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ENTRAPMENT OF MICROBIAL CELLS WITHIN POLYURETHANE HYDROGEL BEADS WITH THE ADVANTAGE OF LOW TOXICITY

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Summary

A new method is described for the entrapment of microbial cells in polyurethane (PUR) hydrogel beads. This hydrogel is produced from a hydrophilic pre-polymer blocked with bisulphite by adjusting the pH between 4 and 6.5. Bisulphite-blocked isocyanate has a substantially lower toxicity against living cells than unblocked (conventional) isocyanates. The poly(carbamoylsulfonate) (PCS) hydrogels have optimal elastic properties and therefore can be used for a matrix of biocatalysts in an agitated reactor as well as in a fluid-bed reactor. The results of ethanol fermentation of *Saccharomyces cerevisiae* entrapped in PCS hydrogel beads, and of the denitrification activity of immobilized *Paracoccus denitrificans* are promising. In contrast, entrapped cells in conventional PUR hydrogels didn't show any activity.

Introduction

Polyurethane (PUR)-pre-polymers have been used for the entrapment of cells since the late 1970s. The resulting biocatalysts became increasingly interesting because of their outstanding chemical and mechanical stability (Fukui *et al.*, 1980; Klein and Kluge, 1981; Fukui *et al.*, 1987). However, the production of PUR hydrogels has a short handling time and, therefore, it is nearly impossible to prepare a large amount of spherical biocatalysts. On the other hand, PUR-pre-polymers contain extremely reactive isocyanate-groups, which are toxic for living microorganisms (Klein and Wagner, 1983).
Our approach to solve this toxicity problem was based on the idea of using chemically blocked isocyanates for the synthesis of polyurethane matrices in order to lower the toxic effect of the isocyanate-groups on the cells. Blocked isocyanates were described for the first time by Petersen (1949).

For the entrapment of living cells it is obviously necessary to build the matrix network under adequate physiological conditions, i.e. at room temperature and in aqueous environment. For

this purpose a hydrophilic blocked isocyanate, which deblocks at room temperature, is needed. Bisulphite and a hydrophilic isocyanate-pre-polymer, based on the isomer 2,4 and 2,6 tolylene diisocyanate 80/20 (TDI) relation and polyol, is optimally suited for these conditions. The principle formation is as follows:

$$R(\text{-NCO})_n + n\ HSO_3^-Na^+ \rightleftharpoons R(\text{-NH-CO-}SO_3^-Na^+)_n$$

The result is a poly(carbamoyl sulfonate), PCS.

In this paper we will describe the production of PCS hydrogel beads, the dependence of the PCS gelation time on pH and the low toxicity of a blocked isocyanate for *Saccharomyces cerevisiae*. We tested the PCS material to immobilize *Saccharomyces cerevisiae* and *Paracoccus denitrificans* in relation to free cells and conventional PUR hydrogels.

Materials and Methods

Microorganisms

Commercial grade baker's yeast (*Saccharomyces cerevisiae*) was used for the toxicity test and for the ethanol production in different beads. *Paracoccus denitrificans* DSM 1403 was used for the denitrification of water.

Chemicals

The PUR-pre-polymers and PCS-pre-polymers are research products of Seus Systemtechnik, Wilhelmshaven, Germany; sodium-alginate Protanal LF 20/60 is a gift from Protan, Drammen, Norway. All other chemicals used are from E. Merck (p.a.), Darmstadt, Germany.

Analytical methods

For ethanol detection a gas-sensor, dip-electrode (GSDE) described by Vorlop *et al.* (1983) was used. Nitrate was determined with a Gynkotek M 300 HPLC with ODS-Hypersil (60 x 4.6 mm) RP column (CS-Chromatographieservice, Langerwehe, Germany) at room temperature and a flow rate of 2.0 ml/min of follow solution at pH 6.5: 900 ml destilled water, 100 ml methanol, 2 ml octylamine, 1 ml H_3PO_4 (concentrated) and 0.5 ml NaOH (10 mol/l). A Pharmacia UV-detector was used at 210 nm.

Toxicity and activity tests

The toxicity tests of *Saccharomyces cerevisiae* against TDI and bisulphite-blocked TDI were carried out as follows: 1 g baker's yeast was added in a 200 ml stirred batch-reactor (700 rpm, 30°C and pH 4) with media (1 % w/v glucose, 0.5 % w/v Bacto-peptone, 0.3 % w/v malt extract and 0.3 % w/v yeast extract). The potential toxic agents were added 5 h after the beginning of fermentation.

Ethanol production was carried out in a 1 l stirred batch-reactor (700 rpm, 30°C and pH 4,3) with the same media as the toxicity tests and 10 g biocatalyst material (wet weight, 10 % w/w cell loading) was added.

The nitrate degradation under autotrophic conditions of *P. denitrificans* after Schmidt *et al.* (1989) was carried out as follows: 20 g biocatalyst material (wet weight, 10 % w/w cell loading) was added to a 400 ml test media, containing drinking water of the city of

Braunschweig at 10°C and pH 7 (maintained constant by titration), 100 mg/l NO_3^- (KNO_3) and a saturation of hydrogen gas (flow > 20 l H_2/h) in a stirred batch-reactor (700 rpm).

Results and discussion

Toxicity test

We selected TDI and his bisulphite derivative as the potential toxic agents and a culture of *S. cerevisiae* as model organism (Fig. 1).

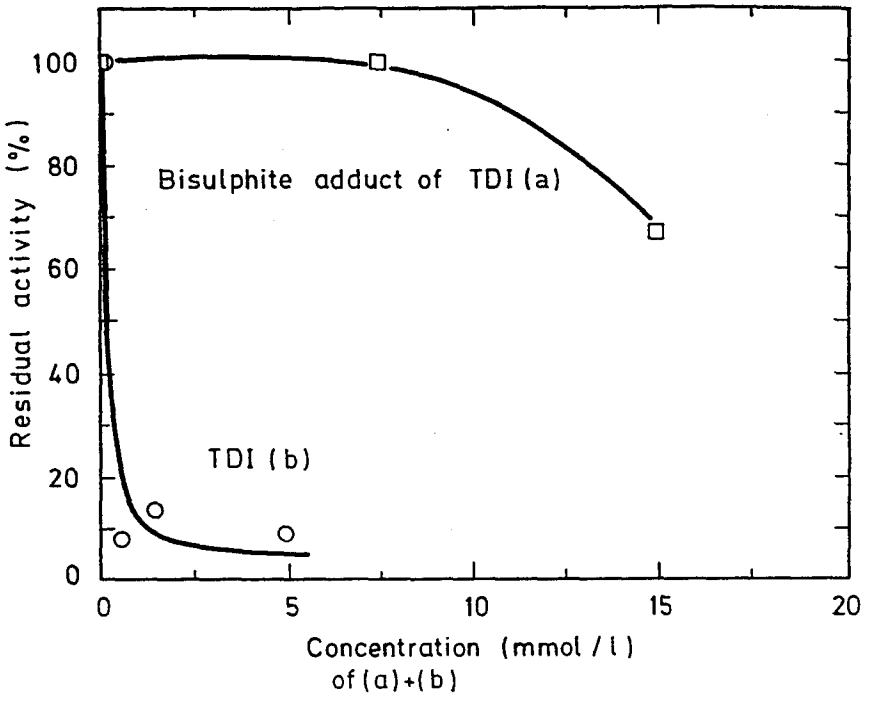

Figure 1. Residual activity of yeast cells after addition of TDI and bisulphite-blocked TDI, 5 h after the beginning of ethanol fermentation (= 100 %)

The inhibition of ethanol production was observed at low concentration of TDI, whereas the ethanol production of *S. cerevisiae* dropped at higher concentration of blocked TDI. The results of these experiments showed that the toxicity of the bisulphite adduct of TDI was significantly lower than that of TDI.

PCS gelation

When mixed with water, conventional PUR-pre-polymers crosslink at once (seconds) to the hydrogel. In contrast to the practise of the PUR-pre-polymer, PCS shows an adjustable gelation time which mainly depends on the pH-value of the solution (Fig. 2): At a pH of 8 - 9.5 an aqueous solution of PCS gelled in < 60 s at room temperature, but at a pH < 5 - 6 the solution can be handled up to 10 h.

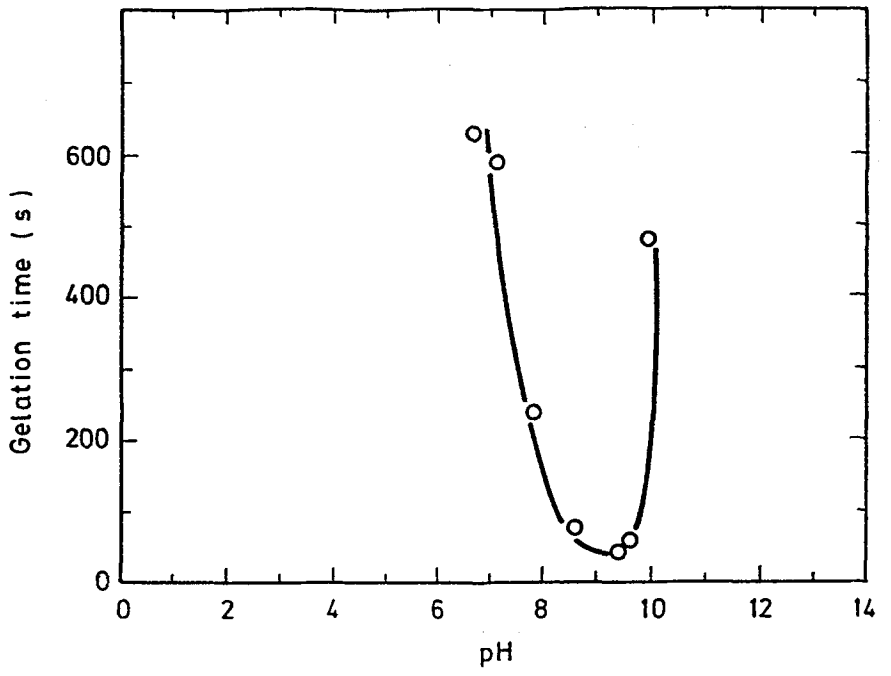

Figure 2. Dependence of the gelation time of an aqueous PCS solution on pH at room temperature

We found that PCS could be stored at a pH of ≤ 2 and a temperature of -20°C for months. It was now possible to prepare an aqueous PCS solution with the physiological adequate pH of 4 - 6.5, containing living cells, and to gel this mixture at once by adjusting the pH to 8.5.

Immobilization procedure

For the production of PCS hydrogel beads with entrapped cells the following method proved to be very practical (Fig. 3):

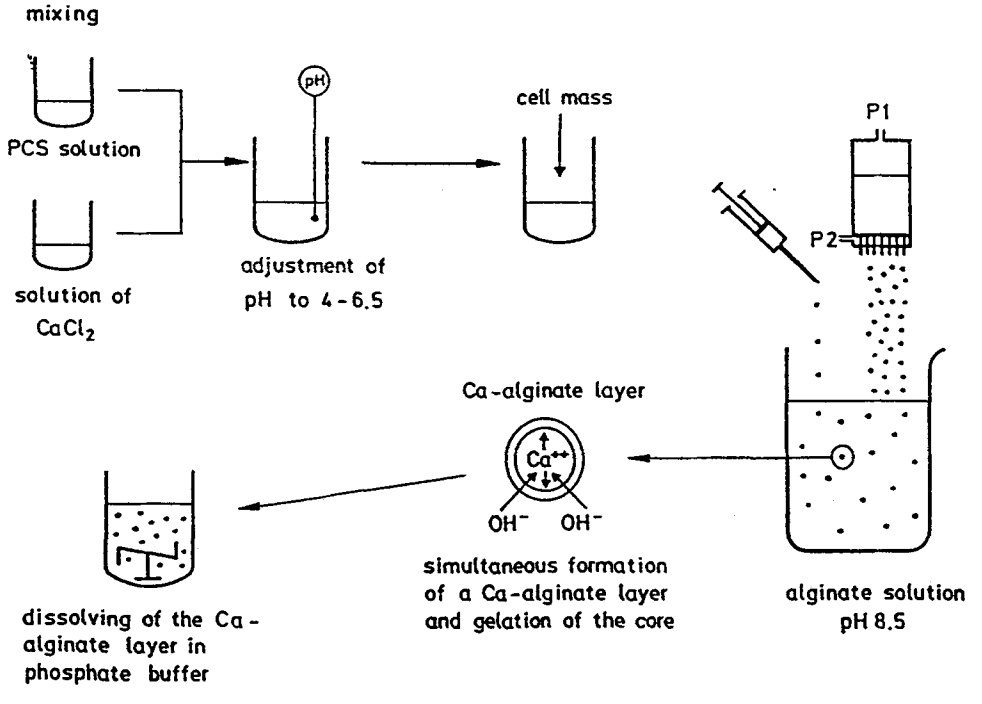

Figure 3. Production of spherical biocatalysts from PCS

4 g of a PCS solution (polymer content: 38 % w/w) is mixed with 5 g solution of $CaCl_2$ (1 % w/v) and adjusted to pH 4. 1 g cell mass is added and this suspension is dropped out from an injection mould or an apparatus for immobilization after Vorlop and Klein (1983) into 100 ml of an alginate solution (0.75 % w/v, pH 8.5). Immediately a calcium-alginate layer is formed by ionotropic gelation (migration of Ca^{2+} from the core to the alginate layer, Spiekermann *et al.*, 1987). The crosslinking reaction (or gelation) of the PCS core take a very fast course at pH 8.5. After a while (< 1 h), the calcium-alginate layer can be dissolved by a sodium-tripolyphosphate buffer (2 % w/v, pH 8.5). The obtained PCS hydrogel beads are highly elastic and have an average diameter of 2 mm.
PUR hydrogels are produced by pouring out a pre-polymer, mixed with water and cells (same polymer content and cell content as described before), on a plate for making cubes (5 x 5 x 1 mm).

Ethanol fermentation

Calcium-alginate can optimally be used for the entrapment of living cells due to the low toxicity of the procedure. The disadvantage is the dissatisfying mechanical stability, especially in stirred reactors. Our PCS hydrogel beads combined the advantage of the calcium-alginate (low toxicity) and PUR matrix (high elasticity): After entrapment of *S. cerevisiae* PCS beads showed nearly the same activity as calcium-alginate beads (Fig. 4), whereas no activity was observed in the case of PUR gel cubes.

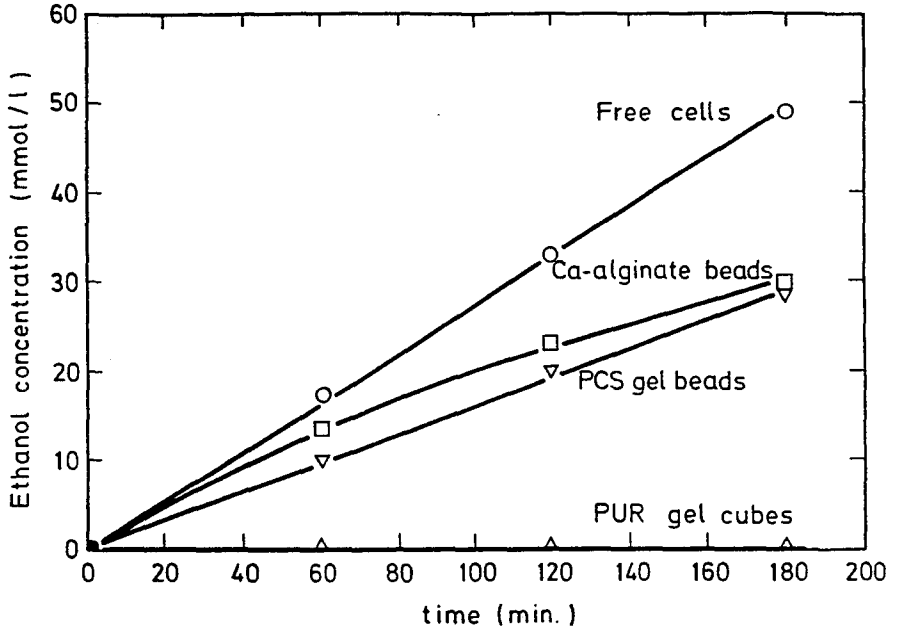

Figure 4. Ethanol formation of *S. cerevisiae* entrapped in calcium-alginate beads, PCS gel beads and PUR gel cubes at 30°C (compared to free cells)

Nitrate degradation

Our experiments with entrapped *P. denitrificans* showed similar results as the ethanol fermentation: The entrapped cells within PUR gel show a total loss of nitrate degradation activity, while entrapped cells within PCS gel possess about 50 % of the nitrate degradation rate of free cells (Fig. 5).

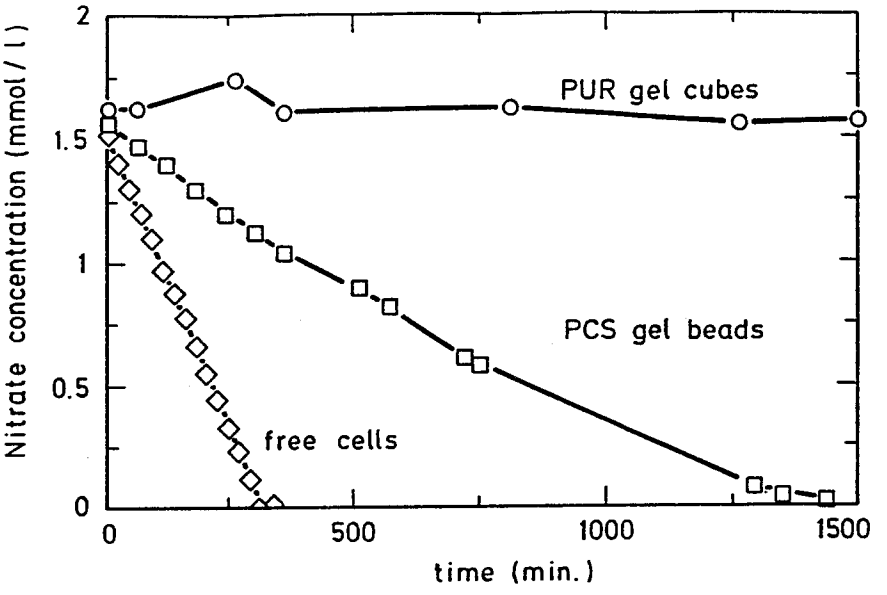

Figure 5. Autotrophic degradation of nitrate with entrapped *P. denitrificans* in PCS gel beads and in PUR gel cubes at 10°C and pH 7 (compared to free cells)

Discussion

PCS can be produced from cheap raw materials without any purification step (as by PUR-prepolymers) because of the low toxicity. Thus PCS hydrogels can optimally be used for the entrapment of living cells. Furthermore, the processing time is adjustable by selecting the pH in a wide range and therefore a spherical forming is easy. The PCS hydrogel biocatalysts show the elastic properties of the classical PUR hydrogel biocatalysts. These advantages of the new PCS hydrogel biocatalysts make use in agitated reactors as well as in fluid-bed reactors possible. Objects for further research are the entrapment of other cells in PCS as well as additional technical applications for PCS.

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