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Biosynthesis of Ergot Alkaloids by *Penicillium citrinum* through Surface Culture Fermentation Process

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Abstract.- The present study deals with the production of ergot alkaloids from *Penicillium* sp. IIB and the optimization of culture conditions for enhanced production of ergot alkaloids. The strain of *Penicillium citrinum* was proved to be considerably useful for biotechnological purposes. The effect of temperature, pH, tryptophan, asparagine, different supplementary nitrogen and carbon sources and KH_2PO_4 on ergot alkaloids production was optimized during the study. Ergot alkaloids were produced between 23°C and 30°C incubation temperatures, with maximum yield (2.01 g/l) at 25°C. Maximum ergot alkaloid production was achieved at pH 5 (2 mg/l) with maximum incubation period of 21 days.

Key words: Ergot alkaloids, culture conditions, *Penicillium* species.

INTRODUCTION

Ergot alkaloids are indole metabolic compounds with specific biological activities (Schardl *et al*., 2006). Human recognition of ergot alkaloids is as old as the civilization. The compounds have been widely used as drugs and in medicines to cure different human diseases. Their ecological role is weakly understood but they have been involved in activities against mammals, nematodes, insects and bacteria (Ball *et al.,* 1997). Many genera have been involved in the biosynthesis of ergot alkaloids *e.g., Claviceps, Balansia, Penicillium,* and *Aspergillus.* There are many species of genus *Penicillium* who have been involved in the production of ergot alkaloids *e.g. P. sizovae, P. roquefortii, P. corylophilum* and *P. chrysogenum. Aspergillus* species are also playing a significant role in the synthesis of these metabolites *e.g., A. fumigatus, A. tamari* and *A. flavus* (Rao *et al.,* 1997; Fleiger *et al.,* 1997; Moussa, 2003). Ergot alkaloids have a wide range of their spectrum of applicability and because of that, this research study was an effort to identify a strain that has the ability to synthesize ergot alkaloids *i.e.*, ergotamine and ergocriptine, through surface culture fermentation technique and to optimize such culture parameters through which one can enhance the yield of ergot alkaloids in the fermentation medium.

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MATERIALS AND METHODS

Fungal strain

Penicillium citrinum was obtained from Institute of Industrial Biotechnology, GC University, Lahore and cultured on 4g MEA medium (2g malt extract and 2g agar) and incubated at 25°C for 15 days.

Preparation of the culture medium

Fungal strain was grown in one litter of fermentation medium containing 5 g sucrose, 0.2 g NH_4Cl, 0.5g yeast extract, 0.5 g succinic acid, 0.5 g asparagines, 0.5 g tryptophan, 0.5 g KH_2PO_4, 0.03 g $MgSO_4$. $7H_2O$, 0.02 g $ZnSO_4$ and 0.01 g $FeSO_4$. pH of the medium was adjusted to 5.2 with the help of 0.1N HCl and ammonia solution and after inoculation all the media was incubated at 25°C for 21 days. Surface culture fermentation technique was applied to determine the synthesis of ergot alkaloids in the fermentation medium.

Determination of ergot alkaloids concentration

A known quantity of fermented broth was centrifuged at 5000 rpm for 10 min and mycelia of all the samples were collected separately in Petri plates. The initial weights of all the mycelia were also noted. The extracts were purified through rotary evaporator.

Alkaloids assay

One ml of each of the culture filtrates was added to 2 ml of Van Urk Reagent in the test tubes and reaction mixture was incubated at 37°C for 30 min (Van Urk, 1929; Smith, 1930). The absorbance was measured at 590 nm by spectrophotometer. The amount of alkaloids produced was measured and compared with dihydroergotamine methane sulfonate standard salt.

Quantitative analysis of ergot alkaloids from mycelium

The mycelia that were separated from the fermentation media of all the fungal cultures were initially weighed and placed in oven at 45°C for 24 h for drying. After 24 h they were weighed again to measure the dry weights of the mycelium. These dry mycelia placed in methanol for 24 h and subjected to cell lysis by sonication process each for 5 cycles of 3 min for all mycelia at 200 rpm/min in an ultrasonic generator. After sonication, cell lysis was done in a homogenizer for 3 cycles of 5 min so that all of the compounds of ergot alkaloids may release from the mycelia of fungi (Linde, 2005). The mixture after homogenization was centrifuged and supernatants were collected. Chloroform from the supernatant was evaporated and the mycelial extracts were measured and assayed with Van Urk Reagent as mentioned above and absorbance was measured by spectrophotometer at 590 nm. Total alkaloids were measured with the help of a standard curve of dihydroergotamine methane sulfonate salt.

Qualitative analysis of ergot alkaloids

Chloroform extraction process was used for the extraction of ergot alkaloids produced by said fungal species. Ergot alkaloids were extracted 3 times in 50 ml of chloroform in a separating funnel. The chloroform extracts were evaporated to dryness at 40^{o}C in a rotary evaporator. The residues were then analyzed by thin-layer chromatography on silica gel strips using the mobile phase of chloroform: methanol:ammonia solution in 80:20: 0.5 ml of proportion. Pure samples of methylergotamine maleate and dihydroergotamine methane sulfonate salt were run as the reference salts to identify the possible ergot alkaloids spectrum. Colored spots were developed on the TLC plates by spraying Van Urk Reagent after the procedures of Stahl (1969). The alkaloid contents of the mycelia were also determined by the same procedure.

Optimization of culture conditions for ergot alkaloids production

Effect of pH and temperature

The ergot alkaloids concentration was determined over a pH range from 3.0 to 8.0 and temperature range from 23°C to 30°C.

Effect of tryptophan and asparagine

The alkaloids production was demonstrated with various concentrations of tryptophan and asparagines *i.e.*, 0.5, 1, 1.5, 2, 2.5 and 3 g in the fermentation media with pH 5.0, 25°C and 21 days of incubation time period.

Effect of supplementary carbon and nitrogen sources and KH_2PO_4

Ergot alkaloids synthesis was also optimized by adding 5g of various supplementary carbon and nitrogen sources *i.e.*, glucose, fructose, maltose, sucrose and mannose yeast extract, peptones, malt extract, meat extract, ammonium chloride and urea in the fermentation medium. Ergot alkaloids production was also determined by adding various concentrations of KH_2PO_4 *i.e.*, 0.5, 1, 1.5, 2, 2.5 and 3 g. pH was maintained at 5.0 and flasks were incubated at 25°C with incubation time of 21 days.

RESULTS AND DISCUSSION

Many species of genus *Penicillium e.g. Penicillim oxalicum, Penicillium italicum, Penicillium digitatum* and *Penicillium citrinum* were obtained from Fungal Culture Bank, University of the Punjab, Lahore and Institute of Industrial Biotechnology, GC University, Lahore. All of them were screened for the best producer of ergot alkaloids and *P. citrinum* strain was determined as the best producer of ergot alkaloids with ergot alkaloid concentration 1.15 mg/ml in the fermentation medium as referred to in Table I.

Effect of pH and temperature on ergot production

Ergot alkaloids concentration from culture

Table I.- Ergot alkaloid production by various *Penicillium* species.

Fungal organism	Fermentation media (mg/ml)									
	M1		M2		M3		M4		M5	
	SAC	MAC	SAC	MAC	SAC	MAC	SAC	MAC	SAC	MAC
P. italicum	0.001	0.002	0.075	0.013	0.143	0.095	0.438	0.095	0.495	0.135
P. oxalicum	0.002	0.001	0.090	0.036	0.251	0.088	0.054	0.101	0.099	0.001
P. digitatum	0.010	0.010	0.095	0.054	0.956	0.087	0.095	0.143	0.143	0.095
P. citrinum	0.014	0.061	0.076	0.086	0.956	0.760	0.981	0.651	1.15	0.64

SAC, supernatant alkaloids concentration; MAC, mycelial alkaloids concentration.

was determined over a pH range from 3.0 to 8.0 and temperature range from 23°C to 30°C. Maximum alkaloid production measured was at pH 5.0 (2 mg/l) and at 25°C (2.01 mg/l) (Fig. 1).

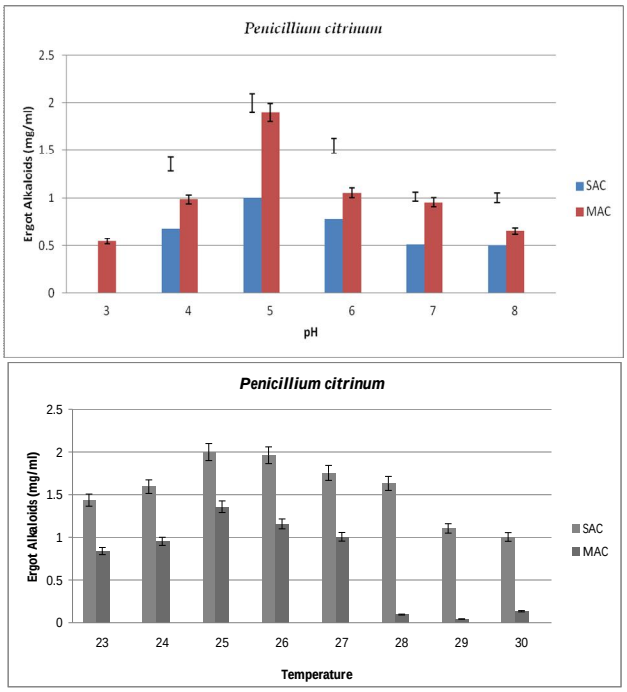

Fig. 1. Effect of pH (top) and different temperatures (bottom) on the production of ergot alkaloids (Where SAC is supernatant alkaloids concentration and MAC is the mycelial alkaloids concentration)

Mizrahi and Miller (1970) and Moussa (2003) also determined various pH levels ranged from 2.0 to 8.0 and obtained the best results at pH 5.5. Incubation temperature is also a significant factor in alkaloids synthesis and it was analyzed that 25°C incubation temperature was most suitable for the optimum production of ergot alkaloids. Similar experiments had been performed by Socic and Garberc-Porker (1992), they described that 24°C and 27°C temperatures supported maximum ergot alkaloid production.

Effect of tryptophan and asparagine on ergot production

Supernatant extracts from the same culture showed maximum alkaloids production when tryptophan and asparagines were added in the fermentation medium. Best alkaloid concentration was measured at 2g/l concentration of tryptophan and 2g/l of asparagine (2.31 mg/l and 1.96 mg/l) respectively (Fig. 2).

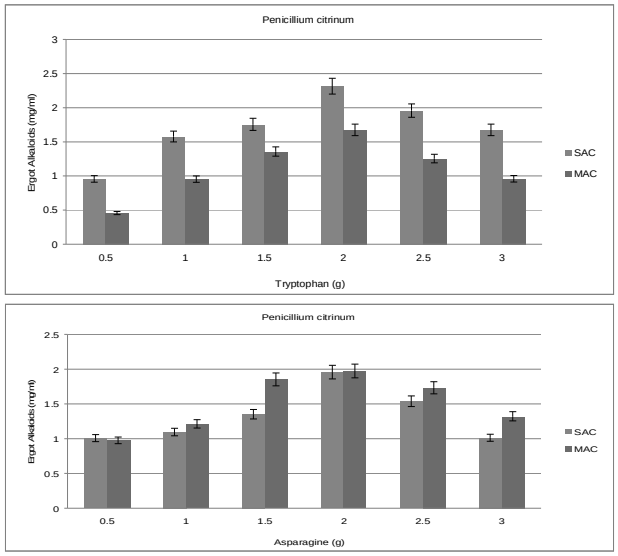

Fig. 2. Effect of tryptophane (top) and asparagine (bottom) on the production of ergot alkaloids (Where SAC is supernatant alkaloids concentration and MAC is the mycelial alkaloids concentration).

Similar studies on the effect of tryptophan have been reported by Christiane and Shu-Ming (2011) in their review in which ergoline moiety have been derived from the tryptophan and DMAPP. Similar experiments had been described by Narayan and Koteswara (1982) that the double carbohydrates along with ammonium succinate had the major effect on the production of ergot alkaloids. Inorganic phosphate in high concentration can inhibit the growth of mycelia and the production of ergot alkaloids (Narayan and Koteswara, 1982). In present research supplementation of KH_2PO_4, 2g/l culture medium supported the maximum yield of ergot alkaloid (Fig. 3). With the further increase in the level of inorganic phosphate there was a decrease in the ergot alkaloid production. Moussa (2003) and Shahid *et al.* (2008) also reported in their studies that inorganic phosphates in the culture medium can be involved in the various cellular metabolic activities e.g. the storage of energies and in the formation of phospho-nucleotide, that's why their higher concentrations of inorganic phosphates can inhibit the ergot alkaloids production.

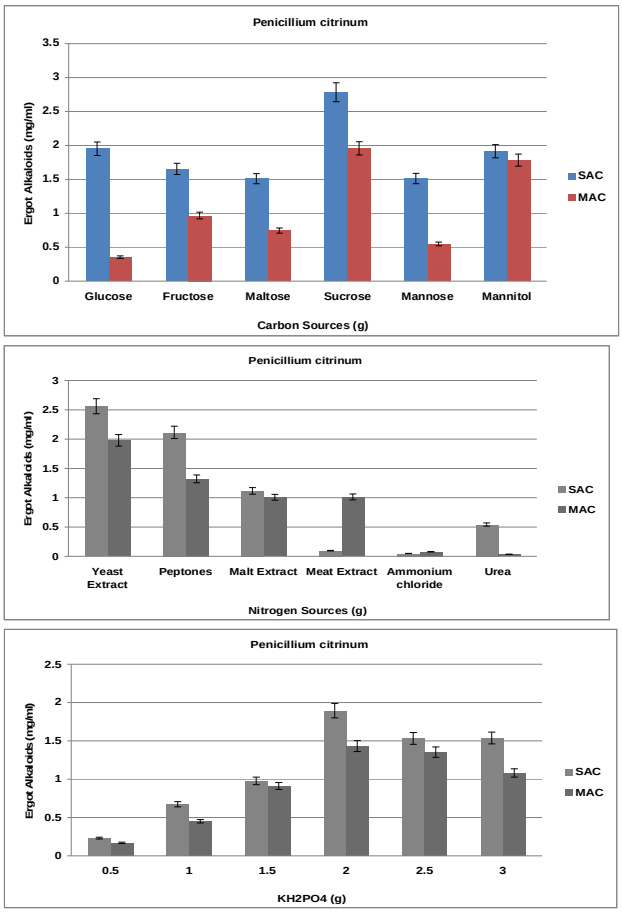

Fig. 3. Effect of various carbon sources (top), nitrogen sources (mill) and nitrogen sources concentrations of KH_2PO_4 (bottom) on the production of ergot alkaloids (Where SAC is supernatant alkaloids concentration and MAC is the mycelia alkaloids concentration).

Penicillium sp. IIB can prove a significant ergot alkaloid producer through surface culture fermentation process. Biosynthesis of ergot alkaloids through such a process is cost effective.

Effect of supplementary carbon and nitrogen sources and KH_2PO_4 on ergot production

Ergot alkaloid yield was obtained at maximum level with 5g/l of sucrose in fermentation medium (2.79 mg/l). Sucrose was determined as the best carbon source for the enhanced production of ergot alkaloids. Yeast extract proved to be the best nitrogen source with ergot alkaloid production of 2.86 mg/l. KH_2PO_4 proved as a significant inorganic phosphate agent for the growth of mycelia and ergot alkaloids yield and maximum alkaloid yield was obtained at 2g/l of KH_2PO_4 (1.90 mg/l) as shown in Figure 3.

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