Screening of *Penicillium* Species and Optimization of Culture Conditions for the Production of Ergot Alkaloids Using Surface Culture Fermentation Process

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Abstract:

The present study deals with the screening of best ergot alkaloids producer for the enhanced production of ergot alkaloids. These were grown on different fermentation media by using surface culture fermentation process. Best production of ergot alkaloids was obtained by *Penicillium commune* when it was grown on M5 fermentation medium (**1.3587mg/ml**). Different culture conditions were also optimized e.g. effect of different Carbon Source (**Sucrose: 2.5811mg/ml**), substrate concentrations (**30g Sucrose: 2.6785mg/ml**), various Nitrogen Sources (**Yeast Extract: 2.8513mg/ml**), different concentrations of yeast extract (**30g Yeast Extract: 2.3510mg/ml**), various concentrations of Inorganic Phosphate (**2g KH_2PO_4: 1.5731mg/ml**), various pH levels (**pH 5: 2.1385mg/ml**) and different inoculums sizes (**15ml spore suspension: 3.3478mg/ml**) were found to be the best for the biosynthesis of ergot alkaloids. Ergot Alkaloids presence was also confirmed by Colorimeteric and Thin Layer Chromatography techniques.

Key words: Ergot Alkaloids, *Penicillium commune*, Culture conditions

Introduction:

The toxicity of ergot alkaloids make them pharmacologically very useful. All of the classes of ergot alkaloids have been used in making different medicines to cure migraine and to reduce post partum bleedings **(Moussa, 2003).** Many of the ergot alkaloids were being used in the induction of labour contractions and for the inhibition of lactation. It can also terminate pregnancy and inhibit mammary tumors **(Floss *et al.*, 1973; Masurker, 1992; Fleiger *et al.*, 1997)**.

Previously, only the members of family *Clavicipitaceae* (Genus: *Claviceps*) were used in the synthesis of Ergot Alkaloids. Other than Genus *Claviceps*, Genus *Balansia* was also used for the biosynthesis of these Alkaloids. Researchers are now focusing on the other members of class Ascomycetes e.g. species of Genus *Penicillium* (*P. aurantiovirens, P. chermisinum, P. ciricavirde, P. sizovae, P. roquefortii, P. corylophyllum, P. kapuscinkii, P. regulosum and P. concavoruglosum*) **(Kozlovsky *et al.*, 1982; Hong and Robbers, 1985)** and some species of *Aspergillus* such as: *A. fumigates* and *A. flavus* have also been reported in literature **(Rao *et al.*, 1977; Flieger *et al.*, 1997).**

A wide range of ergot alkaloids are being used in pharmaceuticals which make research on these species very interesting. The present study focused on the screening for the best fungal strain and the optimization of fermentation conditions for the enhanced production of ergot alkaloids using surface culture fermentation process.

Materials and Methods:

Fungal Species

Different species of Genus *Penicillium* i.e. *Penicillium commune, Penicillium italicum, Penicillium oxalicum* and *Penicillium digitatum* were obtained from Fungal Culture Bank, Institute of Agricultural Sciences, University of the Punjab, New Campus, Lahore and one species *Penicillium*-IIB was collected from Institute of Industrial Biotechnology, GC University, Lahore.

Maintenance of the Fungal Cultures

The cultures were maintained on Malt Extract Agar (2g Malt Extract and 2g Agar) slants. Spores from 7-10 days old slant was transferred to the freshly prepared slants and placed in incubator at 25^{o}C for 10 days for the growth of mycelium.

Screening of Fungal Species and Fermentation Medium for optimum growth

Species of Genus *Penicillium* i.e. *Penicillium commune, Penicillium italicum, Penicillium oxalicum, Penicillium digitatum* and *Penicillium* sp. 1 were screened for the best production of ergot alkaloids. For this purpose, different fermentation media M1, M2, M3, M4 and M5 were selected for maximum production of ergot alkaloids with each fungal species.

Medium M1 contained (g/100ml) Mannitol, 5; Succinic acid, 0.05; $MgSO_4.7H_2O$, 0.01; K_2HPO_4, 0.01; $ZnSO_4$, 0.004.

Medium M2 contained (g/100ml) Glucose, 5; Peptones, 0.1; K_2HPO_4, 0.1; FeSO4, 0.001, $ZnSO_4$, 0.002; $MgSO_4.7H_2O$, 0.15; NH_4OH, 0.01.

Medium M3 contained (g/100ml) Sucrose, 5; Yeast Extract, 0.3; KH_2PO_4, 0.1; $FeSO_4$, 0.001; ZnSO4, 0.001; MgSO4.7H2O, 0.001.

Medium M4 contained (g/100ml) Mannitol, 5; NH_4Cl, 0.2; Succinic Acid, 0.54; Tryptophane 0.025; KH_2PO_4, 0.5; $MgSO_4$ 0.03; $FeSO_4$ 0.001; $ZnSO_4$, 0.004.

Medium M5 contained (g/100ml) Sucrose, 5; NH_4Cl, 0.2; Yeast Extract, 0.5; Succinic Acid, 0.5; Asparagine, 0.5; KH_2PO_4, 0.5; $MgSO_4$, 0.03, $FeSO_4$, 0.001; $ZnSO_4$, 0.002.

pH of all the fermentation media was adjusted to 5.2 and after autoclaving 5ml of spore suspension (10 $^{6-7}$ spores/ml) of each fungal species was transferred to the respective flasks containing fermentation media. After inoculation these flasks were incubated at 25^{o}C for 21 days.

Optimization of Culture Conditions

The selected fungal species and the fermentation media after preliminary screening was further optimized to enhance the ergot alkaloids production and following parameters were also studied.

1. **Effect of different carbon sources**

   In order to get the maximum production of ergot alkaloids by *Penicillium commune,* different carbon sources were used e.g. Glucose, Fructose, Maltose, Sucrose, Mannose and Mannitol.

2. **Effect of Different concentrations of sucrose**

   Different concentrations of sucrose i.e. 10g, 15g, 20g, 25g, 30g, 35g and 40g were used to optimize the best substrate concentration for the highest yield of ergot alkaloids.

3. **Effect of different nitrogen sources**

Various organic and inorganic nitrogen sources were used to optimize the best nitrogen source for the maximum production of ergot alkaloids e.g. Yeast Extract, Peptones, Malt Extract, Meat Extract, Ammonium Chloride and Urea.

4. **Effect of different concentrations of yeast extract**

   Different concentrations of yeast extract i.e. 5g, 10g, 15g, 20g, 25g and 30g were used to optimize the best yeast extract concentration for the highest yield of ergot alkaloids.

5. **Effect of different concentrations of phosphate**

   Different concentrations of KH_2PO_4 were used i.e. 0.5g, 1g, 1.5g, 2.0g, 2.5g, and 3g in selected medium to optimize the best concentration of KH_2PO_4 for the highest production of ergot alkaloids.

6. **Effect of different pH levels**

   The pH was adjusted to different pH values ranging from 3 to 8 by using ammonia solution and 0.1N HCl.

7. **Effect of different inoculum sizes**

   Different inoculum sizes i.e. 5ml, 10ml, 15ml, 20ml, 25ml and 30ml were used to get the maximum production of ergot alkaloids in the fermentation medium.

Determination of Ergot Alkaloids

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

Assaying of Alkaloids: 1ml of each of the culture filtrates was added to 2ml of Van Urk Reagent in the test tubes and reaction mixture was incubated at 37^{o}C for 30 min **(Van Urk, 1929; Smith, 1930).** The absorbance was measured at 590 nm by Spectrophotometer. The amount of alkaloids present was measured by a standard curve of Dihydroergotamine methane sulfonate salt.

Quantitative Analysis of Ergot Alkaloids from Mycelium: The mycelia that were separated from the fermentation media of all the fungal cultures were initially weighted and placed in oven at 45^{o}C for 24 hours for drying. After 24 hours they were weighed again to measure the dry weights of the mycelium. These dry mycelia then placed in methanol for 24 hours and then subjected to cell lysis by sonication process for 5 cycles each of 3 minutes for each mycelium at 200 rpm /min in an Ultrasonic Senerator. After sonication, for further cell lysis, all the sonicated material was homogenized in a homogenizer each for atleast 3 cycles of 5 min so that all of the compounds of ergot alkaloids may release from the mycelium of fungi **(Linde, 2005).** The mixture after homogenization was again centrifuged and the supernatants were collected. Chloroform from the supernatant was evaporated and the mycelia extracts were measured and then assyed with Van Urk Reagent as mentioned above and absorbance was measured on 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 made by two fungal species. Ergot alkaloids were extracted 3 times in 50ml of chloroform in a separating funnel by separating the layers. 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 to identify the possible alkaloids spectrum. Colored spots were developed on the TLC plates by spraying Van Urk Reagent after the procedures of **Stahl (1969).** The alkaloids contents of the mycelia were also determined by the same procedure as mentioned above.

Results

Species of Genus *Penicillium* i.e. *Penicillium commune, Penicillium italicum, Penicillium oxalicum* and *Penicillium digitatum,* were obtained from Fungal Culture Bank, Institute of

agricultural Sciences, University of the Punjab, New Campus, Lahore and one unidentified species *Penicillium*-IIB was collected from the Institute of Industrial Biotechnology, GC University, Lahore.

Penicillium commune and M5 fermentation medium was recorded as the highest ergot alkaloids producer and best fermentation medium resoectively (**1.3587mg/ml**). Mycelial dry weights were also noted during the study (Table 1).

Table 1: Optimization of Fungal Species and Fermentation Media for the production of Ergot Alkaloids

Sr. No.	Fungal organism	Fermentation Media									
		M1		M2		M3		M4		M5	
		Supernatant Alkaloids Activity (mg/ml)	Mycelium Alkaloids Activity (mg/ml)	Supernatant Alkaloids Activity (mg/ml)	Mycelium Alkaloids Activity (mg/ml)	Supernatant Alkaloids Activity (mg/ml)	Mycelium Alkaloids Activity (mg/ml)	Supernatant Alkaloids Activity (mg/ml)	Mycelium Alkaloids Activity (mg/ml)	Supernatant Alkaloids Activity (mg/ml)	Mycelium Alkaloids Activity (mg/ml)
1.	*P. commune*	0.0134	0.0193	0.0958	0.1351	0.3095	0.0985	0.7985	0.5139	1.3587	0.9581
2.	*P. italicum*	0.0014	0.0021	0.0751	0.0131	0.1431	0.0951	0.4385	0.0956	0.4951	0.1356
3.	*P. oxalicum*	0.0019	0.0016	0.0895	0.0356	0.2514	0.0875	0.0543	0.1013	0.0986	0.0019
4.	*P. digitatum*	0.0108	0.0103	0.0954	0.0543	0.956	0.0871	0.0956	0.1431	0.1436	0.0951
5.	*Penicillium* sp. 1	0.0145	0.0610	0.0759	0.0856	0.9563	0.7591	0.9815	0.6514	1.1543	0.6351

Optimization of some Culture conditions for the production of ergot alkaloids

1. **Effect of various carbon sources**

   In order to obtain the maximum yield of Ergot Alkaloids, various carbon sources, i.e. Glucose, Fructose, Sucrose, Maltose, Mannose and Mannitol were used in the 100ml of M5 fermentation medium. Sucrose was optimized as the most favorable carbon source for the mycelium growth (2.156g/100ml) and ergot alkaloids production (2.5811mg/ml) (Fig. 1).

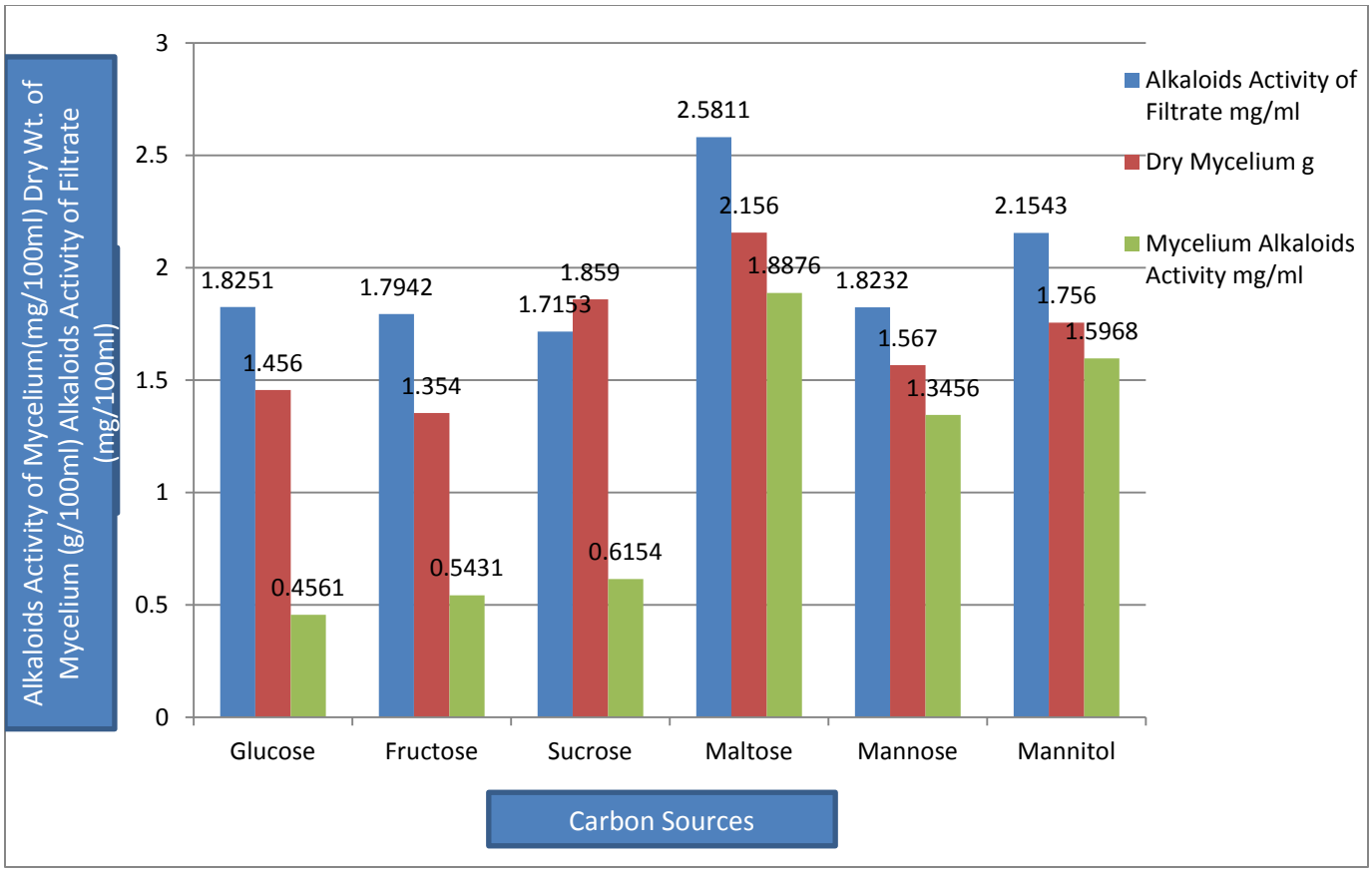

Fig. 1. Effect of various Carbon Sources on the growth of Mycelium and the Production of ergot alkaloids in supernatant and in mycelium of *Penicillium commune*

2. Effect of different concentrations of sucrose

Various concentrations of sucrose i.e. 10g, 15g, 20g, 25g, 30g, 35g and 40g were used in the 100ml of M5 fermentation media and the best growth of mycelium (2.543g/100ml) and the highest yield of alkaloids production (2.6785mg/ml) was obtained at 35g of sucrose as described in Fig. 2.

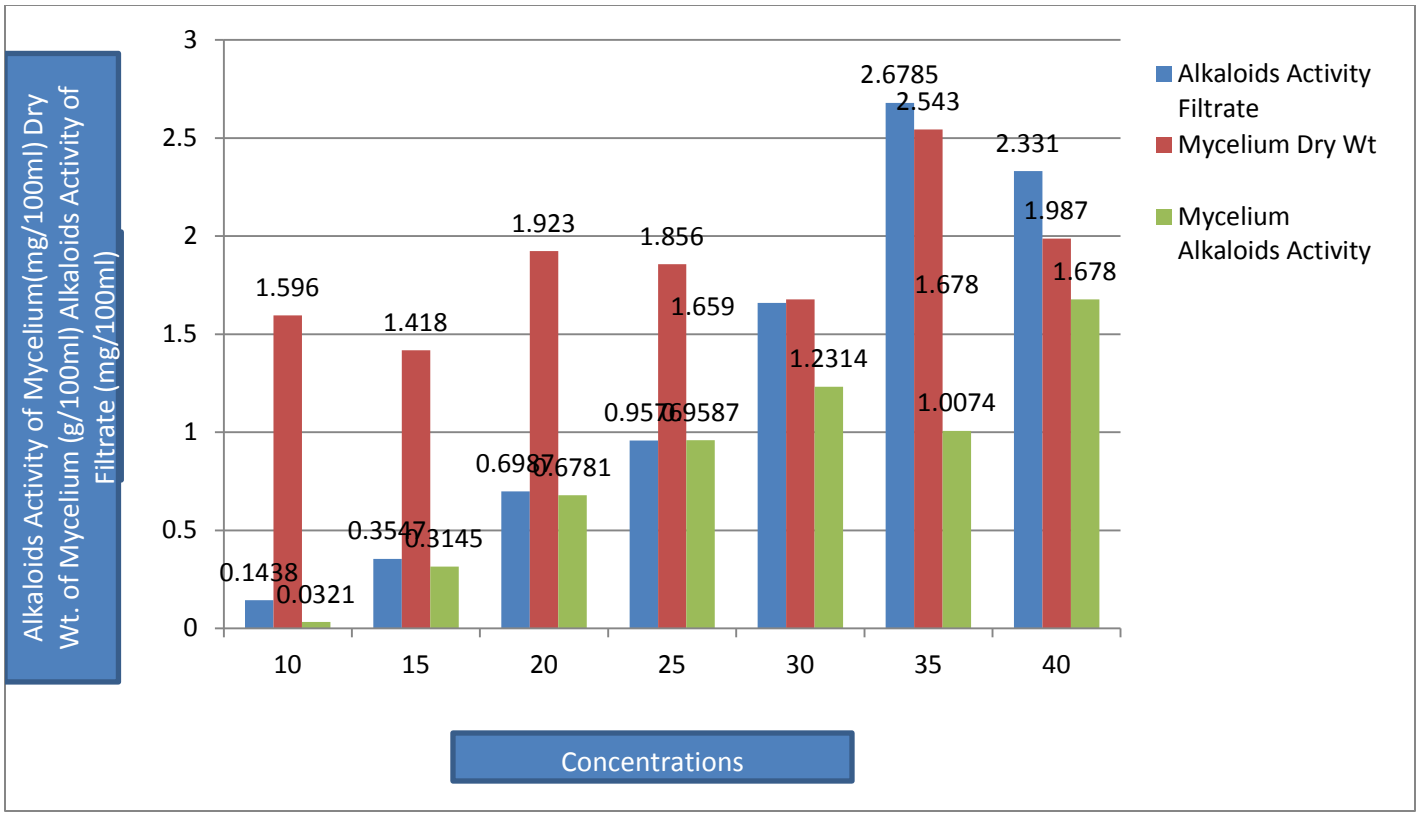

Fig. 2. Effect of Substrate concentrations on the growth and production of Ergot Alkaloids by *Penicillium commune*

3. Effect of various nitrogen sources

In order to obtain the maximum yield of Ergot Alkaloids, various nitrogen sources i.e. Yeast Extract, Peptones, Malt Extract, Meat Extract, Ammonium Chloride and Urea were used in the 100ml of M5 fermentation medium. Yeast Extract was the most favorable nitrogen source for the mycelium growth (2.578g/100ml) and ergot alkaloids (2.8513mg/ml) (Fig. 3).

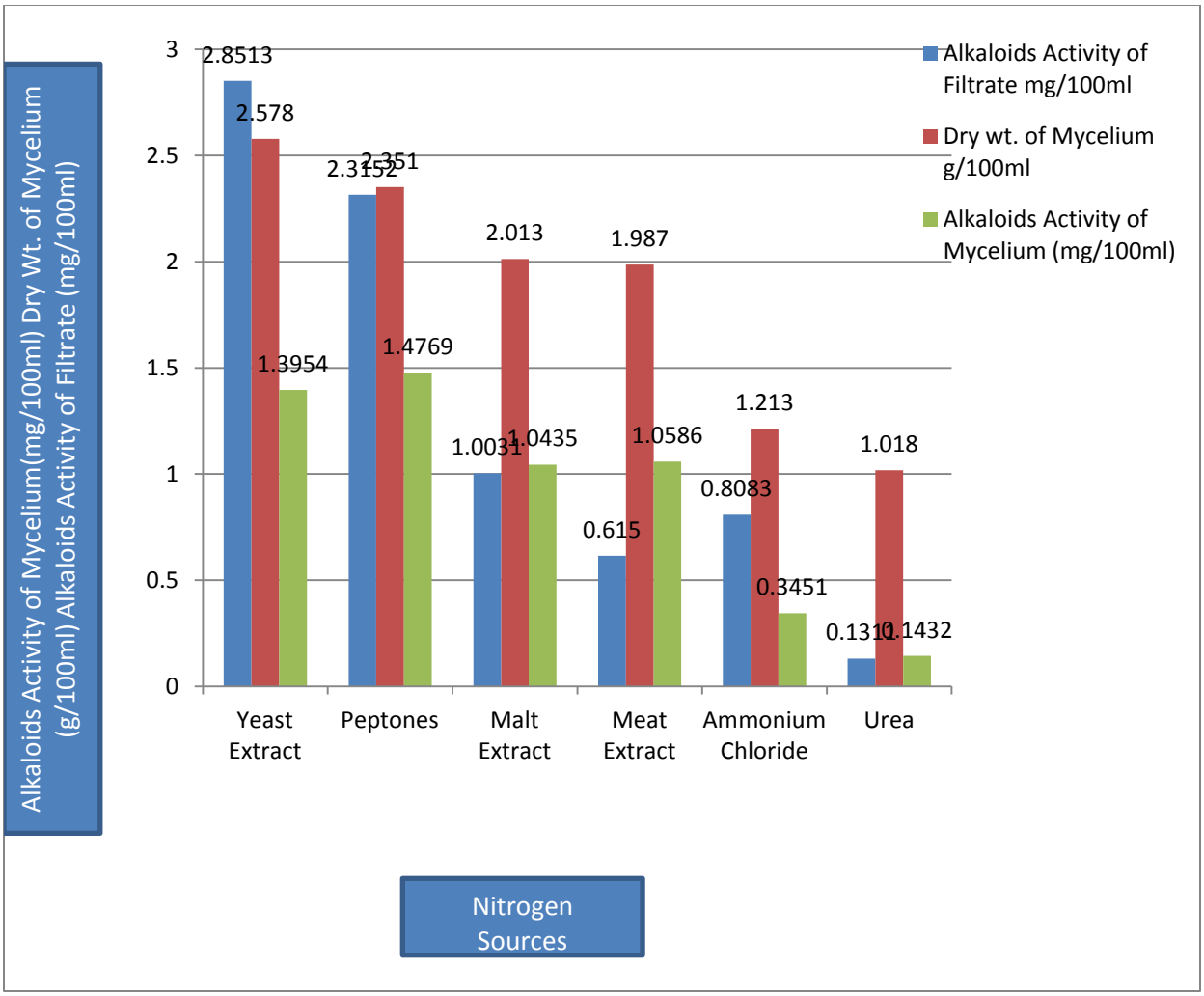

Fig. 3. Effect of various Nitrogen Sources on the growth of Mycelium and the Production of ergot alkaloids in supernatant and in mycelium of *Penicillium commune*

4. **Effect of different concentrations of yeast extract**

Different concentrations of yeast extract i.e. 5g, 10g, 15g, 20g, 25g and 30g were used to optimize the best yeast extract concentration for the highest yield of ergot alkaloids in the selected optimized fermentation medium. 30g of the yeast extract gave maximum yield of alkaloids (2.3510mg/ml) and the highest growth of mycelium (2.150g/100ml) as described in the fig. 4.

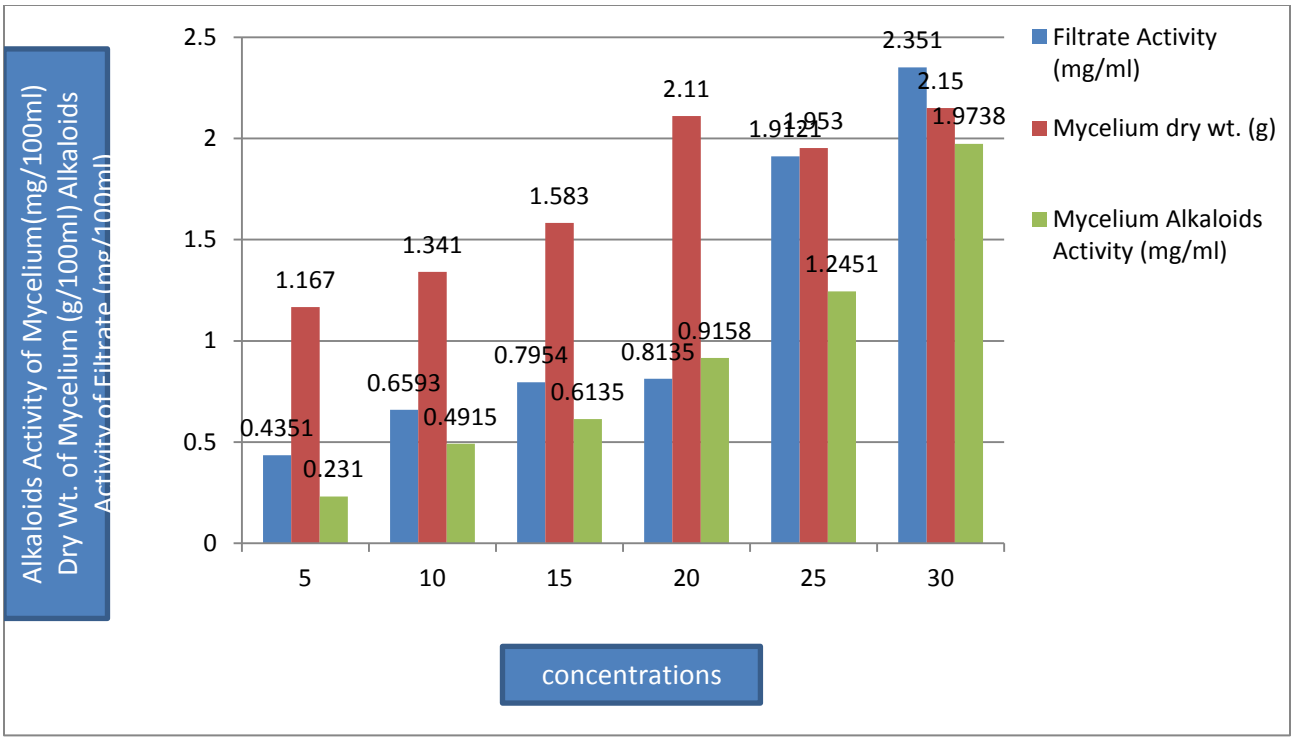

Fig. 4. Effect of various concentrations of yeast extract on the growth of Mycelium and the Production of ergot alkaloids in supernatant and in mycelium of *Penicillium commune*

5. Effect of various concentrations of Phosphate

Different concentrations of KH_2PO_4 were used i.e. 0.5g, 1g, 1.5g, 2.0g, 2.5g, and 3.0g in selected M5 medium. 2g of KH_2PO_4 gave maximum production of alkaloids (1.5731mg/100ml) fungus growth (2.458g/100ml) and alkaloids in mycelium (0.9812mg/100ml) as shown in the Fig. 5.

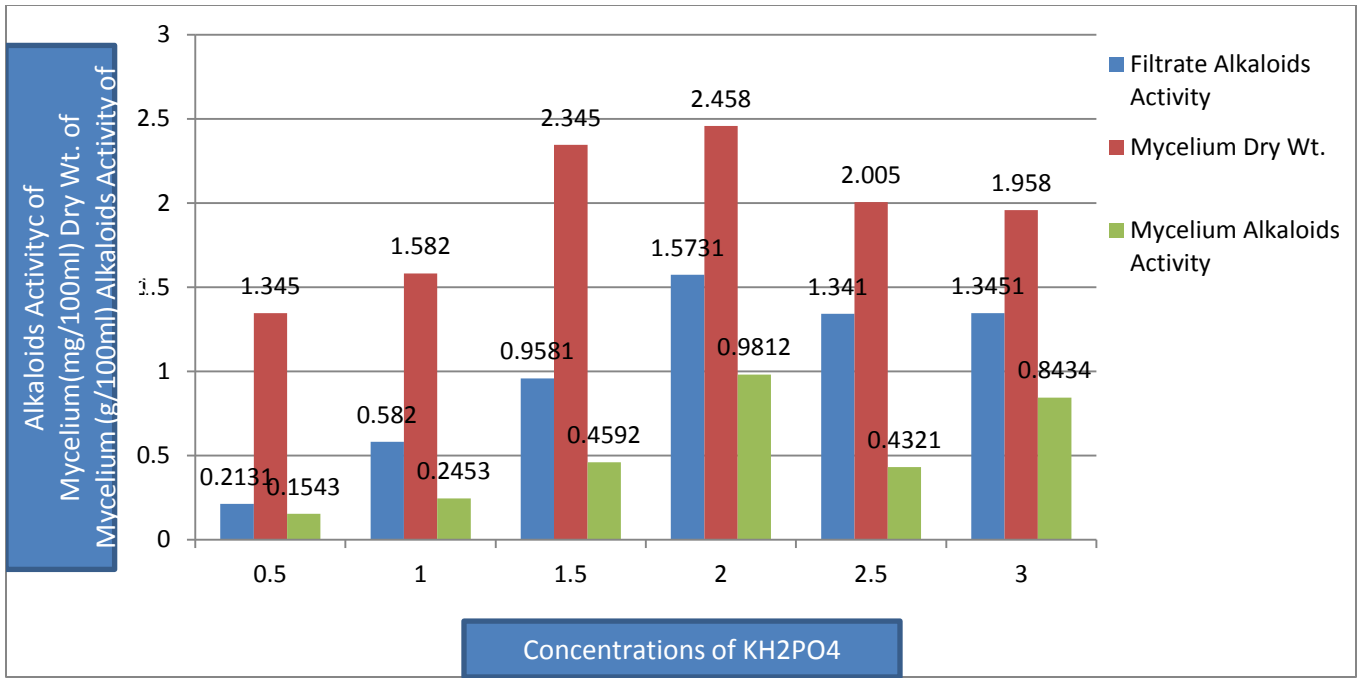

Fig. 5. Effect of different concentrations of KH_2PO_4 on the growth of Mycelium and the Production of ergot alkaloids in supernatant and in mycelium of *Penicillium commune*

6. Effect of different pH levels

The pH of the fermentation medium had a great influence on the growth of fungal mycelia and the production of ergot alkaloids by *Penicillium commune*. pH of the medium ranged from 3-8. Ergot Alkaloids production (2.1385mg/ml) and the growth of fungal mycelium was found maximum at pH 5 as described in the Fig. 6.

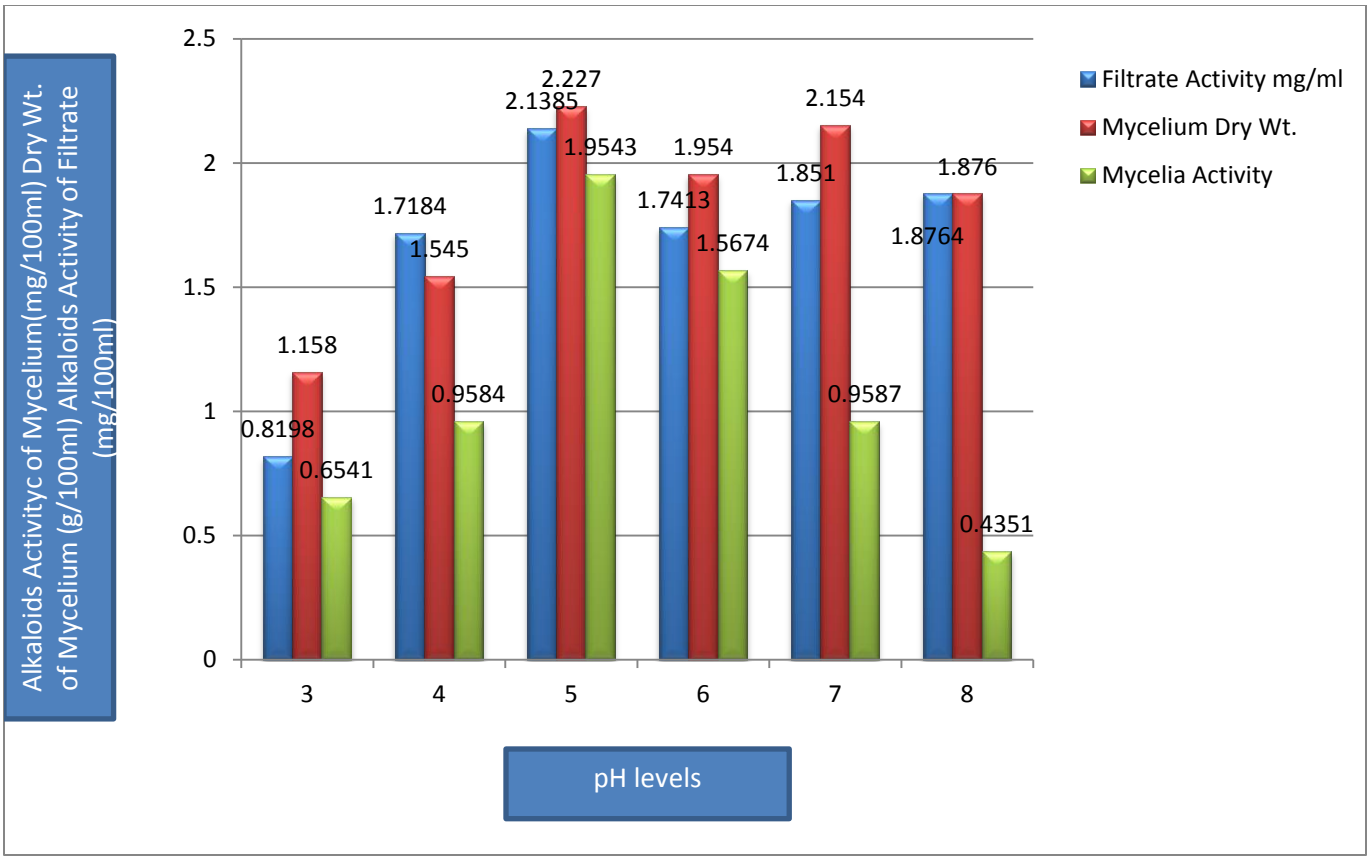

Fig. 6. Effect of different pH on the growth of Mycelium and the Production of ergot alkaloids in supernatant and in mycelium of *Penicillium commune*

7. Effect of different Inoculum Sizes

Different sizes of inoculums i.e. 5ml, 10ml, 15ml, 20ml, 25ml and 30ml, were used in the M5 fermentation media and the best inoculum size 15ml was optimized as the best alkaloids producing inoculum size (3.3478mg/ml) fungal mycelium growth (2.945g/100ml) and the best alkaloids production in mycelium of the fungi (2.7567mg/ml) as described in Fig. 7.

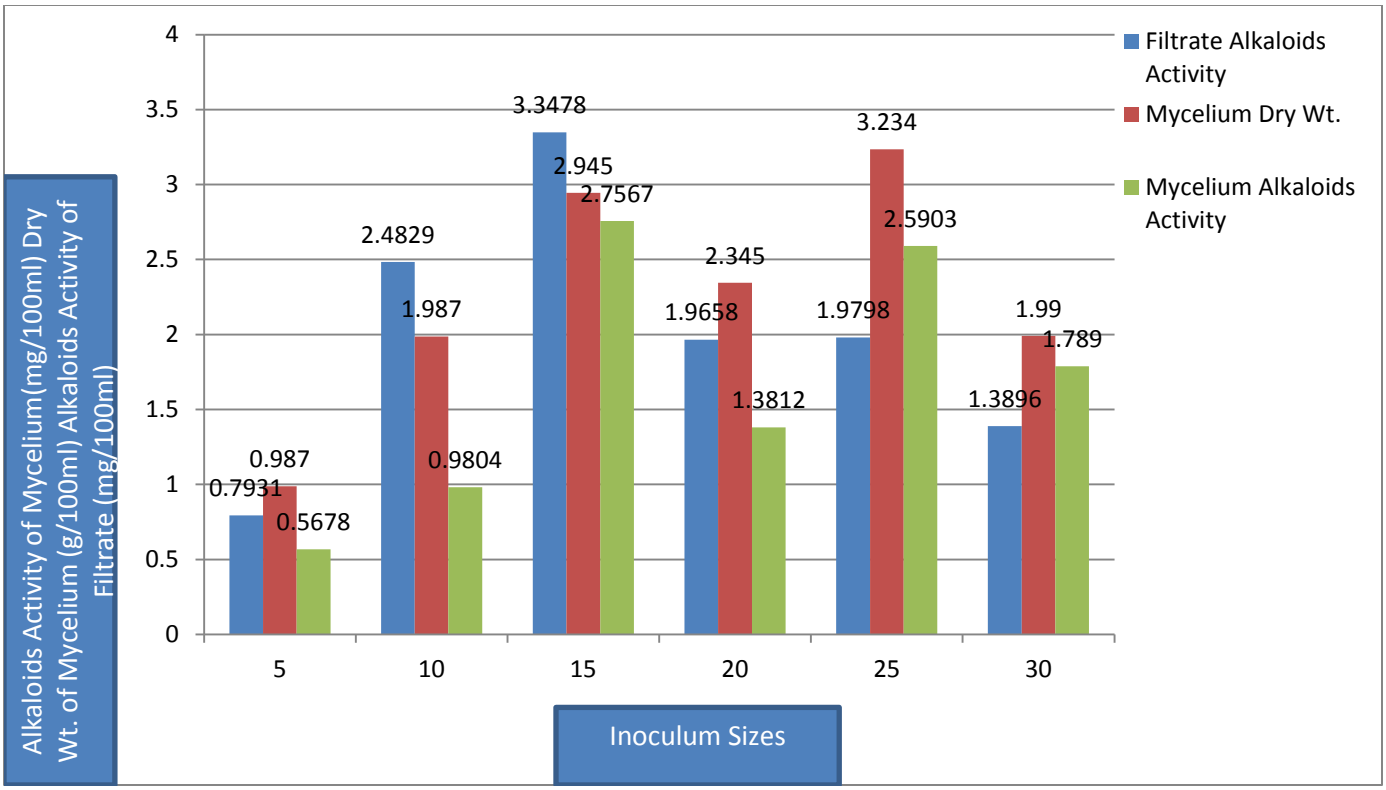

Fig. 7. Effect of different inoculum sizes on the growth of Mycelium and the Production of ergot alkaloids in supernatant and in mycelium of *Penicillium commune*

Discussion

Penicillium commune was screened as the best species amongst all above mentioned species for the production of ergot alkaloids by surface culture fermentation process. Production of ergot alkaloids is also achieved by *Penicillium concavo-rugulosum* **(Abe *et al.* 1969)** and *Penicillium chermesinum* **(Agurell, 1964).**

The optimum conditions for the growth of fungal mycelium and the yield of ergot alkaloids were found to be the (a) Sucrose: 35g/100ml, (b) Yeast Extract: 30g/100ml, (c) Carbon Source (Sucrose: 20g/100ml), (d) Nitrogen Sources (Yeast Extract: 5g/100ml), (e) pH 5, (f) KH_2PO_4: 2g/100ml and (g) Inoculum Size: 25ml/100ml, best for the production.

The fungal organism must be provided with a healthy medium in which it can grow and sporulate well and can produce maximum amount of ergot alkaloids. *Penicillium commune* produced a high amount of ergot alkaloids when it was grown in M5 fermentation medium. Different fermentation media were also used for the production of ergot alkaloids when they were inoculated by species of *Balansia* described by **Bacon *et al.* (1975).**

In terms of sucrose and other carbon sources utilization is concerned sucrose was best amongst all the carbon sources and the by fungal species. It was seen that with the increase in the amount of sucrose there was a remarkable increase in the growth of mycelium and production of ergot alkaloids. Glucose, Fructose, Maltose suppressed the growth of fungus and yield of alkaloids in the fermentation medium. These results are in the harmony of **Moussa (2003)** that has also described sucrose as the best carbon source for the production of ergot alkaloids giving a maximum yield of 0.800mg/l in the fermentation medium. Similar results were also found by **Arcamone *et al.* (1961)** and **Socic and Gaberc-Porekar (1992).** Glucose and Fructose has suppressed the yield of ergot alkaloids in one of the experiments by **Drew and Wallis (1983)**.

The nitrogen level of the culture medium proved to be an important factor influencing the growth and yield of alkaloids. That's why different nitrogen sources were also used in the experiment and they also influenced the growth of the mycelium and the production of ergot alkaloids in the fermentation medium by surface culture fermentation process. Yeast Extract showed a remarkable effect on the growth of the mycelium and on the yield of ergot alkaloids. Ammonium Chloride and Urea had a negative effect on the fungal mycelium production and ergot alkaloids production. **Taber and Vining (1958)** and **Moussa (2003)** also described a negative effect of Ammonium Chloride on growth of mycelium and as well as on the yield of ergot alkaloids.

The pH value of the fermentation medium exhibited a great influence on the growth of the mycelium and production of ergot alkaloids. It was shown that with the increase in

pH value there was an increase in the growth of the fungus and yield of the alkaloids. pH 5 was remarkably best amongst all pH values for the best fungal growth and production of alkaloids. Similar results were also described by **Moussa (2003)** and **Mizrahi and Miller (1970)** who reported that optimum pH 5 was good for the maximum yield of alkaloids.

Variation of KH_2PO_4 clearly affected the fungal growth and the production of ergot alkaloids. It was noted that with the increase in the phosphate concentration there was an increase in the mycelium growth and the production of ergot alkaloids but with the maximum increase in the phosphate there was a decrease in the fungal growth and the alkaloids production. Similar results were also described by experiments on *Claviceps* species by **Rehacek *et al.* (1971). Windish and Bornn (1960)** also described the less quantity of phosphate used in the carbohydrate rich medium gave the best ergot alkaloids production and the increase in phosphate amount limit the production of ergot alkaloids. **Brady and Tyler (1960)** also determined that phosphate is the important nutrient for alkaloids production. These results are also in harmony with the results of **De Waart and Taber (1960), Mary *et al.* (1965)** and **Taber (1967)**. It can be described that the excess phosphate, required for the production of protein and nucleic acids, prolongs the growth phase of the organism and during this amino acids e.g. tryptophane is used for the protein synthesis **(Weygand and Floss, 1963; Robbers *et al.*, 1972).**

Different inoculums sizes in the form of spore suspension material markedly effect the production of ergot alkaloids and mycelial production in the fermentation medium. With the increase in the spore suspension size, there was an increase in the production of ergot alkaloids and when this was increased more in the fermentation media then there was a decrease in the production of alkaloids. This can be due to the limited nutrients availability in the fermentation media for the fungal spores. These results are similar with the results of **Meyrath (1973)** in which they described that with the increase in the inoculums there was the increase in the alkaloids production.

Conclusion

Ergot alkaloids are pharmaceutically very significant but synthetic alkaloids are very expensive and are not cost effective. In the present study ergot alkaloids were produced from fungus and ergocryptine alkaloid was identified that use in the cure of migraine and to stop post partum bleedings. This is a sustainable and a very cost effective way of the production of these useful alkaloids.

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