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Channelling of glucose by methanol for citric acid production from *Aspergillus niger*

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Citric acid produced by *Aspergillus niger* was increased from 4.6 g l^{-1} to 7.8 g l^{-1} by supplementing basal medium with methanol (30 ml l^{-1}). While stimulating citric acid production, methanol did not improve membrane permeability of the fungus for citric acid. Methanol inhibited the germination of *Aspergillus* spores. An increase in glucose concentration from 50 g l^{-1} to 100 g l^{-1} in the presence of methanol (30 ml l^{-1}) improved citric acid production (1.6-fold) while at higher levels of glucose concentration methanol had no effect on citic acid production.

Key words: Channelling, stimulating, extracellular and intracellular citric acid, fungus.

Bioconversion of sugars to citric acid could be enhanced by using *Aspergillus* sp. mutants and incorporating specific stimulators into one culture medium. Such stimulators reported for citric acid production are methanol, quaternary ammonium compounds, oximes and fluoroacetate (Garg & Sharma 1977; Rohr & Kubicek 1983) and it was suggested that the stimulatory effect of some of these agents could be due to the removal of heavy metal ions present as contaminants in the medium (Moyer 1953b; Garg & Sharma 1977). Further, addition of organic solvents such as lower alcohols improved membrane permeability (Kapoor *et al.* 1982) and would thus facilitate transport of the metabolite across the membranes. Use of methanol to enhance citric acid production by *Aspergillus niger* was first reported by Moyer (1953a). Ethanol at 10–20 g l^{-1} concentration showed no effect on citric acid production but its higher concentrations reduced both growth and citric acid yield (Chaudhary *et al.* 1978). *n*-Propanol at concentrations of 10 g l^{-1} and above reduced growth and citric acid yield while *n*-butanol at 10 g l^{-1} concentration was toxic to moulds (Chaudhary *et al.* 1978). Marchal *et al.* (1977), used *Saccharomycopsis lipolytica* to produce citric acid from *n*-paraffins. Considering the above facts, this study was carried out to find the role of methanol in citric acid production.

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Materials and Methods

Organism

Locally isolated *Aspergillus niger* was used in this study. The culture was maintained on potato–dextrose agar (PDA) slants at room temperature and subcultured every 2 weeks (Navaratnam *et al.* 1996).

Analytical Methods

Reducing sugars in the medium were estimated by the dinitrosalicylic acid method (Miller 1959). Citric acid was estimated by the pyridine–acetic anhydride method (Marier & Boulet 1958). Citric acid produced by the organism is presented in two ways as extracellular (in the medium) and intracellular (in the mycelium).

To estimate the intracellular citric acid, the bottom part of the mycelial mat (wet weight 12 g) was washed with 20 ml and 5 ml of distilled water in succession. Excess water was drained by placing the mycelium on a glass plate kept in slant position for 15 min and homogenized in a mortar and pestle with 1 g acid-washed sand and 10 ml distilled water. The homogenate was centrifuged (3000 rev/min, 10 min) and the supernatant was collected. The residue was washed with 10 ml distilled water and centrifuged. The supernatants were pooled and assayed for citric acid (Marier & Boulet 1958).

The mycelia obtained under different experimental conditions were washed with 0.01 M phosphate buffer-saline (pH 7.0) and dried at 80 °C to constant weight.

Medium

Basal medium contained (g l^{-1}): glucose, 50.0; NH_4NO_3, 0.5; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; peptone, 7.0; $ZnSO_4 \cdot 7H_2O$, 0.1×10^{-3}; ferrous ammonium sulphate, 0.1×10^{-3} and $CuSO_4 \cdot 5H_2O$, 0.06×10^{-3}.

Effect of Different Concentrations of Methanol
To evaluate the effect of methanol on citric acid production, basal medium was supplemented with varying concentrations of methanol (0, 20, 30 and 40 ml l^{-1}). The tests had their respective controls with additional glucose (0, 18.2, 27.3 and 36.4 g l^{-1}) instead of methanol to equalize the total carbon content. The media were inoculated with spore suspension (7×10^6 spores ml^{-1}) from a 6-day-old culture and incubated at 30 °C under diffused light. Extracellular citric acid and reducing sugar in the media were monitored.

Role of Methanol on Citric Acid Production
In this experiment, four different media were prepared: basal medium (medium A); basal medium with optimized amount of methanol (medium B); medium containing mineral solution of basal medium and methanol equivalent to the elemental carbon content contributed by glucose and methanol of medium B (medium C); medium containing mineral solution of basal medium and methanol equivalent to the carbon content of medium A (medium D). The experiment was carried out as above. In addition the intracellular citric acid was estimated. At the end of the experiment the dry weight of the mycelium was determined.

Effect of Glucose on Citric Acid Production
Basal medium with three different concentrations of glucose (50, 100 and 150 g l^{-1}) were taken with and without the addition of optimized amount of methanol. The experiment was run on as above. When the reducing sugar was about 1 g l^{-1} the intracellular citric acid was estimated.

Results and Discussion

Effect of Methanol
In basal medium the fungus produced 4.6 g extracellular citric acid l^{-1} at 96 h (Figure 1). As the production of extracellular citric acid was low, the medium was supplemented with different concentrations of methanol (20, 30 and 40 ml l^{-1}). Citric acid production increased with increased methanol concentration up to 30 ml l^{-1} and any further increase in methanol concentration did not improve citric acid production. Maximum citric acid secreted into the medium containing 30 ml methanol l^{-1} was 7.8 g l^{-1} (Figure 1). Therefore it can be assumed that methanol has a stimulatory effect on citric acid production or excretion or both, or that methanol could have been utilized as carbon source.

Citric acid produced in the media containing glucose equivalent to different concentrations of methanol in basal medium was very much less than that produced in basal medium and in the respective test media (Figure 1). These results indicate that increase in glucose concentration from 50 g l^{-1} to 68.2, 77.3 and 86.4 g l^{-1} had decreased citric acid production. This decrease in citric acid production with the increase in glucose concentration could have been due to the osmotic effect of glucose. However the addition of methanol did not show a similar effect and seemed to improve citric acid production. Therefore to confirm the effect of methanol, an optimized

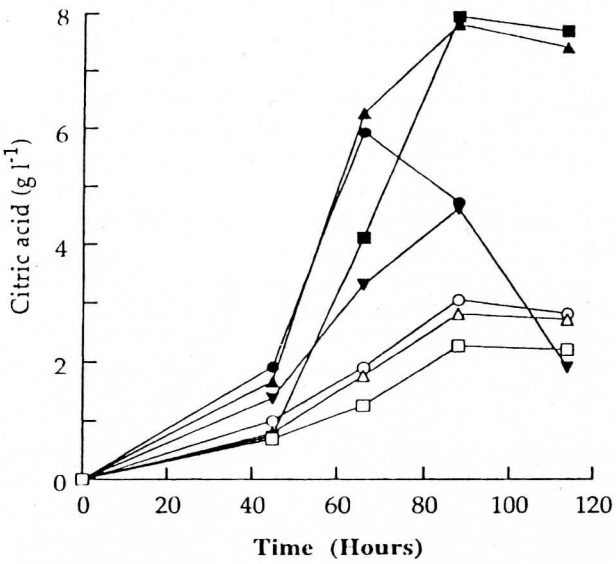

Figure 1. Effect of different concentrations of methanol on citric acid production by *Aspergillus niger*. Test media contained: ▼, 0; ●, 20; ▲, 30; ■, 40 ml methanol l^{-1}. Open symbols indicate the respective controls.

amount of methanol (30 ml l^{-1}) should be used in basal medium with different concentrations of glucose.

Role of Methanol in Citric Acid Production
From the previous experiment it was observed that the addition of methanol to basal medium increased citric acid production (medium A and B; Figure 2). To determine whether methanol was being used as a carbon source, a medium (medium C) containing methanol equivalent to the elemental carbon content (30.9 g l^{-1}) of medium B was prepared. This medium contained 85 ml of methanol l^{-1} (the optimized amount of methanol was 30 ml l^{-1}). In medium C no citric acid production and surface film formation were observed. The measurement of mycelial dry weight proved that the fungus did not grow in medium C, showing that the fungus does not use methanol as carbon source. When medium C inoculated with spores was inspected under the light microscope (× 400) after 2 days, germination of spores was not observed; this suggested that 85 ml methanol l^{-1} in the basic medium without glucose inhibited the germination of fungal spores. This amount of methanol must be toxic to the fungus in the absence of glucose. High methanol concentration could have also been a reason for the inhibition of germination of the spores because in medium B a lower concentration of methanol (30 ml l^{-1}) was used. Therefore methanol equivalent to the amount supplemented to the basal medium (as in medium B, 30 ml l^{-1}) was used as the carbon source with all the other nutrients similar to that in basal medium (medium

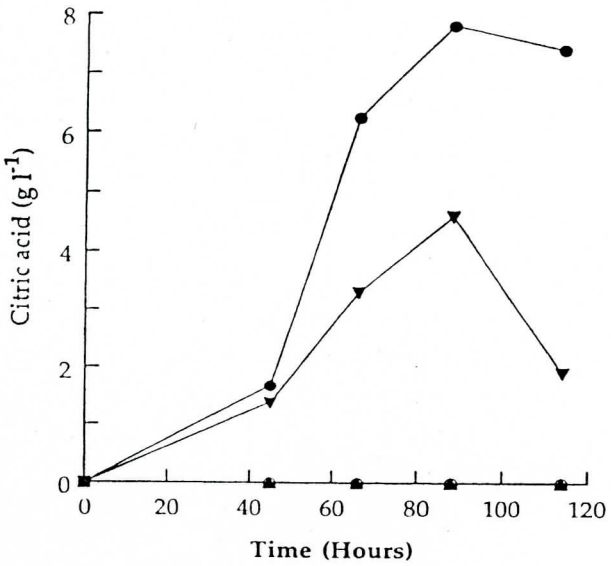

Figure 2. Effect of methanol (30 ml l^{-1}) on citric acid production by *Aspergillus niger*. ▼, Basal medium (medium A); ●, basal medium with 30 ml methanol l^{-1} (medium B); ■, glucose-free basal medium containing methanol to equalize elemental carbon contributed by glucose and 30 ml methanol l^{-1} (medium C); ▲, glucose-free basal medium supplemented with 30 ml methanol l^{-1} (medium D).

D). In medium D both growth of the fungus and the citric acid production were not observed (Figure 2). These results show that methanol was not utilized as a carbon source by the fungus for growth and production of citric acid in the absence of glucose. In addition it can be said that not only 85 ml methanol l^{-1} had inhibited the germination of the spores but also 30 ml methanol l^{-1} gave the same effect. Moyer (1953a) has suggested that ethanol could be assimilated and converted to citric acid while methanol cannot be assimilated. Therefore methanol cannot promote citric acid production in the absence of glucose under these experimental conditions. Thus methanol must be either stimulating citric acid production or increasing the secretion of citric acid into the medium. Hence to find the effect of methanol, the intracellular and extracellular citric acid concentrations were determined.

As observed for extracellular citric acid, the intracellular citric acid was also high in the mycelium grown in medium B (0.322 g l^{-1}) than in medium A (0.228 g l^{-1}). If the percentage of citric acid secreted into the medium was compared with that of the total produced, in the two media the citric acid excreted into the medium was almost the same (95.2–96.0%). Therefore we can conclude that the methanol added was stimulating citric acid production and was neither used as a carbon source nor improved the membrane permiability for citric acid. Our results are contradictory to those reported by Kapoor *et al.* (1982). A stimulatory effect of methanol has been reported by Chaudhary *et al.* (1978) and Hang & Woodams (1989). The increase in citric acid production in medium B compared with that in medium A could be due to the decreased mycelial growth (from 15.6 to 13.7 g l^{-1}). Therefore it can be assumed that methanol channels glucose for citric acid production by decreasing the utilization of glucose for cell multiplication. Methanol retards growth and sporulation, and prolongs the vegetative phase (Chaudhary *et al.* 1978). Methanol alone has an inhibitory effect on the germination of the spores and citric acid production; this is evident from the studies made with media C and D.

Effect of Different Concentrations of Glucose

Considering the positive effect of methanol on citric acid production, methanol (30 ml l^{-1}) was supplemented to basal medium containing different concentrations of glucose (50, 100 and 150 g l^{-1}). A prominent difference in citric acid production was observed with respect to their controls (Figure 3). This again showed the stimulatory effect of methanol on citric acid production. In two of the test media containing 100 and 150 g glucose l^{-1} with 30 ml methanol l^{-1}, maximum citric acid (12.1 g l^{-1}) was produced at day 7 while in the medium containing 50 g glucose l^{-1}, only 7.6 g of citric acid l^{-1} was produced on day 4 (Figure 3). The efficiency of citric acid production was 16.9, 13.2 and 10.3% when 50, 100 and 150 g glucose l^{-1} were respectively used along with 30 ml methanol l^{-1} (efficiency (%) = citric acid produced/expected citric

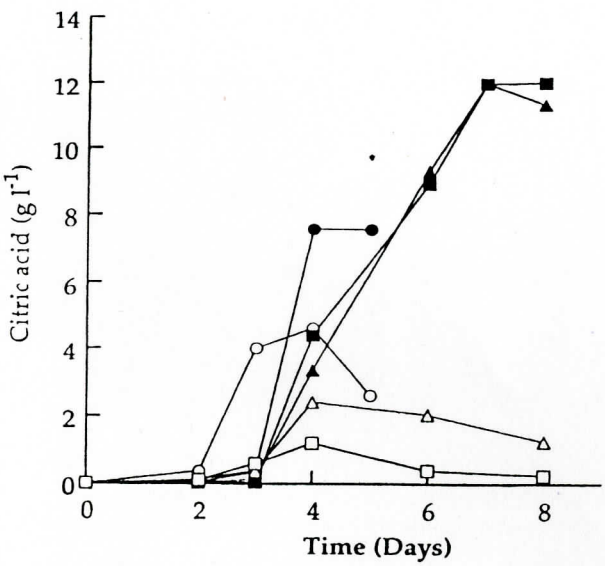

Figure 3. Effect of different concentrations of glucose on citric acid (extracelluar) production by *Aspergillus niger*. ●, 50; ■, 100; ▲, 150 g glucose l^{-1}. Closed and open symbols indicate the media with and without 30 ml methanol l^{-1} respectively.

Table 1. Effect of different concentrations of glucose on intracellular and extracellular citric acid production and dry weight of mycelium.

Initial glucose (g l^{-1})	Citric acid (g l^{-1})				Dry weight (g l^{-1})	
	Intracellular Methanol (ml l^{-1})		Extracellular Methanol (ml l^{-1})		Methanol (ml l^{-1})	
	0	30	0	30	0	30
50	0.0228	0.322	4.6	7.6	15.6	3.7
100	0.0210	2.900	2.4	12.1	16.0	16.5
150	0.0050	2.700	1.2	12.1	16.2	17.4

acid × 100). Therefore an increase in glucose concentration from 50 to 100 g l^{-1} would be useful to increase citric acid production from 7.6 to 12.1 g l^{-1} in the presence of 30 ml methanol l^{-1}. Any further increase in glucose concentration showed no useful effect. However citric acid production in media containing a higher glucose concentration than 50 g l^{-1} even in the presence of methanol was delayed (from day 4 to day 7). This could be due to the osmotic effect exerted by glucose.

When the intracellular and extracellular (secreted) citric acid concentration in the presence (tests) and absence (controls) of methanol were compared, intracellular citric acid in presence of methanol increased with the increase in concentration of glucose upto 100 g l^{-1} and further increase in glucose concentration in test media decreased the intracellular citric acid concentration under our experimental conditions (Table 1). Maximum intracellular citric acid obtained in the test medium containing 100 g glucose l^{-1} was 2.9 g l^{-1} whereas in the control medium the intracellular concentration of citric acid decreased with a concomittant increase in glucose concentration. From this result it can be concluded that intracellular citric acid was increased by the stimulatory or channelling of glucose by methanol (30 ml l^{-1}) but this effect of methanol was not seen with a further increase in glucose concentration. However if the percentage of citric acid excreted into the medium in the presence and absence of methanol was compared there was no significant difference. Therefore this again confirmed that methanol did not improve the membrane permeability for citric acid excretion.

When the reducing sugar concentration in the medium was considered, sugar uptake was almost the same in tests and the respective controls (Figure 4). As the rate of glucose uptake in tests and the respective controls were same (Figure 4), it can be assumed that methanol did not affect the rate of sugar uptake but may have channelled the glucose more into citric acid production than into growth.

Dry weights of the mycelia were almost the same indicating that the growth in all the cases was similar

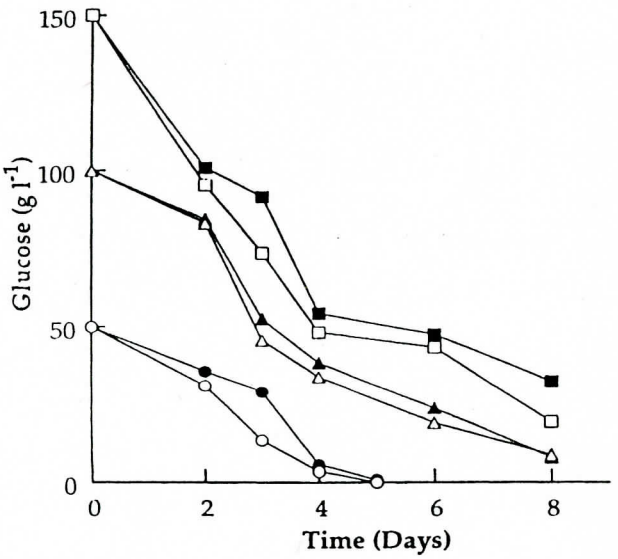

Figure 4. Effect of different concentration of glucose on glucose uptake in the presence and absence of methanol by *Aspergillus niger*. Test media contained: ●, 50; ■, 100; ▲, 150 g glucose l^{-1} and 30 ml methanol l^{-1}. Open symbols indicate the respective controls without methanol.

except that in the medium having 50 g glucose l^{-1} and 30 ml methanol l^{-1} (Table 1). These results indicated that the effect of methanol is more evident in the presence of lower concentrations of glucose than in the presence of higher glucose concentrations.

Conclusions

The results indicate that incorporation of methanol had channelled glucose for citric acid production. Methanol was not used as a carbon source in the absence of glucose and it did not improve the membrane permeability to citric acid. Experiments should be performed to find the specific reaction step/s where methanol shows its stimulatory or channelling effect.

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