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Micro-Elements Work for the Growth and Total Soluble Protein Production in *Aspergillus niger* at Different Concentrations

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Aspergillus niger **van Tieghem. is an industrially important soil-borne fungus. In biotechnological industries, it has been used for the production of many enzymes. It is well-known that the production of enzymes and its activity depends on co-factors. Some micro-elements act as cofactor of some enzymes involved in the production and function of industrially important enzymes or proteins in different ways. The micro-elements may also act on gene expression, protein folding and maintaining the activity of enzymes or proteins. In this study, Zn^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+} were used for the study of the effect of these micro-elements on the growth and the production of total soluble proteins of *Aspergillus niger*. This experiment suggests that few of Zn^{2+}, Mg^{2+} and Mn^{2+} have important role in promoting growth and high production of total soluble proteins. Fe^{2+} has inhibitory effect on the growth as well as total protein production.**

Key words*: Aspergillus niger*, Micro-elements, Co-factors, Microbial growth, Total soluble protein.

Aspergillus niger van Tieghem. is cultured for the industrial production of many substances like citric acid and gluconic acid. Many useful enzymes are produced using industrial fermentation of *A. niger*. *Aspergillus niger* glucoamylase is used in the production of high fructose corn syrup and pectinases used in cider and wine clarification. β-galactosidase, an enzyme that breaks down certain complex sugars, is a component of Beano and other medications where it can decrease flatulence. The effect of different media and pH on the formation of amylase by *Aspergillus oryzae* (Ahlburg) E. Cohn El 212 was found to secrete a α or β-amylase, or both (Kundu and Das, 1970). Fermentation with *A. niger*, however, significantly increased the albumin and globulin content of the *Terminalia catappa* L. seed meal to 38.83%, while it significantly reduced the gliadin fraction. The glutelin fraction was,

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however, not significantly ($p>0.05$) affected by the treatment. There were significant reductions ($p<0.05$) in some of the essential amino acids (like leucine, isoleucine and methionine), while the non-essential amino acids like glutamic acid was significantly increased ($p<0.05$) (Muhammad *et al.,* 2009). Optimal sources and concentrations of carbon (soy flour-25 g/L) and nitrogen (pepton-7.5 g/L), dry borine blood (in concentration of 1%) and a number of guanidine derivatives (guanidine carbonate-0.25%, nitroaminoguanison dimethylaminobenzaldehyde-0.05%, nitroaminoguanison of salicylic aldehyde-0.1%) play significant role for the synthesis of alpha-N-acetylgalactosaminidase and alpha-galactosidase by *A. niger (*Borzova *et al.,* 2001). In these instances, the culture is rarely grown on a solid substrate, although this is still common practice in Japan, but is more often grown as a submerged culture in a bioreactor. In this way, the most important parameters can be strictly controlled, and maximal productivity can be achieved. It also makes it far easier to separate the chemical or enzyme of importance from the medium, and is therefore far more cost-effective. Sorghum pomace medium significantly ($P<0.05$) induced higher level of raw and extracellular amylase than soluble starch medium. Mixed culture media recorded higher ($P<0.05$) level of raw starch degrading amylase than monoculture media (Abu *et al.,* 2005). Maximum growth rate of *Aspergillus ochraceus* G.Wilhelm was found after 5 days of incubation at 30°C, but maximum amylase production was obtained after 2 days with lactose, maltose, xylose and starch as carbon sources. The extracellular amylase production and mycelial growth were influenced by the concentration of starch (Nahas and Waldemarin, 2002). The maximum production of β-glucosidase and CM-cellulase was achieved by *Aspergillus fumigatus* Fresenius grown on H_2SO_4 and HCl pretreated wheat straw substrate in comparison to HNO_3 and $HCIO_4$ pretreated wheat straw (Dahot and Noomrio, 1996). The culture filtrate *A. niger* exhibited relatively highest activity of cellulose, endoglucanase and β-glucosidase and extracellular protein content at 7-day interval during the course of its growth on Czapek-Dox medium supplemented with 1.0% (w/v) cellulose. Urea as a nitrogen source and pH 5.0 were found to be optimal for growth and cellulase production by *A. niger* (Narasimha *et al.,* 2006). Lignocellulolytic enzyme production by *A. niger* was compared both in submerged fermentation (SF) and biofilm fermentation (BF) at varying water activities (Villena and Gutiérrez-Correa, 2007). *A. niger* GH1 produced the highest tannase level (2291 U/L) in SSF at 30 °C during the first 20 h of culture at tannic acid concentration of 50 g/L, and under these conditions enzyme production was entirely extracellular (Cruz-Hernández *et al.,* 2006).

The essential trace elements (e.g. Iron, manganese, cobalt, copper, zinc, selenium, and molybdenum etc.) reflect their role as cofactors. They play essential role in regulation of growth and other protein formation and function in organisms. Free amino acid pools have been investigated in a citric acid accumulating strain of *A. niger* during batch growth under manganese sufficient and deficient conditions by means of an improved chromatographic method. Studies on the mycelial content of several nitrogenous compounds under manganese sufficient and deficient conditions showed that manganese deficiency resulted in lower amino acid pool sizes during trophophase and considerable accumulation during idiophase, and in a reduction of the protein and nucleic acid contents. Addition of cycloheximide to mycelia grown with sufficient manganese also caused an elevation of free amino acid pool sizes, thus indicating that impairment of protein synthesis by manganese deficiency is responsible for the observed rise in amino acid concentration. Furthermore, the manganese deficient mycelia excreted high amounts of all amino acids suggesting that manganese deficiency may also affect membrane permeability (Kubicek *et al.,* 1979). D-galactonate dehydratase has a requirement for Mg^{2+} and Mn^{2+}, however $ZnSO_4$ and $HgCl_2$ caused a complete inhibition of the enzymatic activity of this enzyme (Elshafei *et al.,* 2001). In the presence of copper, the significant induction of citric acid overflow was observed, while concomitantly lower levels of total lipids were detected in the cells. Its effect was more obvious in a medium with magnesium as sole divalent metal ions, while in a medium with magnesium and manganese. The addition of copper had a less pronounced effect. Since the

malic enzyme was recognised as a supplier of reducing power in the form of reduced nicotinamide adenine dinucleotide phosphate for lipid biosynthesis, its kinetic parameters with regard to different concentrations of metal ions were investigated. Some inhibition was found with Fe^{2+} and Zn^{2+}, while Cu^{2+} ions in a concentration of 0.1 mM completely abolished malic enzyme activity. The same metal ions proportionally reduced the levels of total lipids in *A. niger* cells. A strong competitive inhibition of the enzyme by Cu^{2+} was seen as it competes with Mg^{2+} and Mn^{2+} for the same binding site on the protein (Jernejc *et al.,* 2002). Zn^{2+} reduced mycelial growth of *A. niger*. The toxicity of zinc to the fungi was unaffected, lessened, or increased by the addition of high concentrations of NaCl. *Aspergillus niger* tolerate higher concentrations of zinc in the presence of NaCl at 37°C than at 25°C (Babich *et al*., 1978). Nature of antagonisms responsible is for Mg^{2+}, Cd^{2+} and Zn^{2+} interaction in the growth of *A. niger* (Laborey *et al.*, 1973). A considerable increase in the biocidal activity of Cu(II), and Zn(II) complexes ligands on being coordinated with metal ions has been reported (Nagar *et al.,* 1989). Complexes of the type Na(6)[M(HL)(2)(H(2)O)(2)], where M= Mn(II), Fe(II), Co(II), Ni(II), Cu(II) and Zn(II) and Na(3)H(2)L= 2-(4-sulphophenylazo)-1,8-dihydroxy 3,6 naphthalene disulphonic acid trisodium salt, have been synthesized and characterized by physico-chemical (elemental analyses, solubility, electrolytic conductance, magnetic susceptibility measurement) and spectral (UV-Visible, IR, ESR, powder x-ray diffraction) techniques for their structure and studied for their antifungal activity against ten fungi including *A. niger, Aspergillus flavus* Johann Heinrich Friedrich *(*Pandey *et al.,* 2005).

The problems which are being faced by the company are the unavailability of data regarding different micro-elements and their proper concentration for the optimal growth and production. In the current study, the micro-elements which have important role in the growth promotion and the production of proteins have been evaluated.

MATERIALS AND METHOD

Medium: Potato Dextrose Agar (PDA) Medium was used for the growth of *Aspergillus niger* (our lab strain). One litre medium was prepared from 15g Agar, 20g Dextrose and 4g Potato Infusion (from 200g potatoes). The pH of the medium was maintained as 5.6 ± 0.2 at 25°C.

Metal cofactors: Zn^{2+}, Mn^{2+}, Mg^{2+} and Fe^{2+} were used to evaluate their function on the growth and total soluble protein production in *A. niger*.100mg, 250mg, 500mg and 1000mg per litre of medium of the above mentioned micro-elements (autoclaved) were taken in culture separately (with triplicate forms along with control culture (not having micro-element).

Growth of Fungi

The cultures (our lab isolated culture) were inoculated by serial dilution method and maintained at 28°C in an incubator. The number of black colonies was calculated by colony counting instruments. Colonies were counted after 3, 6 and 9 days of culture initiation.

Estimation of Protien

The concentration of total soluble protein secreted in the medium by the strain of *A. niger* was determined by Folin-Lowry method at 660nm using UV-spectrophotometer after 3, 6 and 9 days of culture initiation. BSA standard solution was used to estimate the soluble protein concentration with different treatments (100mg, 250mg, 500mg and 1000mg microeliment per litre of medium).

RESULT AND DISCUSSION

Zn^{2+} shows varying growth pattern

The maximum numbers of colonies were found in 500mg and 1000 mg $ZnSO_4$ after 3 days of inoculation But, the number of colonies was dramatically decreased after 6 days and 9 days (Fig. 1a) This shows that high growth is observed at 500 mg and 1000 mg up to 3 days while the growth of *A. niger* at 100 mg and 250 mg was not significant. $ZnSO_4$ may be used at 100 mg and 250 mg to promote growth of *A niger* in steady condition. For getting dramatic increase of growth, one may use 500 mg and 1000 mg for 3 days only due to its depletion after 3 days of inoculation.

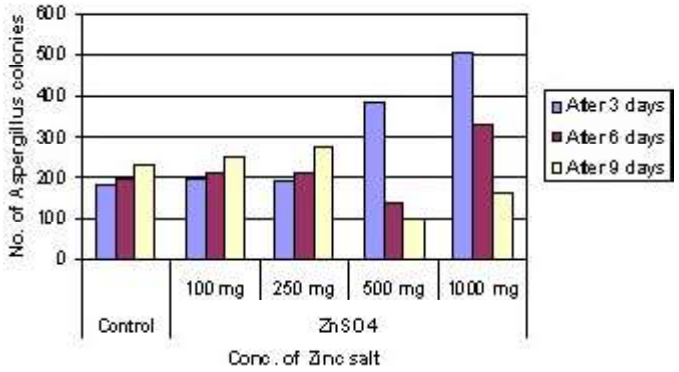

Fig. 1(a): Average growth of *Aspergillus niger* in Zn^{++} medium after 3 days, 6 days and 9 days of inoculation

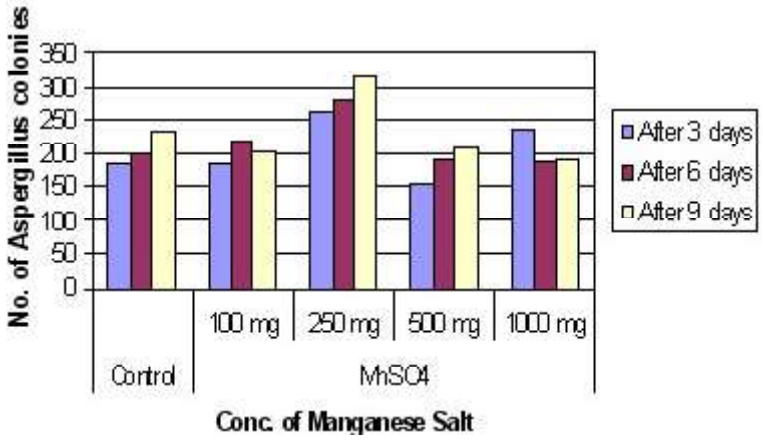

Fig. 1(b). Average growth of *Aspergillus niger* in Mn^{++} medium after 3 days, 6 days and 9 days of inoculation

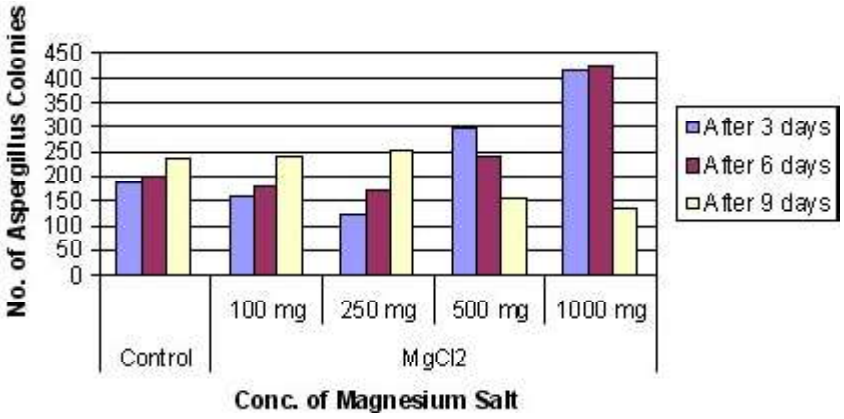

Fig. 1(c): Average growth of *Aspergillus niger* in Mg^{++} medium after 3 days, 6 days and 9 days of inoculation

Fig. 1(a)-1(c). Graphs showing comparative growth of *Aspergillus niger* with different cofactors

Mn^{2+} shows optimum growth at low concentration

$MnSO_4$ at different concentration shows growth fluctuation. $MnSO_4$ promote growth at 250 mg while 100mg, 500mg and 1000 mg showed little effect on the growth (Fig. 1b). 250 mg of $MnSO_4$ is recommended for enhancing the growth of *A. niger*.

Mg^{2+} helps in growth at high concentration

$MgCl_2$ also shows a significant growth at 1000mg followed by 500 mg. 100 mg and 250 mg did not show any significant change in growth pattern (Fig. 1c). So, this salt at 1000 mg can be used to enhance growth with supplementation after each 6 days as it showed decrease in growth after 6 days of inoculation.

Fe^{2+} shows strong fungicidal activity

$FeSO_4$ inhibits growth totally at 100 mg, 250 mg, 500mg and 1000 mg. $FeSO_4$ cannot be used at any concentration for its fungicidal activity.

Zn^{2+} and Mn^{2+} increases total soluble protein

Total soluble protein production was increased in all concentration of $ZnSO_4$. The maximum protein production was observed in 500 mg followed by 1000 mg of $ZnSO_4$. 250 mg and 1000 mg $MnSO_4$ showed significant increase in total soluble protein production (Fig. 2). Any concentration of $MgCl_2$ did not show any significant change in total soluble protein production, where as $FeSO_4$ showed fungicidal activity. Therefore, 500 mg of $ZnSO_4$ and 250 mg of $MnSO_4$ may be recommended as these are capable to increase the concentration of total soluble protein.

The total protein production increases in a regular manner but that is not reflected in growth with any particular concentration of micro-element. So any cofactor which promotes total protein production but they doesn't promote growth in the same manner. Further research

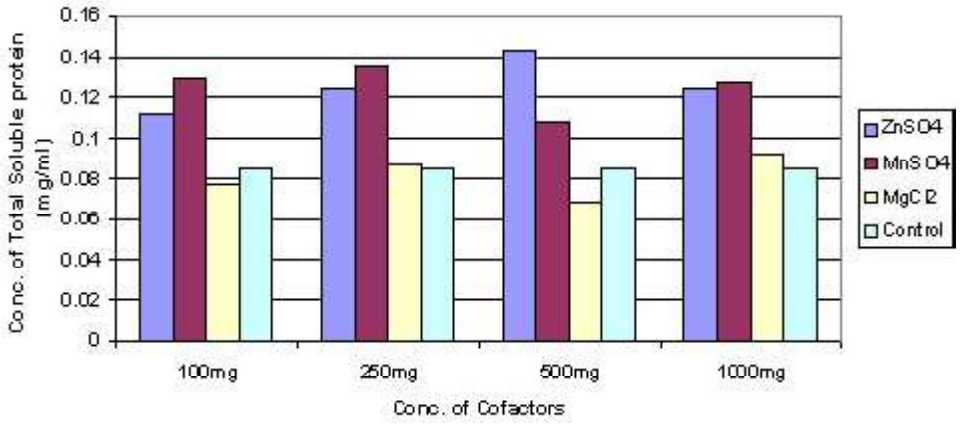

Fig. 2: Graphs showing comparative production of total soluble protein in *Aspergillus niger* with different cofactors after 9 days of inoculation

based on this project work may help the industries to understand the most important micro-elements and their suitable concentration for culture of *Aspergillus niger* and the production proteins from it. Then it would be easy for the companies to establish efficient strategy for maximum production of important enzymes and therapeutic proteins.

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