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Studies on the Physical Parameters for Cephalosporin Biosynthesis from *Acremonium chrysogenum* by Submerged Fermentation

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ABSTRACT

The present study was performed to optimize physical parameters for the maximum production of antibiotic (cephalosporin) by isolated fungal strain *Acremonium chrysogenum* (IIB-10). The strain IIB-10 was cultivated on medium containing (%, w/v): Baker's flour, 1.5; corn meal, 2; Methyl oleate, 1.6; calcium carbonate, 0.3 and ammonium sulphate, 0.1. The cultural conditions such as incubation temperature (30°C), pH (6.5) and inoculum size and age (2%, w/v, 48 h) were optimized. The maximum cephalosporin production was achieved after 144 h of incubation. The dry mycelial mass was found to be 39.46 mg/ml. The antimicrobial activity was assessed by disc diffusion method. Cephalosporin production was estimated by using spectrophotometric technique.

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Authors' Contribution
UFG AN and cunducted the research and wrote the manuscript. HM and IUH supervised the research. AM analysed the data.

Key words
Cephalosporin, *Acremonium chrysogenum*, Submerged fermentation, Antibiotic, Disc diffusion method.

INTRODUCTION

Antibiotics are low molecular weight microbial metabolites and their derivatives; this at low concentration can kill or retard the development of most of the microorganisms. An ideal antibiotic exhibit selective toxicity *i.e.,* the drug is harmful to the parasite without causing any harmful effect to the host. In the remote past meat, bread and moldy cheese were used in the traditional medicine to cure the injuries and infectious diseases (Demain, 2005). More than 25,000 antimicrobial compounds have been separated from microorganisms. In 1945 Brotzu isolated *Cephalosporium acremonium* from sea water. Later, Abraham *et al*. (1955-56) had isolated cephalosporin C, cephalosporin P and penicillin N from culture of *C. acremonium* (Bhat *et al*., 2005).

Cephalosporins cause the breakage of cell wall of bacteria by interacting synthesis of peptidoglycan layer of bacterial cell wall. Cephalosporins are most frequently used in the treatment of infections caused by bacteria *i.e.* infection of respiratory tract, infections of skin and infections of urinary tract (Dasari *et al*., 2008). Cephalosporins generally cause few side effects. The patients, who are allergic to penicillin, may be allergic to cephalosporin. About 5 to 10% incidence of cross sensitivity is observed.

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Some strains of *Cephalosporium* species such as *Emericellopsis* species, *Acremonium chrysogenum*; *Paecilomyces* species like *Paecilomyces carnius* and *Streptomycetes* species *i.e. Streptomycetes sclavuligerus* are frequently used in the fermentation to produce the starting material for the synthesis of cephalosporin (Higgins and Hamill, 1974). *A. chrysogenum* mostly occurs in soil organic matter and plant debris. *A. chrysogenum* mostly form white loose cottony hyphae producing white and gray colonies and over grows in moist environment at 25°C-28°C.

A. chrysogenum shows four different morphological forms *i.e.,* conidia, hyphae, wide swollen hyphal fragments named yeast like forms and metabolically inactive arthrospores during fermentation (Bartoshevic *et al*., 1990). The wild strain of *A. chrysogenum* predominantly formedconidia. While the strains which tend to differentiate into yeast like forms *e.g.* swollen hyphal fragments show high and medium productivity of cephalosporin (Bartoshevic *et al*., 1990). The highest amount of cephalosporin produces when hyphal filaments convert into swollen hyphal fragments (Huber and Nash, 1971). Yeast like morphological form of the fungus has high tendency to produce cephalosporin as compared to any other morphological form (Bartoshevic *et al*., 1990).

The cephalosporin production is carried out by submerged fermentation and solid state fermentation (Kim *et al*., 2007; Cuadra *et al*., 2008). Many workers have reported submerged fermentation in shake flask for the production of cephalosporin (Cruz *et al*., 2004; Kim *et al*.,

2007; Srivastava *et al*., 2007). The maximum productivity of cephalosporin has been obtained by pH control, temperature control, incubation period and inoculum (Cuadra *et al*., 2008).

The incubation period markedly effect the production of cephalosporin by *A. chrysogenum* as it results into the differentiation of fungal mycelium into different morphological forms which then affect the biosynthesis of cephalosporin. The most suitable morphological form for the production of cephalosporin is the swollen hyphal fragments which are formed usually after 5-6 days. So the most suitable time for the production of the cephalosporin is 5-6 days (Nigam *et al*., 2007). After that there is decline in the production of the cephalosporin due to the conversion of swollen hyphal fragment into conidia. The conidia are not associated with cephalosporin production.

Among the external factors, which influence the sporulation and growth of the fungi, temperature plays an important role. It affects almost every function of the organism. So the incubation temperature also has an important effect on the development of *A. chrysogenum* and synthesis of antibiotic. The optimum temperature for growth and antibiotic production by *A. chrysogenum* lies in the range of 25-30°C. An optimum temperature also favors the formation of the swollen hyphal fragments in it which is an important morphological form concerned with the production of cephalosporin (Nigam *et al*., 2007). In general fungi are capable of growing within a wide range of hydrogen ion concentration of the culture medium however; most of the fungi grow best in neutral or acidic medium. The initial pH value of the medium can affect the growth and sporulation of the fungus (Madan and Thind, 1998). The most suitable initial pH value for the production of the cephalosporin is 6.0 to 7.2 (Kuenzi, 1980; Nigam *et al*., 2007). The pH may also affect the stability of different medium constituents in the culture broth (Kuenzi, 1980) so making them unavailable to fungus.

MATERIALS AND METHODS

Fungal strain

The fungal strain IIB-10 (*A. chrysogenum*) used in the study was obtained from Institute of Industrial Biotechnology, GC University, Lahore.

Fermentation experiments

Preparation of inoculum

Vegetative inoculum was used in the present study which was prepared in 250ml conical flask containing 50ml of vegetative medium. The vegetative medium was consisted of (g/l); Peptone, 20; malt extract, 20; corn steep liquor, 5; magnesium sulphate, 0.25; di potassium hydrogen phosphate, 0.5; potassium dihydrogen phosphate, 1.0 and calcium chloride, 0.1 (pH 6.5). The flask was inoculated with fungal spores from the slant and incubated at 28ºC for 4 days in incubator shaker rotating at a speed of 200 rpm. One ml of this inoculum was used to inoculate the fermentation flasks.

Fermentation batch

Submerged fermentation was carried out in 250ml conical flasks containing 50ml culture medium for the production of cephalosporin from selected fungal cultures. The culture medium was consisted of (%, w/v): Baker's flour, 1.5; corn meal, 2; methyl oleate, 1.6; calcium carbonate, 0.3 and ammonium sulphate, 0.1. One ml of the vegetative inoculum was transferred to each sterilized flask and the fermentation was carried out in an incubator shaker at 28ºC at 200 rpm for 7 days. After that, the fermentation broth was centrifuged at 6000 rpm for 10 min and the antibacterial activity of the cell free culture supernatant was determined.

Analytical procedures

Antimicrobial assay

Agar plate disc diffusion method (Masuda and Tomioka, 1978) was used to test the antimicrobial activity of the fermentation broth. A disc of blotting paper (5mm diameter) was dipped in the clear supernatant and placed in the test plate. The plates were incubated at 37ºC for 24 h. A clear zone of inhibition of bacterial growth was set as a criterion to assess the antibiotic titer of cell free culture supernatant.

Assay of cephalosporin

Spectrophtometeric method, using hydroxyl amine nickel reagent and iron III reagent, was used for the assay of cephalosporin (Mays *et al*., 1975). The absorbance was taken at 470 nm.

Dry mycelial mass

The fermentation broth was filtered using preweighed filter paper (Whatman-44) to obtain the mycelial mass on the filter paper. The cell mass of the fungus was dried in a hot air oven at 80°C for 24 h and weighed again. The dry mass was calculated by subtracting preweight from after weight.

Optimization of physical parameters

Physical parameters of fermentation were optimized for the production of cephalosporin by *Acremonium chrysogenum* using submerged fermentation process.

The effect of the incubation time on the cephalosporin C production by *Acremonium chrysogenum* was

determined by carrying out fermentation experiments for different time intervals ranging from 4-8 days. After specific period of incubation, antimicrobial activity was determined by agar plate disc diffusion method.

The temperature effect on the fermentation process was determined by incubating the fermentation flasks at temperatures as 26ºC, 28ºC, 30ºC, 32ºC, 34ºC, 36ºC, 38ºC and 40ºC in incubator shaker at 200 rpm for 7 days. The increase or decrease in quantity of antibiotic production was determined by bioassay of culture broth.

The effect of initial pH of the culture medium on CPC production by *A. chrysogenum* was determined by preparing fermentation medium with different pH values ranging from 5.5 to 8.5. These media were inoculated with the microorganism and kept in incubator shaker at 28ºC for 7 days. After that the fermentation broth was checked for antimicrobial activity by agar plate disc diffusion method. The effect of the size and age of the inoculum was determined by changing the size (1%-5%) and age (1-4 days) of the inoculum to inoculate the fermentation flasks and the activity was determined by agar plate disc diffusion method.

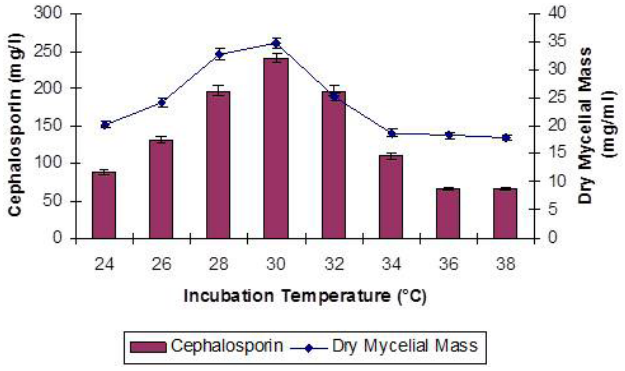

Fig. 1. Optimization of the incubation temperature for the production of cephalosporin by *Acremonium chrysogenum* in shakes flasks. All the values are means of three parallel replicates. Y-error bars indicate the standard error from mean. Fermentation conditions: incubation period= 144 h.

RESULTS

The incubation temperature for the production of cephalosporin by *Acremonium chrysogenum* was optimized by incubating the fermentation flasks at different temperatures ranging from 24-38°C (Fig. 1). As the temperature was increased, the amount of cephalosporin was also increased and the maximum amount of cephalosporin (240.41 mg/l) was produced at an incubation temperature of 30°C. On further increasing the temperature, the production of cephalosporin was decreased to its minimum (65.59 mg/l) at an incubation temperature of 38°C. The fungal growth was also increased from low temperature towards the maximum at temperature of 30°C. Later on there was a sharp decline in the growth of the fungus from maximum value of 34.80 mg/ml to the minimum value of 17.80 mg/ml of the culture as the temperature was increased from 30 to 38°C. Incubation period of the fermentation was optimized by carrying out fermentation experiments for different time intervals ranging from 24 to 192 h and the production of cephalosporin was recorded (Fig. 2).

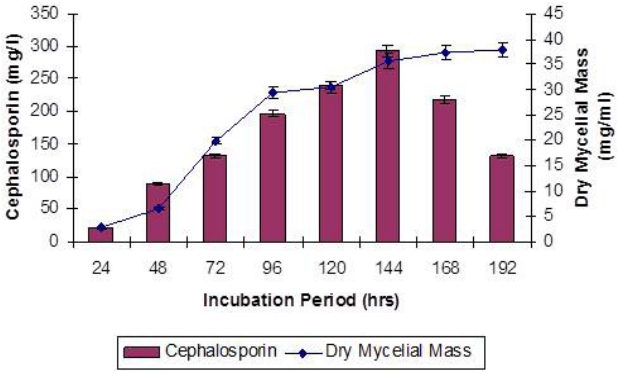

Fig. 2. Optimization of incubation period for the production of cephalosporin by *Acremonium chrysogenum* in shake flasks. All the values are means of three parallel replicates. Y- error bars indicate the standard error from mean. Fermentation conditions: incubation temperature = 30°C, initial pH = 6.6.

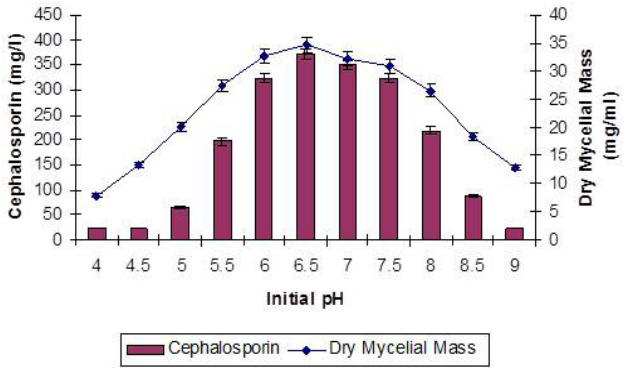

Fig. 3. Effect of the initial pH of culture medium on the production of Cephalosporin by *Acremonium chrysogenum* in shake flasks. All the values are means of three parallel replicates. Y-error bars indicate the standard error from mean. Fermentation conditions: incubation period=144 h; incubation temperature = 30°C.

The results showed that the maximum amount of cephalosporin (292.79 mg/l) was produced after 144 h

(6 days) of incubation. As the fermentation started, the production rate of cephalosporin was gradually increased till the maximum amount of cephalosporin (292.79 mg/l) was produced after 144 h of incubation. Further increasing the time interval resulted in the decreased production of cephalosporin. The biomass was minimum in the beginning (2.70 mg/ml) but as the fermentation proceeded, the production of biomass was also increased and gradually reached maximum (37.90 mg/ml) after 192 h (8 days). Therefore, an incubation period of 144 h was optimized for the production of cephalosporin by *A. chrysogenum.* It is also evident from the results that maximum production of cephalosporin did not correspond to the maximum growth of the fungus, *i.e.,* the growth was continuously increasing while the cephalosporin production was started to decreased after 144 h of incubation.

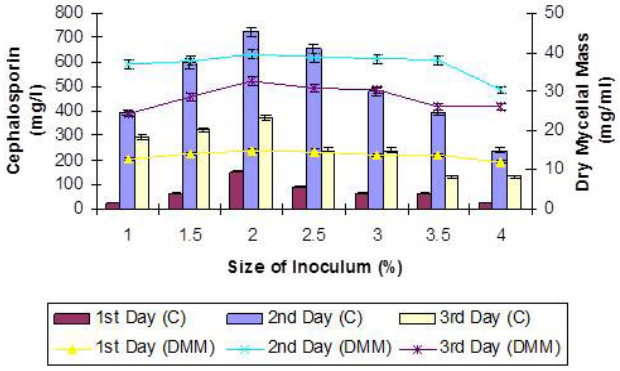

Fig. 4. Effect of inoculum of various sizes and ages on the production of cephalosporin by *Acremonium chrysogenum* in shake flasks. All the values are means of three parallel replicates. Y- error bars indicate the standard error from mean. Fermentation conditions: Incubation period = 144 h; incubation temperature = 30 °C; pH = 6.5. C, amount of cephalosporin; DMM, dry mycelial mass.

Figure 3 shows the effect of initial pH of the culture medium (4.0-9.0) on the biosynthesis of cephalosporin by *A. chrysogenum.* The most favorable pH, at which maximum amount of cephalosporin (372.06 mg/l) was observed, was 6.5. Above and below the pH value of 6.5, cephalosporin production was decreased with a rapid decline above pH 7.5. The maximum biomass (34.70 mg/ml) was also observed at pH 6.5. However, at different pH values, fungal growth did not match the antibiotic production as reasonable growth of *A. chrysogenum* was found at all the pH values ranging from 4-9. So, the initial pH of 6.5 was found to be the most suitable for maximum production of cephalosporin by *A. chrysogenum.* The experiments were performed to determine the effect of age and size of inoculum on the biosynthesis of cephalosporin by *A. chrysogenum* in shake flasks fermentation. Figure 4 shows the effect of inoculum of various ages (1-3 days) and various sizes (1-4%) on production of cephalosporin. The maximum cephalosporin (721.89 mg/l) was produced when fermentation flasks were inoculated with 48 h old inoculum at level of 2% (v/v %). While minimum amount of cephalosporin (21.89 mg/l) was produced by the 1% inoculum of 24 h age. The highest amount of biomass (39.46 mg/ml) was also produced by the 2% inoculum of the age of 48 h.

DISCUSSION

The incubation temperature can affect the morphological differentiation of the fungus during the course of fermentation. During growth, *A. chrysogenum* shows four different morphological forms, of which the swollen hyphal fragment is the most favorable morphological form, which is responsible for the production of cephalosporin (Huber and Nash, 1971; Nigam *et al*., 2007). The temperature of 27-30°C had been reported most favorable incubation temperature for the formation of swollen hyphal fragments (Nigam *et al*., 2007; Sabbagh *et al*., 2007). The other morphological forms are filamentous hyphae, arthrospores and conidia which show lesser production of cephalosporin. High temperature can also denature the fungal enzymes thus retard the growth of the fungus which results in the lesser biosynthesis of secondary metabolites. Incubation period is the one of the most important factors which determines the metabolic pathway for the biosynthesis of secondary metabolites by microorganisms during the course of fermentation. *A. chrysogenum* shows various morphological changes during growth and fermentation (Huber and Nash, 1971). The swollen hyphal fragments are the most important morphological forms suitable for the biosynthesis of cephalosporin (Huber and Nash, 1971). The maximum number of swollen hyphal fragments is usually achieved during 120-144 h of incubation (Lim *et al*., 2002; Nigam *et al*., 2007). On further increasing the incubation period, swollen hyphal fragments are converted into conidia which are not involved in the synthesis of the cephalosporin. So, after 144 h (6 days), the production rate of cephalosporin would also be decreased (Huber and Nash, 1971). In the present study, the maximum amount of cephalosporin was produced after 144 h of fermentation due to formation of maximum number of swollen hyphal fragments. The initial pH value of the fermentation media has an important bearing on the production of cephalosporin. A specific pH value is required for the production of antibiotics which can be affected by variation in the pH value (Kysilka, 1993). Probably the pH of the culture medium might

favor the conversion of filamentous hyphae to the swollen hyphal fragments which in turn affect the antibiotics production by *A. chrysogenum* (Huber and Nash, 1971). The production of the cephalosporin has been studied by different workers using fermentation medium having initial pH of 6.5-7.2 (Kuenzi, 1980; Nigam *et al*., 2007) which is also supporting our findings. The pH may also help to maintain the media constituents in dissolved form thus affecting nutrient availability (Kuenzi, 1980). The size and age of the inoculum is very important parameter for the growth of the microorganism and production of secondary metabolites. The physiological conditions of the inoculum when it is transferred to the production stage have a major effect on the performance of the fermentation for the production of secondary metabolites (Hockenhull, 1980). A lag period is required for the significant growth of the fungus. The pattern of growth and morphogenesis of this growing mycelium is very important for the production of secondary metabolites during fermentation (Hunt and Stieber, 1986). The vegetative inoculum at a level of 2-8% is normally used for the better growth of the fungus in the fermentation medium (Hunt and Stieber, 1986). An optimized size of the inoculum is used to reduce the lag phase and maximize the production rate. The fungus *A. chrysogenum* in the seed culture shows different morphological characteristics on changing the age of the culture (Lee *et al*., 2001). 16 have also used 2% inoculum of 48 h for the highest production of cephalosporin. So the present study has a good agreement with pervious findings.

CONCLUSION

In the present study the isolate IIB-10 identified as *A. chrysogenum* had shown significant amount of antibiotic (cephalosporin) production, which was active against gram positive and gram negative bacteria. A 2.83 fold enhancement in the production of cephalosporin was observed after optimizing the cultural conditions such as temperature, medium pH and incubation period. During the course of study, a moderate amount 721.89 mg/l of the cephalosporin production was observed.

Statement of conflict of interest

The authors declare no conflict of interest.

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