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Selection of *Aspergillus niger* Strains for Improved Production of Humic Acid

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By adopting the total isolation procedure following mutagenic treatment with ethylene imine or X-ray irradiation, a strain of *A. niger* was obtained which gave improved production of humic acid. The evolved strain gave as high an yield as 9.8 g/l against 3 g/l of humic acid by the parent strain. Conditions favouring maximum production, that is, 14g/l. of humic acid in stationary liquid cultures were: Dextrose —4%, asparagine —0.8%, *p*H —7.4 and incubation at 25°C for 5 weeks.

Key Words: *Aspergillus niger*, **Humic acid, Mutagenic treatment**

Introduction

Humus is a complex aggregate of brown to dark-coloured substances which is produced during the decomposition of plant and animal residues by microorganisms under aerobic and anaerobic conditions usually in soils, composts etc. Humic acid has beneficial effect on the physical properties of the soil, i.e. it improves the soil texture, increases its water-holding capacity and heat regime and determines to a large degree other physico-chemical properties such as the exchange and buffering capacities. These properties of humic acid are of great importance in regulating the supply of nutrients to plants. The slow but gradual decomposition of organic matter by microorganisms results in the production of a continuous stream of CO_2 of available N as NH_3^- which is changed into nitrate and liberation of potassium, phosphorus and other elements. All these substances have a positive effect on the growth and development of the plant.

Humic acid is reported to be among important metabolic products of *Aspergillus niger*, *Epicoccum*, *nigrum*, *Stachybotrys chartarum* and *Hendersonula toruloidea*. Williams (1914) postulated that both the formation and decomposition of humic acid are the result of the enzymatic activity of microorganisms. Mutagenic improvement of industrial microorganisms has become a common practice (IAEA 1971). A project for the development of superior strains was

undertaken in this laboratory with the primary objective of improving the yield of humic acid by strain selection. A strain of *Aspergillus niger* was treated with various mutagenic agents and series of experiments were conducted to investigate the problem. The optimum concentration of dextrose and asparagine and the effect of *p*H, time of fermentation, and temperature to improve the yield of humic acid by the mutant, *A. niger* AB 1705, are also reported in this paper.

Materials and Methods

Development of the mutant strains of A. niger

In the course of mutation studies with ethylene imine and X-rays of *A. niger* strain about 2160 mutant strains were isolated. Out of these only *A. niger* AB 1706 which produced 3.0 g/l humic acid was selected for further studies. The parent strain *A. niger* AB produced only 1.4 g/l humic acid.

Medium and cultural conditions

The parent culture, *A. niger* strain AB, and mutant strains were maintained on malt extract and yeast extract agar slants at 4°C. The Czapek-Dox agar medium was used for the mutation studies. The medium I was used for the fermentative production of humic acid consisting of 30g dextrose, 0.1g yeast extract, 0.5g $MgSO_4.7H_2O$, 2.0g K_2HPO_4, 0.25g KCl and 7.5g asparagine in one litre of distilled water (*p*H 7.4). When the cultures were needed for mutation studies, they were transferred to slants of malt extract and yeast extract agar and incubated at 25°C for 5 days for sufficient sporulation. Spare crops were harvested by washing the slant with sterile distilled water and filtering the resulting spore suspension through several layers of sterile absorbent cotton. The spore density was adjusted to 12×10^6/ml. This spore suspension was used both for mutation studies and for the inoculation of the fermentation medium. Surface culture fermentation was carried out using 500 ml flat flasks each containing 150 ml of the medium. The flasks were then incubated at 25°C for 4 weeks.

Factors affecting the production of humic acid

The optimum cultural conditions for the production of humic acid by *A. niger* AB 1705 were worked out by keeping all the factors constant except the one which was varied. The factors studied were (a) different concentrations of dextrose, (b) different concentrations of asparagine, (c) *p*H of the medium, (d) incubation period, (e) temperature. Fermentation conditions were the same as described before.

Determination of humic acid concentration

To obtain the humic acid, the cultures were filtered and the filtrate concentrated to about 50% of volume at 60°C. The evaporation procedure did not appear to alter the properties of the final product. The liquid was again filtered and then dialysed against frequent changes of distilled water for 48 hr. The humic acid was precipitated by adjusting the *p*H of the solution to 2.0 with HCl. After standing overnight, the supernatant was decanted and the remaining suspension centrifuged. After decantation, the humic acid was resuspended in distilled water and the process repeated. The humic acid was then dried at 60°C and ground (Martin et al. 1967, Schnitzer et al. 1973).

Results and Discussion

Selection of mutants of A. niger: The parent culture used in this experiment was *A. niger* AB, selected out of 55 isolates of the fungus from various sources. Spore suspension of this parents strain, containing 12×10^6/ml was then treated with ethylene imine and X-rays. Ethylene imine treatment

was observed to have maximum mutagenic effect at a concentration of 1 : 5000. This concentration was, therefore, used for the treatment of spores of the parent cultures. After treatment for 1, 2, 3, 4 and 5 hr the spore suspension in each case was diluted and plated out in Czapek-Dox (CD) agar medium. In all 1,250 isolates after treatment with ethylene imine were tested for humic acid production. *A. niger* AB 660 gave higher yields of humic acid (6.0 g/l) in medium I. The spores of the mutant *A. niger* AB 660 were then exposed to X-rays (35 KV and 10 mÅ) at the distance of 10 cm for 20, 30, 40, and 60 min. The treated spores were plated out in Czapek-Dox agar medium. Colonies were then transferred to malt extract and yeast extract agar slants. Again 910 isolates were selected after different treatments with X-rays for humic acid production. It was observed that the mutant *A. niger* AB 1705 gave the highest yield of humic acid (9.8 g/l) in medium I this strain was studied to standardize the cultural condition for fermentation.

Effect of different concentration of dextrose

Dextrose was the best carbon source for the humic acid production. The optium level of dextrose in the medium I for the production of humic acid was 4% (table1).

Effect of different concentrations of asparagine: Among the different nitrogen sources tried, such as, asparagine, urea, sodium nitrate, ammonium-nitrate, ammonium sulphate, ammonium chloride, peptone, diammonium hydrogen phosphate, asparagine appeared as a superior source for the production of humic acid. The optimum level of asparagine for the humic acid production was 0.80 (table 2).

Effect of pH of humic acid production: The initial *p*H of the medium was adjusted to 6.5, 7.0, 7.4, 7.7, 8.0 with IN HCl or NaOH. The optimum *p*H for the humic acid production was 7.4 (table 3).

Table 1 *Effect of different concentrations of dextrose on humic acid production by* Aspergillus niger *AB 1705*

Dextrose concentration (%)	Cellular growth (Dry wt g/l)	Humic acid* (g/l)
2.5	7.7	7.8
3.0	8.2	9.8
3.5	8.6	10.4
4.0	8.8	12.0
4.5	9.2	10.2
5.0	10.4	9.2

*Values are averages of triplicates

Table 2 *Effect of different concentrations of asparagine on humic acid production by* Aspergillus niger *AB 1705*

Asparagine concentration (%)	Cultural growth (Dry wt g/l)	Humic acid* (g/l)
0.60	7.4	7.5
0.70	8.0	8.8
0.75	8.6	9.8
0.80	8.8	13.2
0.85	9.3	11.8
0.90	10.2	9.0

*Values are averages of triplicates

Table 3 *Effect of initial pH on the humic acid production by* Aspergillus niger *AB 1705*

Initial *p*H	Cultural growth (Dry wt g/l)	Humic acid* (g/l)
6.5	8.0	8.6
7.0	8.2	11.0
7.4	8.8	13.2
7.7	8.0	10.8
8.0	7.5	8.4

*Values are averages of triplicates

Effect of temperature on the humic acid production: A temperature of 25°C optimally favoured the production of the humic acid (table 4).

Effect of fermentation time on the humic acid production: The optimum period required for the fermentation of humic acid was 5 weeks (table 5).

The final composition of the medium and cultural conditions for the humic acid production by *A. niger* AB 1705 maintained as follows:

Dextrose, 4.0%; Yeast extract, 0.01%; $MgSo_4.7H_20$, 0.05%; K_2HPO_4, 0.2%; KCl, 0.025%; asparagine, 0.8%; *p*H, 7.4; fermentation time, 5 weeks and temperature 25°C.

Further studies are in progress to assess the strain for industrial use for the production of humic acid.

Table 4 *Effect of different temperature on the humic acid production by* Aspergillus niger *AB 1705*

Temperature (°C)	Cultural growth (Dry wt g/l)	Humic Acid* (g/l)
23	8.2	11.1
25	8.8	13.2
27	9.0	12.0
30	8.3	10.3

*Values are averages of triplicates

Table 5 *Effect of fermentation period on the humic acid production by* Aspergillus niger *AB 1705*

Time of fermentation (weeks)	Cultural growth (Dry wt g/l)	Humic acic* (g/l)
3	8.0	10.8
4	8.8	13.2
5	9.0	14.0
6	9.5	12.4

*Values are averages of triplicates

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