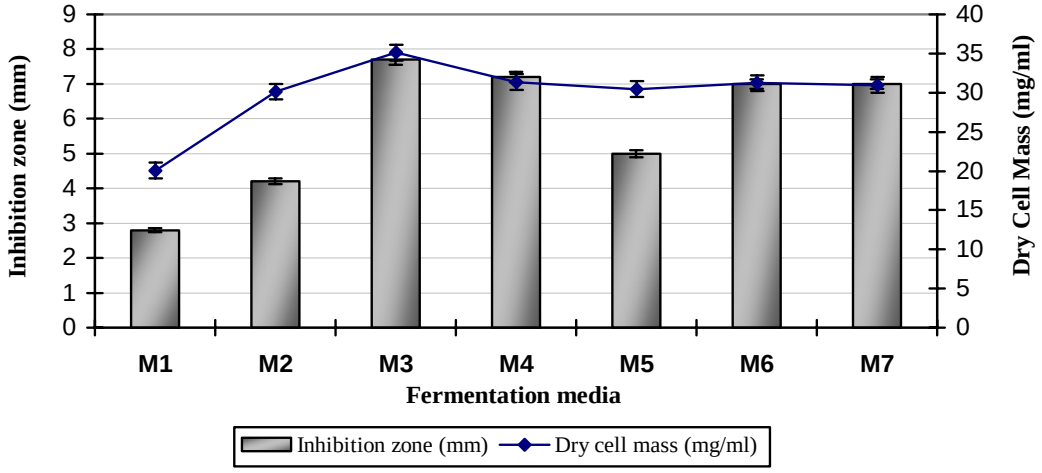
M7 (g/L): glucose, 4; starch, 5; malt extract, 10; yeast extract, 4 (Martins & Souto- Maior, 2003).

Maximum amount of dry cell mass and maximum production of antibiotic was observed when M3 medium was used for fermentation. Streptomycetes commonly grow at remarkably variable nutritional conditions (Kontro *et al.,* 2005) and antibiotic production by them is also influenced by the medium composition (Theobald *et al.,* 2000; Maria *et al.,* 2001 and Schimana *et al.,* 2001). The maximum growth of *Streptomyces capoamus* and maximum antibiotic production in M3 was due to the reason that this complex medium provided all the necessary components e.g. carbon, energy sources, minerals and growth factors for the growth of this organism in the right quantity. Glucose has been found to increase the growth of *Streptomyces* (Emerson *et al.,* 1946). Perlman & Langlykke (1949) have shown that NaCl helps in the release of bound antibiotic from the mycelium. The minimum growth in M1 might be due to the reason that it contains much less nutrients.

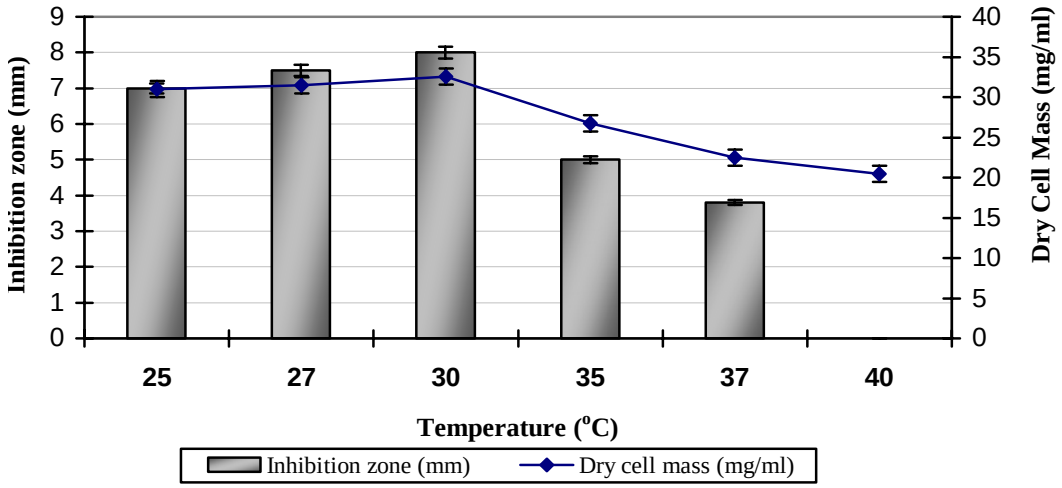
Effect of Incubation temperature: The optimum temperature for the growth of microorganism and antibiotic production was found as 30°C (Fig. 2). Gradual increase in the amount of dry cell mass and diameter of inhibition zone was observed when the incubation temperature was increased from 25-30°C. Above 30°C, there was a rapid decline in both the growth and antibiotic production with no antibiotic production at 40ºC. Different workers have reported that optimum temperature for the growth of *Streptomyces* is 25-35°C (Buchanan & Gibbons, 1974). Lesser growth of microorganism at higher temperature is due to the fact that high temperature retards the metabolic processes of the microorganism by denaturing enzymes, transport carriers and other proteins.

Effect of medium pH: The growth of the microorganism in the acidic side of pH was much less with no production of antibiotic but when the pH value was kept at 6.5, there was sudden increase in dry cell mass. At this pH, antibiotic was also produced as shown by 5 mm diameter of inhibition zone. The maximum dry cell mass i.e. 36.99 mg/ml was obtained at pH value of 7.5 (Fig. 3). Changes in pH affect both the timing and extent of antibiotic production by *Streptomyces* spp. (James and Edwards, 1988). Although the pH value of 7.5 has been found to be the best for maximum growth of *Streptomyces capoamus* and antibiotic production yet this organism also showed considerable dry cell mass at the pH value of 9.0.

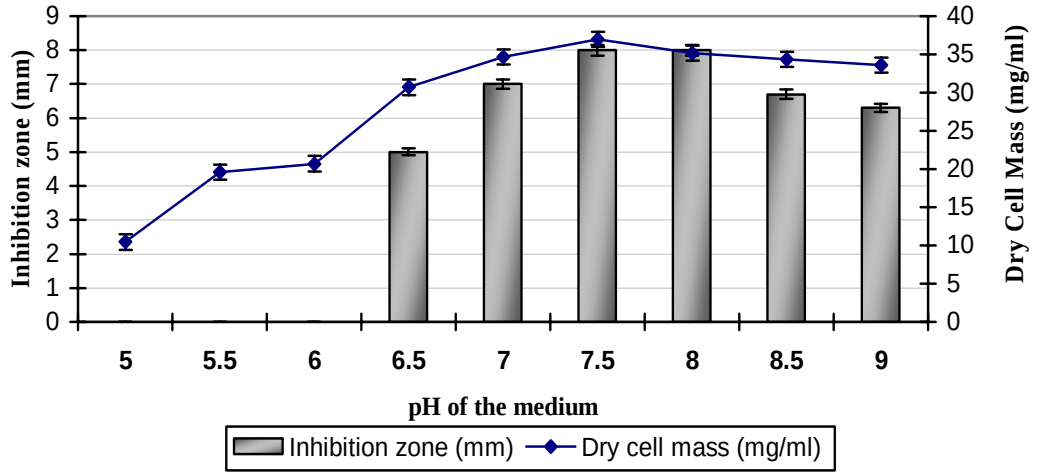
Effect of incubation period: The growth of microorganism was started to increase gradually as the fermentation batch was started. After 24 hrs of incubation, very small amount of dry cell was obtained with no antibiotic production. The maximum dry cell mass i.e. 36.83 mg/ml and highest production of antibiotic as shown by 7 mm diameter of inhibition zone was obtained when the fermentation flasks were incubated for 72 hrs (Fig. 4). There was a decline in the biomass and yield of antibiotic as the incubation period was increased above 72 hrs. Maximum production of antibiotic after 72 hrs of incubation may be due to the fact that organism has entered the stationary phase of growth and it has been reported that the antibiotic production by *Streptomyces* takes place in stationary phase of the growth (Gramajo *et. al.*, 1993). The decrease in the production of antibiotic after 72 hrs can be attributed to the decrease in the supply of nutrients to the microorganism or the accumulation of toxic byproducts.



Each value is a mean of three replicates. Y – error bars indicate the standard error from the mean.
Incubation temperature = 30°C; Initial pH = 7.2; Incubation period = 48 hrs.
Fig. 1. Screening of fermentation media for the production of antitumor antibiotic by *Streptomyces capoamus*

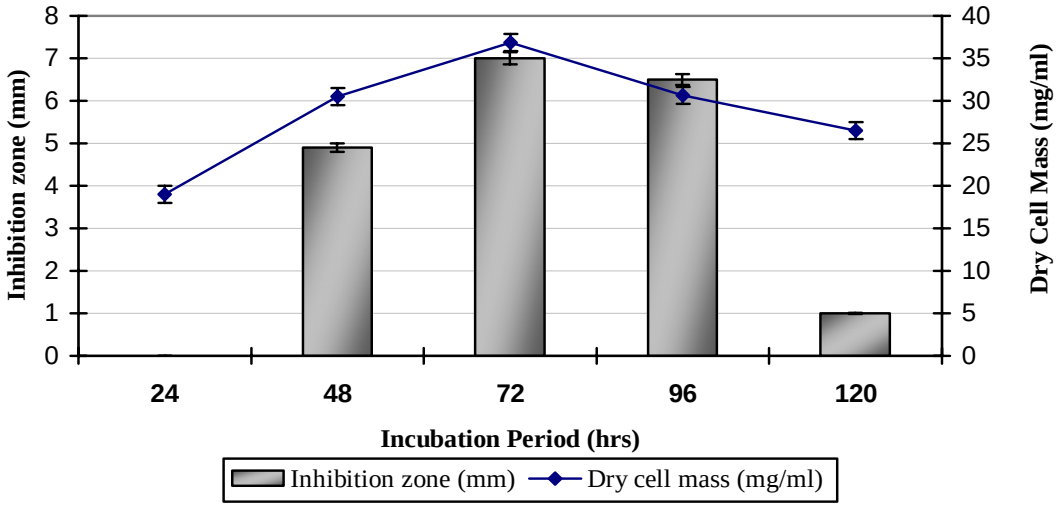


Each value is a mean of three replicates. Y – error bars indicates the standard error from the mean.
Initial pH = 7.2; Incubation period = 48 hrs; Fermentation medium = M3
Fig. 2. Effect of incubation temperature on the growth of *Streptomyces capoamus* and antitumor antibiotic production.

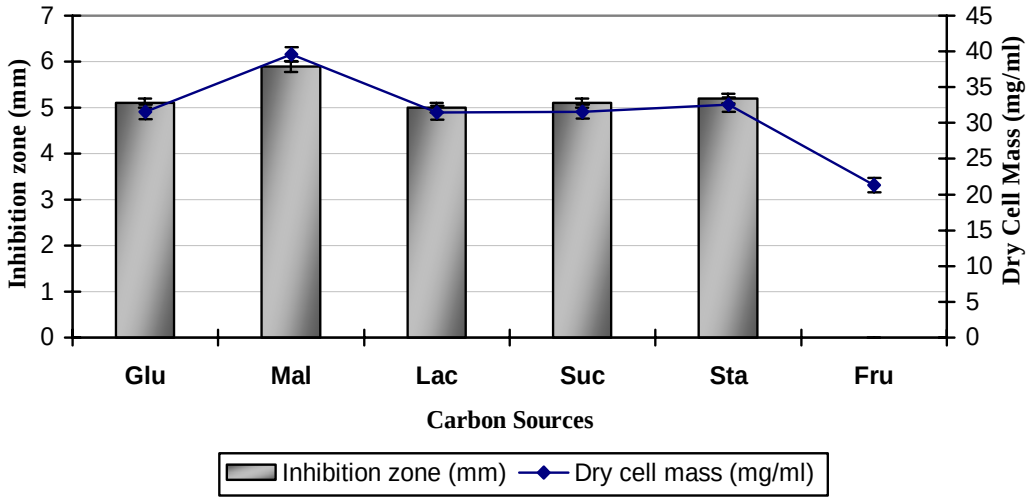


Each value is a mean of three replicates. Y – error bars indicates the standard error from the mean.
Incubation temperature = 30°C; Incubation period = 48 hrs; Fermentation medium = M3
Fig. 3. Effect of medium pH on the growth of *Streptomyces capoamus* and antitumor antibiotic production.

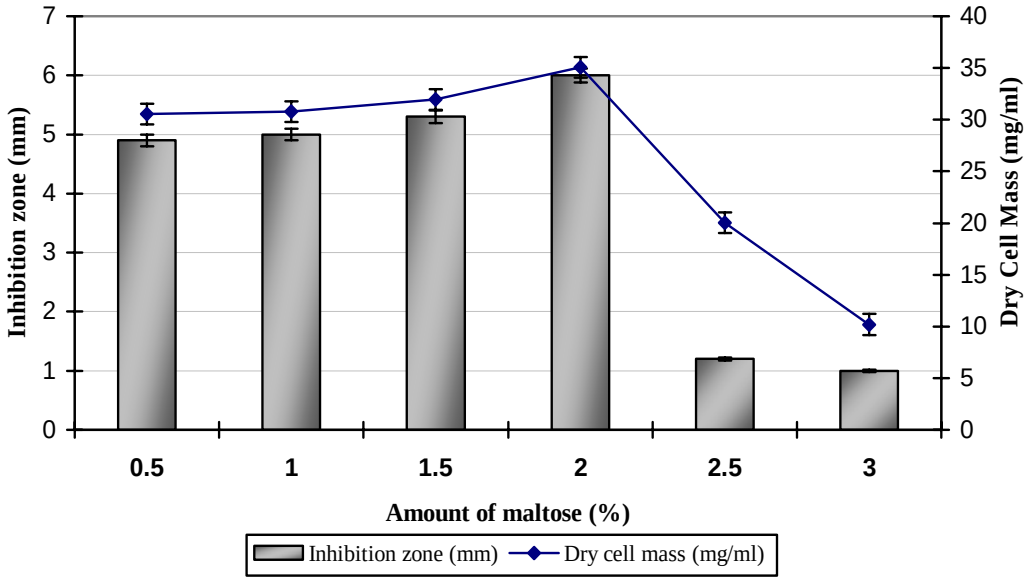
Effect of carbon source: The growth of microorganism and production of antibiotic was considerable in all the carbon sources tested except fructose. However, the best results were obtained with maltose (Fig. 5) at a level of 2% (w/v) (Fig. 6). The need for carbon sources varies from organism to organism. The amount of carbon source has also a marked effect on the metabolism of *Streptomyces capoamus.* Low yield of antibiotic in lower amounts of carbon source might be due to the fact that it could not fulfill the needs of organism to grow and produce maximum antibiotic. Dekleva *et. al.* (1985) have described that carbon and energy source which had been completely utilized during growth would prove unsatisfactory for subsequent antibiotic production; conversely, a compound which is only partly consumed during cell growth may be more suitable for antibiotic formation. In the present work, maltose has been found to be the most suitable source for the growth of *Streptomyces capoamus* and antibiotic production. Such results have also been found by Motkova *et. al.*, (1981). According to Dekleva *et al*., (1985), the carbon sources which support best growth and highest anthracycline titres include fructose, maltose and soluble starch but our organism did not show good results with fructose. Less amount of carbon could not fulfill the needs of organism to grow and then to produce maximum antibiotic, that is why yield of antibiotic in lower amounts of carbon source is low.



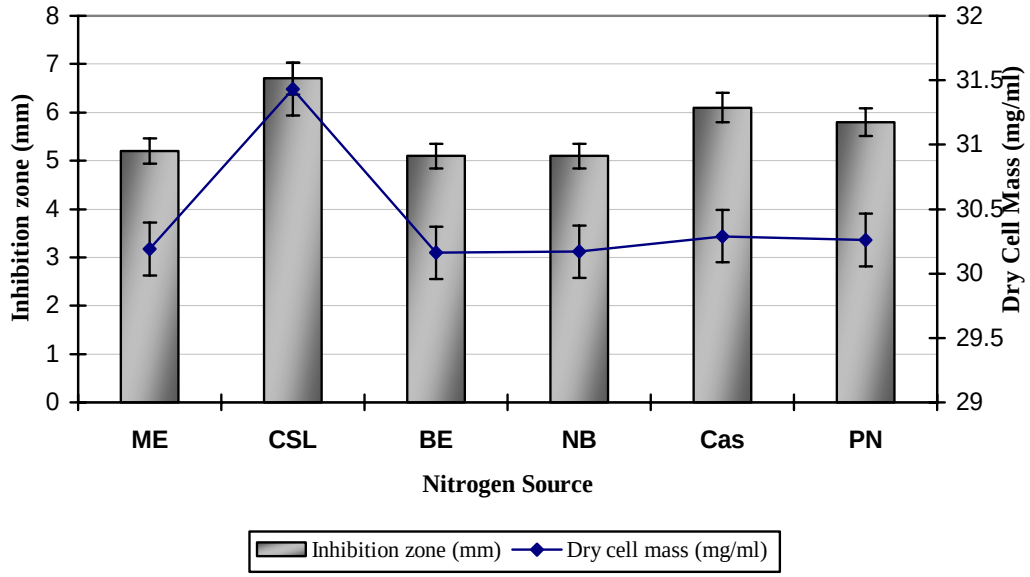
Each value is a mean of three replicates. Y – error bars indicates the standard error from the mean.
Incubation temperature = 30°C; Initial pH = 7.5; Fermentation medium = M3
Fig. 4. Effect of incubation period on the growth of *Streptomyces capoamus* and antitumor antibiotic production.



Each value is a mean of three replicates. Y – error bars indicates the standard error from the mean.
Incubation temperature = 30°C; Initial pH = 7.5; Incubation period = 72 hrs; Fermentation medium = M3
Abbreviations: Glu = Glucose; Mal = Maltose; Lac = Lactose; Suc = Sucrose; Sta = Starch; Fru = Fructose.
Fig. 5. Effect of type of carbon source on the growth of *Streptomyces capoamus* and antitumor antibiotic production.



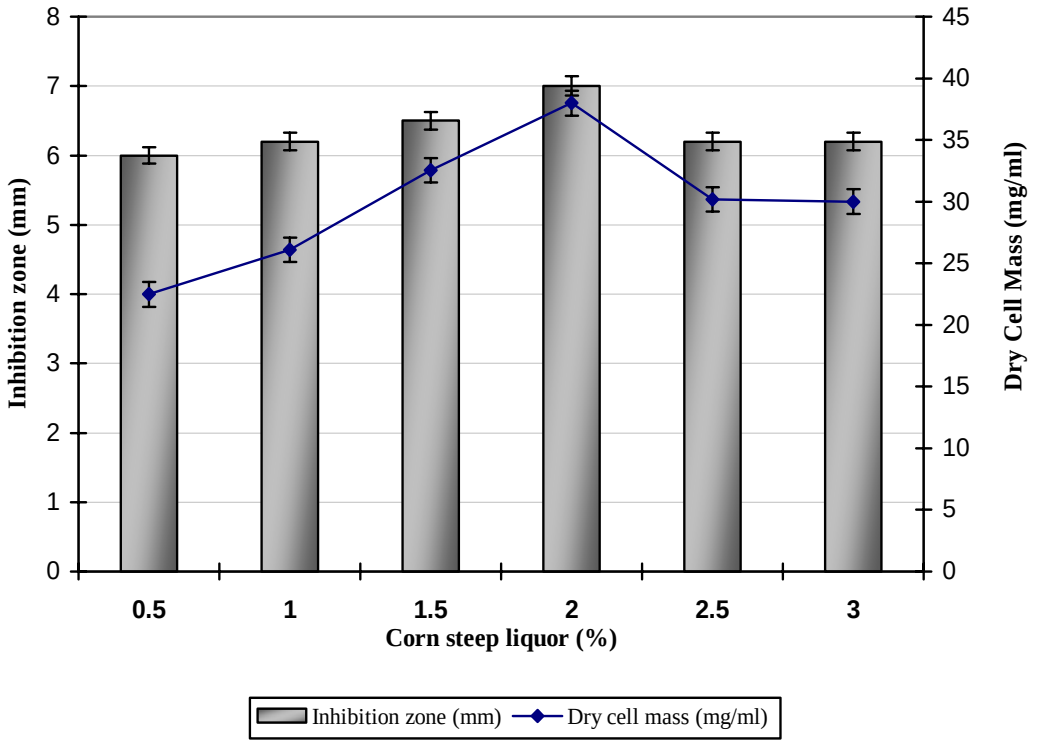
Each value is a mean of three replicates. Y – error bars indicates the standard error from the mean.
Incubation temperature = 30°C; Initial pH = 7.5; Incubation period = 72 hrs.
Fig. 6. Effect of amount of carbon source on the growth of *Streptomyces capoamus* and antitumor antibiotic production.



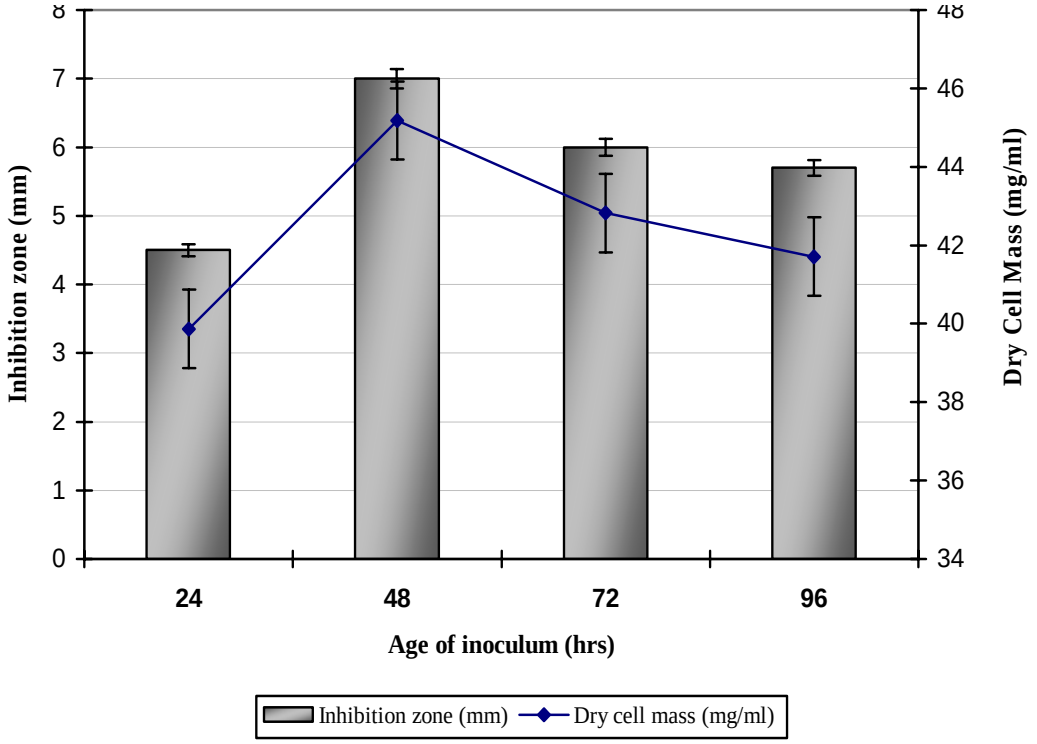
Each value is a mean of three replicates. Y – error bars indicate the standard error from the mean. Incubation temperature = 30°C; Initial pH = 7.5; Incubation period = 72 hrs; Fermentation medium = M3 + 2% Maltose
Abbreviations: ME = Mal Extract; CSL = Corn Steep Liauor; BE = Beef Extract; NB = Nutrient Broth; Cas = Casein; PN = Potassium Nitrate
Fig. 7. Effect of type of nitrogen source on the growth of *Streptomyces capoamus* and antitumor antibiotic production.

Effect of nitrogen source: The nitrogen source supplied to an organism has a marked influence on the quantitative nature of the antibiotic produced (Katz *et al.,* 1957; Theobald *et al.,* 2000). Figures 8 & 9 explain that growth of microorganism was high and production of antibiotic was maximum when corn steep liquor was used as nitrogen source at an amount of 2% (w/v). The same nitrogen source was optimized by Motkova *et al*., (1981) during production of tobramycin from *S. ceremeus var. tobramycini* and by Malina (1983) while producing cephamycin from *S. griseus.*

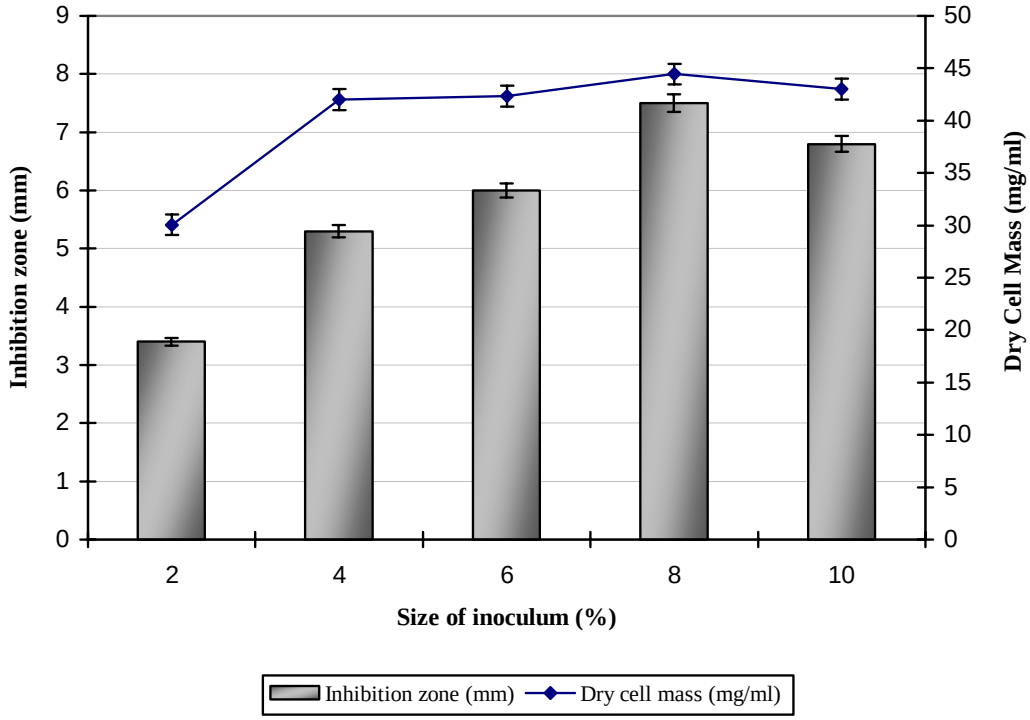
Effect of age and size of inoculums: It was found that 48 hrs old inoculum at a size of 8% (v/v) gave best antibiotic production (Figs. 9 & 10). The quantity and quality of inoculation material play a crucial role in the bioprocess results (Ettler, 1992). It has been found that 8% inoculum of the cells at stationary phase yielded the best growth and most consistent anthracycline production which is very close to the results reported by Dekleva *et. al.,* 1985. However, El-Enshasy *et. al.,* (2000) have reported that spore inoculum yielded higher concentration of antitumor antibiotic as compared to the vegetative cells.



Each value is a mean of three replicates. Y – error bars indicates the standard error from the mean.
Incubation temperature = 30°C; Initial pH = 7.5; Incubation period = 72 hrs; Fermentation medium = M3 + 2% Maltose
Fig. 8. Effect of amount of Corn steep liquor on the growth of *Streptomyces capoamus* and antitumor production.



Each value is a mean of three replicates. Y – error bars indicates the standard error from the mean.
Incubation temperature=30°C; Initial pH=7.5; Incubation period=72 hrs; Fermentation medium=M3 + 2% Maltose + 2% Corn Steep Liquor.
Fig. 9. Effect of age of inoculum on the growth of *Streptomyces capoamus* and antitumor antibiotic production.



Each value is a mean of three replicates. Y – error bars indicates the standard error from the mean.
Incubation temperature = 30°C; Initial pH = 7.5; Incubation period = 72 hrs.
Fermentation medium = M3 + 2% Maltose + 2% Corn Steep Liquor.
Fig. 10. Effect of the size of inoculum on the growth of Streptomyces capoamus and antitumor antibiotic production.

Conclusion

It is concluded by the present study that *Streptomyces capoamus* NRRL B3632 showed good titers of antitumor antibiotic production under optimized conditions during batch fermentation. The optimization of process substantially enhanced the antibiotic yield.

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(Received for publication 13 September 2009)