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# ITACONIC ACID PRODUCTION BY IMMOBILIZED *ASPERGILLUS TERREUS* FROM XYLOSE AND GLUCOSE

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## SUMMARY

*Aspergillus terreus* NRRC 1960 spores were entrapped in calcium alginate gel beads or alternately the fungal mycelium was immobilized either on Celite R-626 or in agar gel cubes, and the biocatalyst was employed both in repeated batch and in continuous column reactors to produce itaconic acid from D-xylose or D-glucose. The highest itaconic acid yield obtained in a submerged culture batch fermentation was 54.5% based on total initial glucose (55 g/l) with a volumetric productivity of 0.32 g/l h, and 44.8% from xylose (67 g/l) with a productivity of 0.20 g/l h. In a repeated batch fermentation mycelium immobilized in agar gel had a productivity of 0.12 g/l h, and mycelium grown from spores immobilized in calcium alginate gel 0.06 g/l h, both from xylose (60 g/l). With the best immobilized biocatalyst system used employing Celite R-626 as a carrier, volumetric productivities of 1.2 g/l h from glucose and 0.56 g/l h from xylose (both at 60 g/l) were obtained in continuous column operation for more than 2 weeks.

## INTRODUCTION

Itaconic acid, methylene succinic acid, is an important intermediate in polymer production. It may be used in styrene butadiene copolymers, in acrylonitrile copolymers for synthetic fibre manufacture, in lattice and in emulsions in general, and to improve fibre properties such as dyeing characteristics. According to Miall (1978) the most important use of styrene copolymers is in paper coating and carpet backing. The overpro-

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duction of itaconic acid by certain fungi was first reported by Kinoshita (1929). It is produced by *Aspergillus terreus*, *A. itaconicus* and *Ustilago zeae* (Miall, 1978), and by a number of *Candida* sp. yeasts (Kawamura, 1983). Itaconic acid has been manufactured by submerged fermentation by Pfizer in the U.S.A. and by Iwata in Japan. Moyer and Coghill (1945), and Lockwood and Reeves (1945) screened a large number of strains discovering good producers *A. terreus* NRRC 265 and NRRC 1960. Lockwood and Ward (1945) reported as best yield 30% on total glucose. In a semi-pilot plant scale in a 20 l fermentor Nelson *et al.* (1952) obtained 45 - 54% itaconic acid by weight from 6% initial glucose. In a pilot scale of 300 - 600 gallons a maximum yield of 64.2% from 6.15% initial glucose was obtained (Pfeifer *et al.*, 1952). Pfizer (1948) patented a process for the production of itaconic acid in a submerged culture, claiming a yield of 27.7% in two weeks from pure sucrose. According to Fries (1966) *A. terreus* fermented beet molasses to itaconic acid in a yield of 66.2%, and technical hydrolyzed starch with a conversion of 60%, both in 6 days, whereas Batti and Schweiger (1960) claimed a yield of 58% on total supplied sucrose. Larsen and Heyden (1956) also obtained similar results.

Horitsu *et al.* (1983) recently reported the production of itaconic acid by polyacrylamide gel entrapped *A. terreus* mycelium from 6% glucose at a yield of 60 mg/h employing 40 g of biocatalyst gel in a continuous reactor fermentation. The only previous investigation based on D-xylose as substrate is to our knowledge the work by Nowakowska-Waszczuk and Marciniak (1974), who claimed a conversion of 51% from D-glucose and of 44% from D-xylose in 50 ml batch fermentations. As part of our research programme on biomass utilization and biotechnical fine chemical production we have investigated the production of itaconic acid both from D-glucose and D-xylose both in submerged culture and with immobilized *A. terreus*.

## EXPERIMENTAL

### *Culture of microorganism*

*Aspergillus terreus* NRRC 1960 used in this investigation was maintained both on a modified Czapek-agar containing D-xylose instead of sucrose and on potato-dextrose-agar. The mycelium was cultivated in a 14 litre Biostat E fermentor (B. Braun AG, Germany), using 10 l of a medium containing 67 g/l D-xylose, 3.3 g/l $(NH_4)_2SO_4$, 0.8 g/l $MgSO_4 \cdot 7\ H_2O$, 0.088 g/l $KH_2PO_4$ and 0.004 g/l $CuSO_4 \cdot 5H_2O$. The initial pH was 3.1, temperature 36°C, and the dissolved oxygen profile 75% for one day, 50% for 3 days and 25% for one day. A 10% inoculum was used, and the mycelium was harvested by centrifuging at 5900×g for 20 min, followed by washing with water.

### *Immobilization*

Celite R-626. The fungal mycelium was grown on Celite R-626 (Manville Co., U.S.A.) in shake flasks containing 100 ml each of the medium described above for 3 days under agitation at 130 rev/min.

Agar gel. Six grams of wet mycelium (0.52 g d.m.) was added to 20 g of 1% agar solution at 45°C and mixed. After the hardening of the agar on cooling the biocatalyst was cut into 3mm cubes.

Calcium alginate gel. Three millilitres of spore suspension with $10^2$- $10^6$ spores per millilitre was mixed with 20 g of 6% sterilized (10 min at 108°C) sodium alginate (BDH Chemicals, England), extruded into 0.5 M calcium chloride, and allowed to harden under mixing for 20 min. The biocatalyst beads were washed with distilled water. The immobilized spores were subsequently germinated for 6 days at 36°C in 100 ml of the medium described above.

*Analytical methods*

Itaconic acid. Itaconic acid was determined by HPLC (Varian 5000) using a UV-detector (Knauer Variable Wavelength Monitor) at 210 nm and an integrator (Hewlett-Packard 3390A). A 300 mm Aminex HPX-87 ion-exchange column (Bio-Rad) at 75°C was used with 0.025 M sulfuric acid as eluent at 0.7 ml/min. Samples were boiled for 20 min and membrane filtered (Gellman Sciences GA6 membrane with 0.45 µm pores) before analyzing.

Reducing sugars. Reducing sugars were determined according to Nelson (1944).

Dry matter. For mycelium dry weight determination a 20 ml sample was filtered through Whatman No 1 paper, washed with 20 ml of distilled water, and dried at 55°C for 24 h.

*Itaconic acid production.*

Batch fermentation. Itaconic acid was produced in 2 and 10 litre batches on 55 g/l glucose with 3.3 g/l $(NH_4)_2SO_4$ or on 67 or 100 g/l xylose with 3.3 g/l $(NH_4)_2SO_4$ or 3 g/l $NH_4NO_3$. The rest of the medium was as described earlier. The initial pH was 3.1, temperature 36°C, and aeration rate of 0.7 l/l min.

Repeated batch fermentation. Repeated batch fermentations were carried out in 250 ml erlenmeyer flasks containing 100 ml of the medium and either free cells (1.5 g wet weight) of immobilized biocatalyst (6.5 g wet weight) at 36°C, 130 rev/min. The media used were as described for batch fermentations, except that in all cases 60 g/l of sugar was used.

Continuous fermentation. Continuous fermentations were carried out with Celite R-626 immobilized mycelium, employing 16 × 70 mm packed-bed column reactors with aeration and 9 h residence time. The medium used was as described for repeated batch fermentations, except that $KH_2PO_4$ was omitted.

## RESULTS AND DISCUSSION

*Batch fermentation with free cells*

The highest itaconic acid yield of 30 g/l (54.5% w/w based on initial glucose of 55 g/l) by *Aspergillus terreus* NRRC 1960 in conventional submerged culture batch fermentation with 2 litres of medium was obtained after 4 days (Fig. 1). At this time all glucose was consumed. The pH decreased during the fermentation from the initial value of 3.1 to 2.3 during the first day, and remained constant thereafter. Nowakowska-Waszczuk and Marciniak (1974) obtained a yield of 51% from 100 g/l glucose in 50 ml cultures in 9 days, and Pfeifer *et al.* (1952) have reported the highest itaconic acid yield of 64.2% from 61.5 g/l glucose in pilot fermentation.

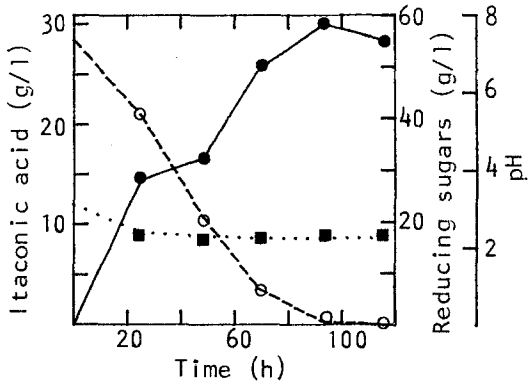


Fig. 1. Itaconic acid production in a 2-litre batch fermentation with free mycelium from glucose (55 g/l); ● itaconic acid, o reducing sugars, ■ pH.

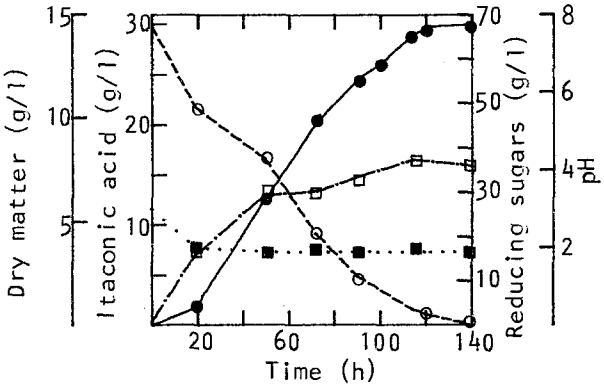


Fig. 2. Itaconic acid production in a 10-litre batch fermentation with free mycelium from xylose (67 g/l); ● itaconic acid, o reducing sugars, ■ pH, □ dry matter.

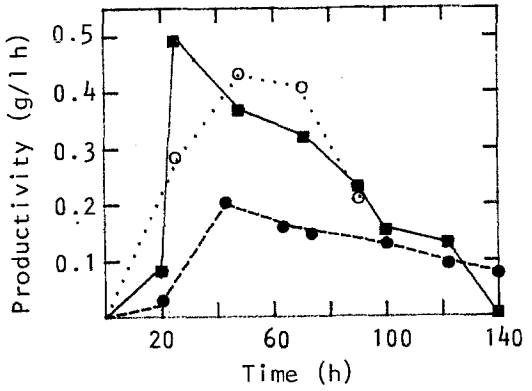


Fig. 3. Itaconic acid productivity; o glucose (55 g/l) and ● xylose (100 g/l) in 2-litre fermentations, and ■ xylose (67 g/l) in 10-litre fermentation.

In 2-litre fermentations the total volumetric itaconic acid productivity from glucose (55 g/l) was 0.32 g/l h, and the maximum between the 2nd and 3rd day of fermentation 0.43 g/l h, agreeing well with the results of Larsen and Hovden (1956) with sucrose. The total productivity from xylose (100 g/l) was only 0.12 g/l h, with the maximum of 0.20 g/l h (Fig. 3). However, in 10-litre fermentations a significantly higher total productivity from xylose (67 g/l) was obtained, with a maximum of 0.50 g/l h reached already after the first day of fermentation (Fig. 3). This corresponded to a yield of 30 g/l (44.8% based on initial xylose of 67 g/l) in 5 days, and a total volumetric productivity of 0.20 g/l h (Fig. 2). The xylose was completely consumed in 6 days. During the fermentation period a total of 3.82 g of itaconic acid was produced per one gram of dry mycelium, significantly more than the 1.44 g reported by Nowakowska-Waszczuk and Marciniak (1974) from xylose (100 g/l) in 50 ml batch fermentation in 9 days.

*Immobilized cells*

Repeated batch fermentation. The agar gel cube immobilized *Aspergillus terreus* NRRC 1960 biocatalyst system in shake flasks with xylose as substrate did not appear to be very stable (Fig. 4). The highest volumetric productivity was in this case 0.12 g/l h, corresponding to a yield of 13.8 g/l (23% based on initial xylose of 60 g/l) in 5 days. In contrast, a very stable itaconic acid production from xylose could be obtained with calcium alginate gel entrapped spores, which on germination formed a very dense mycelium layer near the surface of the biocatalyst beads. After the 2nd batch a relatively constant itaconic acid production of about 7 g/l (23%) and a volumetric productivity of 0.06 g/l h could be maintained for at least 16 batches. The initial spore quantity on immobilization did not significantly affect results. The inclusion of $MgSO_4$ and the omission of $KH_2PO_4$ was necessary for stable production. Consequently, the complete nutrient medium was reduced after the 2nd batch to contain only xylose, ammonium nitrate and magnesium sulphate.

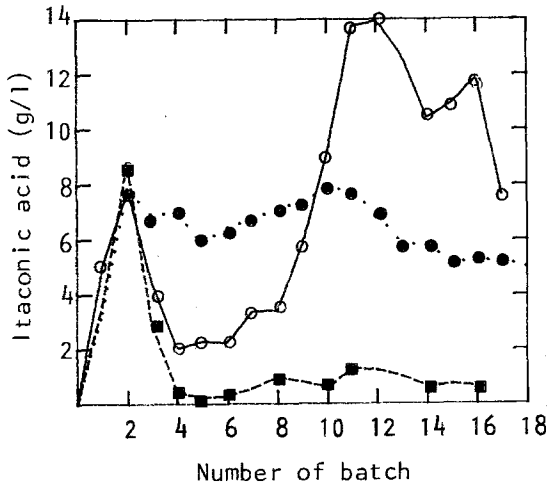


Fig. 4. Itaconic acid production in repeated batch fermentations from xylose (60 g/l) by immobilized biocatalyst; o mycelium in agar gel, ● spores in alginate gel, ■ free cells.

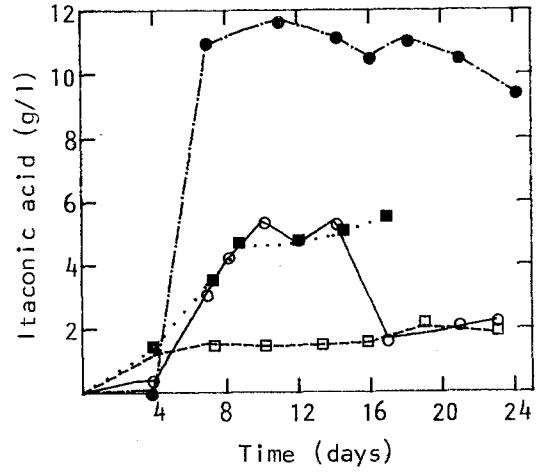


Fig. 5. Itaconic acid production in continuous column reactor with mycelium immobilized on Celite; o● glucose, □■ xylose, ●■ with $NH_4NO_3$, o□ with $(NH_4)_2SO_4$.

Continuous fermentation. *Aspergillus terreus* NRRC 1960 mycelium immobilized on Celite R-626 produced from glucose about 11 g/l of itaconic acid (18% based on initial glucose of 60 g/l) at a residence time of 9-10 h for more than 2 weeks, if ammonium nitrate was used as the nitrogen source (Fig. 5). The productivity was about 1.2 g/l h, almost 4 times that obtained in conventional batch fermentation with free mycelium and equal to or better than that obtained by Horitsu *et al.* (1983) with polyacrylamide gel entrapped mycelium. The corresponding production from xylose was about 5 g/l, with a yield of about 8% and a productivity of 0.56 g/l h. Also in this case the productivity was about twice that obtained in conventional batch fermentation. The production decreased sharply when the aeration was discontinued after 13 days. Further, a significantly poorer production was obtained both from glucose and xylose when ammonium sulphate was used as the nitrogen source.

ACKNOWLEDGEMENT

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