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IMMOBILIZED *ASPERGILLUS TERREUS* IN ITACONIC ACID PRODUCTION FROM GLUCOSE

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Summary

The production of itaconic acid by immobilized *Aspergillus terreus* TKK 200-5-1 was studied both in shake flask cultures, and in continuous column bioreactors. The effect of glucose and ammonium nitrate concentrations, and of pH were examined using a statistical experimental plan. The highest itaconic acid product concentration could be reached at the highest investigated glucose concentration of 150 g/l and the highest initial pH of 3.75, in the absence of ammonium nitrate. In a continuous packed bed column system operated for 4.5 months itaconic acid was obtained at a productivity of 328 mg/d per gram of polyurethane foam carrier.

Introduction

Itaconic acid (methylene succinic acid), a valuable intermediate for copolymers, was first studied with an immobilized fungus by Horitsu *et al.* (1983). Polyacrylamide gel-immobilized *Aspergillus terreus* G-26 produced itaconic acid from 6% glucose at a rate of 60 mg/h. After this Kautola *et al.* (1985, 1987, 1989 and 1990) and Kautola (1990) have investigated itaconic acid production with immobilized *Aspergillus terreus* TKK 200-5-1 (NRRC 1960), TKK 200-5-2 and TKK 200-5-3 immobilized on different carriers such as calcium alginate, agar and polyurethane gels, polyurethane foam, nylon net and Celite. Most work was done on xylose or sucrose as the carbon source, with some continuous experiments on glucose. With the immobilized mycelium the highest volumetric productivity of 1.2 g/l•h on glucose was reached with continuous Celite-immobilized packed bed column bioreactors (Kautola *et al.*, 1985). More recently, Kautola *et al.* (1990) investigated systematically the influence of a number of salts on itaconic acid production. In the present work the influence of ammonium nitrate and the initial pH together with glucose on itaconic acid production was similarly studied.

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Materials and methods

Microorganism. The itaconic acid fermentations were carried out using the fungus *Aspergillus terreus* TKK 200-5-1, maintained on potato-dextrose-agar slants.

Media. The growth medium contained 60 g/l glucose, 4 g/l ammonium nitrate, 0.95 g/l magnesium sulphate, 0.004 g/l copper(II)sulphate and 0.088 g/l potassium dihydrogenphosphate. The production medium contained 50 to 150 g/l glucose, 0 to 4 g/l ammonium nitrate, 0.004 g/l copper(II)sulphate and 0.95 g/l magnesium sulphate.

Immobilization and fermentation procedure. In the *repeated batch fermentation* 0.5 g of one cm^3 polyurethane foam (pore size 1.5-1.7 mm, Espe, Finland) prewashed cubes submerged in 100 ml of growth medium in a 250 ml erlenmeyer flask were sterilized at 121 °C for 20 min. The carrier cubes inoculated with 6 day-old *A. terreus* spores from one slant were germinated for 3 days and washed with sterile distilled water, and transferred to 100 ml of the production medium in 250 ml erlenmeyer flasks. The immobilized mycelium was transferred every 14th day into fresh production medium corresponding the previous batch. The fermentation was carried out at 36 °C under agitation at 200 rev/min. The initial pH was 2.9 for the germinating phase and 1.72 to 3.75 for the production phase (Kautola *et al.*, 1987).

In *the continuous fermentation* a 130 ml water jacketed glass column with 0.95 g of polyurethane foam cubes was sterilized in an autoclave and inoculated spores from one slant adding 95 ml of growth medium. The packed bed bioreactors were aerated at 100 ml/min for one week after which the continuous production with a residence time of 190 h and aeration rate of 100 ml/min was started with the production medium (Kautola *et al.*, 1990).

Analytical methods. Itaconic acid was determined with a Varian 5000 HPLC with a 30 cm Aminex HPX-87 (Bio-Rad) ion exchange column at 65 °C, and at a flowrate of 0.7 ml/min of 0.4 mM sulphuric acid as the mobile phase. A Knauer UV-detector was used at 210 nm. Samples were boiled for 20 min and filtered through 0.2 μm membranes (Kautola *et al.*, 1985).

Statistical experimental design. A statistical experimental design (Box and Hunter, 1957) was used in the systematic investigation of the influence of the independent variables glucose (X_1, 50, 64.7, 100, 135.3 and 150 g/l), ammonium nitrate (X_2, 0, 0.59, 2, 3.41 and 4 g/l) and initial pH (X_3, 1.72, 2.04, 2.75, 3.46 and 3.75) in repeated batch fermentations. Itaconic acid concentration (Y_1, g/l) and volumetric productivity (Y_2, g/l•d) at the end of each batch were chosen as the dependent output variables. The variables X_i were coded as x_i for the statistical calculations according to $x_i = (X_i - X_0) \cdot \Delta X^{-1}$, where x_i = coded value of the variable X_i, X_0 = the value of X_i at the center point of the investigated area, and ΔX = the step change (Kautola *et al.*, 1991). A 2^3-factorial experimental design with 6 star-points (α = 1.415) and four replicates at the center point was employed (Kautola *et al.*, 1989). To estimate the responses of the dependent variables second degree polynomials were calculated using the SPSS/PC+ data programme (Thompson, 1982).

Results and discussion

Repeated batch fermentations

The effects of glucose and ammonium nitrate concentrations, and of the initial pH were studied in repeated batch shake flask fermentations using polyurethane foam immobilized *Aspergillus terreus* mycelium. At the end of each batch the calculated multiple correlation coefficient of the obtained polynomial models for the itaconic acid concentration increased from 0.69 in the first batch to 0.81 in the third batch, depending on the variation in the inoculum, germination, and the variations in the repeat tests. The behaviour of the volumetric productivity was similar. This is in a good accordance with results obtained by Kautola *et al.* (1991) when investigating the effects of metal additions. One can clearly see from Figure 1 that the increase in glucose concentration in the absence of ammonium nitrate increased itaconic acid product concentration more than at the level of 4 g/l of ammonium nitrate. This suggests that at high nitrogen levels more glucose is used for growth and maintenance. The knowledge that an increased glucose concentration in the absence of nitrogen results in a higher itaconic acid concentration, was valuable in designing the experiments for the use of immobilized biocatalyst systems in continuous fermentations.

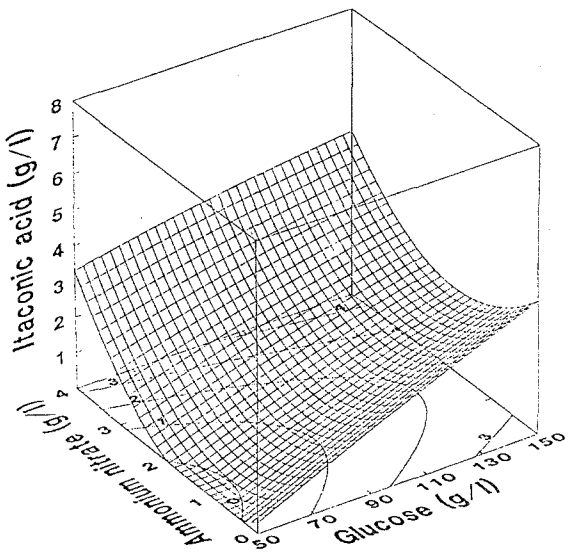

Figure 1. Itaconic acid concentration (g/l) in a repeated batch 250 ml shake flask fermentation by immobilized *A. terreus* mycelium, as a function of glucose and ammonium nitrate at pH 2.75.

According to Rychtera and Wase (1981) the highest itaconic acid product concentrations are reached with freely decreasing pH during the fermentation. Accordingly, the pH was not controlled in this work. Nevertheless, the initial pH was of great importance. According to Larsen and Eimhjellen (1955) a low pH closer to 2 than 6 is necessary for the formation of at least a part of the enzymic system for itaconic acid production with the free mycelium. Consequently the pH was varied between 1.72 and 3.75. Figure 2 shows that the increase in glucose concentration needed an increase in the initial pH for maximum itaconic acid

production, in this case about 7 g/l. This is in a good agreement with the results obtained on sucrose (Kautola *et al.*, 1989), but with xylose the optimum initial pH was lower, 2.5 (Kautola, 1990). Figure 3 illustrates that at a high nitrogen level pH had very little effect on itaconic acid production in the investigated area but in the absence of nitrogen a higher pH results in a higher product concentration. The highest actual itaconic acid concentration obtained in the investigated area was 6.1 g/l with a volumetric productivity of 0.433 g/l·d. This was slightly higher than the calculated 5 g/l in Figure 1 when glucose was at 150 g/l, ammonium nitrate at 4 g/l and initial pH at 2.75.

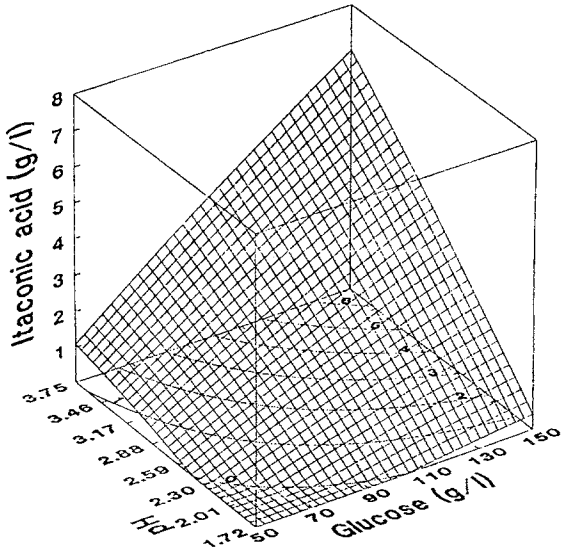

Figure 2. Itaconic acid concentration (g/l) in repeated batch 250 ml shake flask fermentation by immobilized *A. terreus* mycelium, as a function of glucose and pH at the zero level of ammonium nitrate concentration.

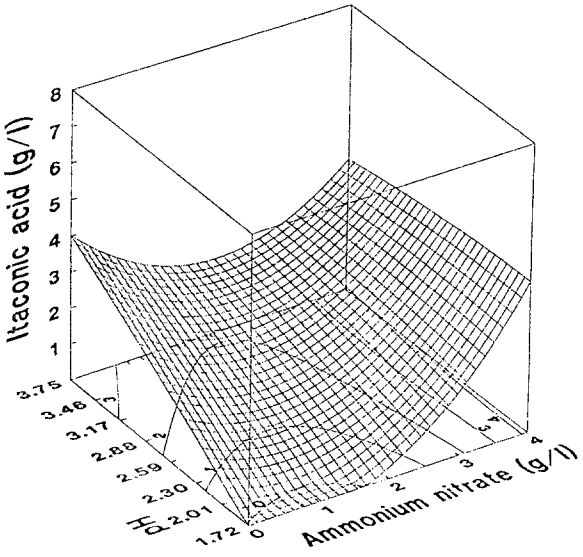

Figure 3. Itaconic acid concentration (g/l) in repeated batch 250 ml shakeflask fermentation by immobilized *A.terreus* mycelium, as a function of ammonium nitrate and pH at glucose concentration of 100 g/l.

In conclusion from the shake flask experiments, the highest itaconic acid product concentration could be reached at the highest glucose concentration of 150 g/l and the highest pH of 3.75, when no ammonium nitrate was added.

Continuous fermentation

In a continuous packed bed bioreactor the itaconic acid production process could be carried out for at least 4.5 months which shows a remarkable stability (Fig.4). The experiment was carried out at pH 3.0 using different medium compositions (Kautola *et al.*, 1990). It could be shown that nitrogen supplementation was not necessary for continuous column itaconic acid production. When the production medium contained only 90 g/l glucose and 0.95 g/l magnesium sulphate the itaconic acid concentration reached was 18 g/l with a productivity of 0.227 g/d per one gram of carrier, whereas in the precense of ammonium nitrate a concentration of about 26 g/l with a productivity of 0.328 g/d per gram of carrier, and a volumetric productivity of 3.5 g/l·d was obtained. According to Kautola *et al.* (1987) the itaconic acid production by the free mycelium in a 14-liter bioreactor as compared with optimized shake flasks experiments gave nearly double product concentration, probably because of a better aeration control. The only likely reason for scale-up problems is that transport processes are very dependent on scale (Kossen and Oosterhuis, 1985). Further, in the present work the continuous column bioreactor which gave the higher itaconic acid concentrations, had almost double amount of immobilized cubes. The aeration conditions were also easier to control. Consequently, the packed bed column fermentation seemed to be a more suitable system for itaconic acid production.

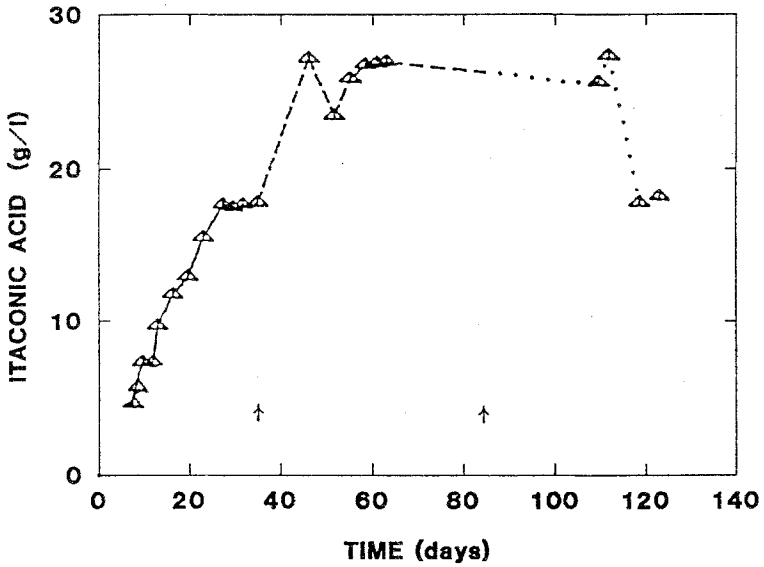

Figure 4. Continuous itaconic acid production with immobilized *A. terreus* mycelium in column bioreactor from 9% glucose with aeration rate of 100 ml/min, residence time of 190 h and initial pH of 3.0 at 36 °C with medium containing 2.5 g/l ammonium nitrate, 0.004 g/l copper(II)sulphate and 0.95 g/l magnesium sulphate (—) or 2.5 g/l ammonium nitrate and 0.95 g/l magnesium sulphate (- -) or 0.95 g/l magnesium sulphate (····). Arrows indicate the change of substrate composition.

References

Box, G.E.P., and Hunter, J.S. (1957). *Ann. Math. Stat.* 28, 195-241.

Horitsu, H., Takahashi, Y., Tsuda, J., Kawai, K., and Kawano, Y. (1983). *Eur. J. Appl. Microbiol. Biotechnol.* 18, 358-360.

Kautola, H. (1990). *Appl. Microbiol. Biotechnol.* 33, 7-11.

Kautola, H., Grönlund, B., Horitsu, H., Linko, Y.-Y., and Linko, P. (1987). Optimization of itaconic acid production from glucose by free and immobilized *Aspergillus terreus*. In: *Proc. 4th Eur. Congr. Biotechnol.*, O.M. Neijssel, R.R. Van der Meer and K.Ch.A.M. Luyben, eds. vol. 1, pp. 106-109, Amsterdam: Elsevier Scientific Publishers.

Kautola, H., Rymowicz, W., Linko, Y.-Y., and Linko, P. (1991). *Appl. Microbiol. Biotechnol.* 35, 154-158.

Kautola, H., Vahvaselkä, M., Linko, Y.-Y., and Linko, P. (1985). *Biotechnol. Lett.* 7, 167-172.

Kautola, H., Vassilev, N., and Linko, Y.-Y. (1989). *Biotechnol. Lett.* 11, 313-318.

Kautola, H., Vassilev, N., and Linko, Y.-Y. (1990). *J. Biotechnol.* 13, 315-323.

Kossen, N.W.F., and Oosterhuis, N.M.G. (1985). Modelling and scaling-up of bioreactors. In: *Biotechnology*, H.-J. Rehm and G. Reed, eds. vol. 2, pp. 571-605, Weinheim: VCH Verlagsgesellschaft.

Rychtera, M., and Wase, D. (1981). *J. Chem. Technol. Biotechnol.* 31, 509-521.

Thompson, D.J. (1982). *Food Process Preserv.* 6, 155-188.

Larsen, H., and Eimhjellen, K. (1955). *Biochem. J.* 60, 135-147.