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Δ′-Dehydrogenation of steroids by *Arthrobacter simplex* immobilized in calcium polygalacturonate beads

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SUMMARY

Arthrobacter simplex ATCC 6946 (viable cells) was immobilized in a calcium polygalacturonate gel. The trapped cells were used for repeated batchwise bioconversion of steroids. Reichstein's compound S and hydrocortisone were dehydrogenated introducing a double bond between C_1 and C_2 of ring A. The products 1-dehydro S and prednisolone, respectively, were identified by high pressure liquid chromatography. Steroid dehydrogenase activity increased in the system when an artificial electron acceptor, such as menadione (vitamin K_3) was present in the reaction mixture. An airlift-type reactor was used to bioconvert up to 90% of substrate in 15 min, under optimal conditions. The gel entrapped cell preparations were used for repeated batch bioconversion during 30 days; 69 batch bioconversions for Reichstein's compound S were performed during 15 days of operation of the reactor. The operational stability of the process and the feasibility of repeated batch bioconversions was shown to be comparable to similar processes.

INTRODUCTION

The introduction of double bonds into the molecule of certain steroids is of a great interest since these are essential for the increase of anti-inflammatory activity of the compound [5]. The double bond between C_1 and C_2 in ring A is introduced by microbial fermentation which is more desirable than the chemical synthesis route. A large number of microorganisms are known for their ability to carry out 1-dehydrogenation [22], but *Arthrobacter simplex* is used in most cases [21]. Microorganisms capable of steroid 1-dehydrogenation are often capable of degrading the side chain and the steroid nucleus, and in some cases unwanted products are accumulated, namely the reduction of the 20-*keto* to 20-*β*-hydroxy product [4,19]. Control of these problems is achieved by changing the fermentation conditions [6,12,18].

Today, the emphasis of research in the steroid field is centered on improving existing processes, rather than a search for new reactions.

The application of immobilized cells for steroid bioconversion has received a great deal of attention in the last decade because the technique has several advantages over immobilized enzymes or free cells; the need for enzyme isolation and purification is avoided, the enzymes are most stable inside the cell, operation stability of immobilized cells is longer, the gel can be re-used, and immobilized cells are less susceptible to microbial attack, mainly when operational conditions are not too time-consuming.

However, other factors affecting immobilized cell performance are: support properties and retention capacities, the method used for cell immobilization, properties of the solvent used, and oxygen concentration.

The immobilized system often requires a cofactor. In this case, 1-dehydrogenation of steroids is an enzyme-cofactor catalysed reaction [23].

Arthrobacter simplex has been entrapped into several different supports: polyacrylamide gel [14], collagen [3,20], adhesion to glass [15], calcium alginate [17]; and used for continuous fermentation in the presence of organic solvents or in repeated batch operations at high substrate concentration [9]. However, the physical stability of the immobilized system in conventional stirred tank reactors and their operation stability is poor.

In the present report, *A. simplex* was entrapped into calcium polygalacturonate beads and the bioconversion capability was studied in an airlift-type reactor for the 1-dehydrogenation of Reichstein's compound 'S' and hydrocortisone, respectively.

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MATERIAL AND METHODS

Microorganism

A. simplex ATCC 6946 was used. The strain was maintained in nutrient agar slants (BBL) at 4 °C in refrigeration. Cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of nutrient broth. These flasks were inoculated with an 18-h-old inoculum (10% v/v) that had been grown in an Erlenmeyer flask, inoculated with a loopful of culture. All flasks were shaken at 150 rpm on a rotatory shaker at 28 °C for 18 h. Both cell growth and liquid medium were used for immobilization.

Immobilization

Cells were immobilized in polygalacturonic acid as described by Ohlson et al. [17]. One volume of cells suspended in culture medium was mixed with one volume of polygalacturonic acid (8% w/v aqueous solution pH 8) under aseptic conditions. The mixture was dropped into a solution containing 0.2 M $CaCl_2$, 0.01 M sodium tetraborate and ethanol to a final concentration of 4% (v/v). The final pH was 8.5. The droplets were made with a syringe equipped with a No. 18 hypodermic needle. The speed of the extrusion of the polygalacturonic acid–cell mixture through the needle in combination with air flow used as propellent determined the size of the beads. The beads are formed immediately in the $CaCl_2$–borate–ethanol solution. The calcium polygalacturonate–cell beads were allowed to cure at 4 °C overnight, washed with distilled water, resuspended in nutrient broth and stored in refrigeration until use.

Activation of the gel-cells

The beads suspended in nutrient broth were incubated at 28 °C with shaking. After 24 h they were washed with a minimum medium (glucose 1%, peptone 0.5%, $CaCl_2$ 1.5%, pH 8, without sterilizing), and finally resuspended in the same medium and used immediately for experimentation.

Induction of the 1-dehydrogenation activity

Induction of 1-dehydrogenase enzyme was achieved by adding the steroid RCS (200 mg dissolved in 0.8 ml of ethanol) to the immobilized cell system, suspended in minimal medium and shaken for 2 h at 28 °C. The beads were separated by filtration, washed with minimal medium until no inducer or products were detected. The induction can be done in flasks or directly in the reactor. No differences were found.

Reactor

An airlift reactor with two external loops and a working volume of 270 ml was used. An air flow was introduced to maintain the system oxygenated and the beads suspended. The reactor was packed with 70 ml of beads suspended in 200 ml of minimal medium. Substrate and cofactor were added at the start of each run and the reaction mixture was incubated at room temperature for 15 min. The supernatant was collected and the steroids were extracted with chloroform and analysed by High Pressure Liquid Chromatography (HPLC).

Analysis

Substrate and products were identified and quantified by HPLC (tracor Mod 970-A, Tracor Inc, Austin, TX). Each sample was analysed on a 250 × 4.6 mm Spherisorb ODS column with 48 : 52 methanol : water as the mobil phase and a flow rate of 1 ml/min and a UV detector with 246 nm and a pressure of 2640 psi.

Chemicals

Reichstein's compound S (RCS), hydrocortisone (HC), prednisolone (PD) and polygalacturonic acid (sodium salt grade II) were purchased from Sigma Chemical Co. (St. Louis, MO). Menadione (Vitamin K_3) from Merck AG (Darmstadt, F.R.G.). Nutrient broth from Beckton (Dickinson, MD). Peptone from Difco Laboratories (Detroit, MI). All other chemicals were analytical grade.

RESULTS AND DISCUSSION

A. simplex has been entrapped in several supports in order to dehydrogenate hydrocortisone [8]. Calcium polygalacturonate resulted in a mild and simple method, providing a system with good mechanical strength. Figure 1 shows the polygalacturonate beads obtained. The average size was 2–3 mm. A polygalacturonate concentration of

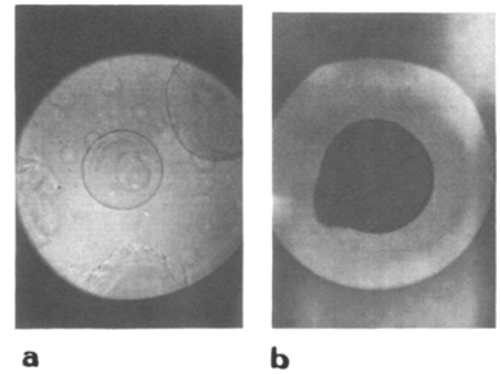

Fig. 1. *A. simplex* distributed in beads during the immobilization: **A.** Polygalacturonate bead without cells; **B.** Immobilized cells stained with Crystal Violet. Microphotography 100 × (Ektachrome).

4–5% (w/v) was suitable for the immobilization. The polymer does not have toxicity effects on the cells like polyacrylamide [8]. Polymerization proceeded quickly, at low temperature, and did not show handling problems as does alginate.

The activation of immobilized cells was carried out by incubation in nutrient broth following a well established technique [11,13]. After activation, the enzyme derepression was carried out using RCS as inducer, as described in MATERIAL AND METHODS.

Preliminary experiments were done in Erlenmeyer flasks with shaking using the induced entrapped cells in order to examine the requirements of added cofactors, pH range, and incubation temperature. Molecular oxygen is the final electron acceptor, as has been reported for this biocatalysed reaction [7]. However, bioconversion of the steroid is increased when artificial electron acceptors are added to the reaction mixture, as indicated by Ohlson et al. [16] using menadione (Vitamin K_3). 1-Dehydrogenase is an enzyme associated with the respiratory chain and the cofactor which transfers the electrons is known to be a quinone [1,16,23]. We assayed several artificial electron acceptors such as 2,6-dichlorophenol-indophenol, phenazine methosulphate and menadione. Both the chlorophenol-indophenol and phenazine methosulphate gave good reaction rates; however, even poisoned, the system was irreversible after washing the cells several times (data not shown). When immobilized cells were used for the dehydrogenation of RCS with increasing concentration of menadione, the bioconversion rate increased up to 0.03 mM (Fig. 2). A further increase to 0.05 mM seems to be toxic.

Figure 3a shows the plot obtained when the cells are

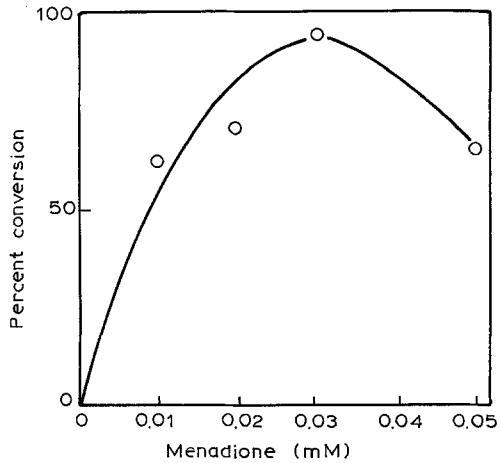

Fig. 2. Effect of menadione concentration on the dehydrogenation of RCS. Reaction mixture: RCS concentration was 5 mg; immobilized cell 5 g (wet weight); pH 8.0; incubation time 2 h, room temperature.

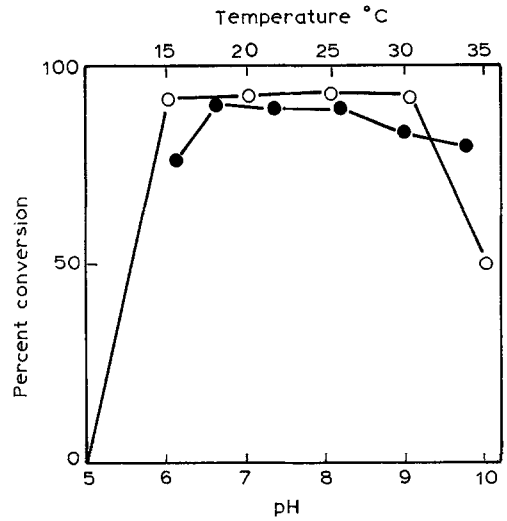

Fig. 3. Influence of the pH on the bioconversion of RCS at room temperature. Influence of the temperature on the bioconversion of RCS at pH 8. Reaction mixture; immobilized cell was 5 g (wet weight); RCS 5 mg; menadione 0.03 mM; incubation time 2 h. ○, pH; ●, temperature.

incubated at different pH values. There is a plateau of maximum dehydrogenating activity in a wide range from pH 6 to 9. Whole free cells are usually incubated in a pH range of 7.6–7.8. The optimum activity pH value for the enzyme in cell free extracts has been reported to be between 9.0 and 9.5 [10], and it loses a significant activity percentage at pH 7.0–7.2 [16]. Figure 3b shows the effect of incubation temperature on the transforming activity of the entrapped cells. It is interesting to observe high bioconversion of the steroid even at low temperatures.

In order to scale up the system, we transferred it to an airlift type reactor. Figure 4 shows the reactor. Polygalacturonate–cell beads, previously induced, were incubated at room temperature with steroid (RCS or HC) and an air flow was passed through the reaction mixture. The time of appearence of product is shown in Fig. 5. The substrate was dehydrogenated at high levels with short incubation times. 15 min was sufficient to convert more than 90% of steroid.

Bacterial concentration in the beads was critical. Gels with few cells were almost void of activity. Gels with high bacterial content had a tendency to produce anaerobic conditions. Figure 6 shows the effect of cell concentration on the conversion of RCS. A cell concentration higher than 1×10^6 cells/ml in the mixture polygalacturonic acid–cells, once beaded and induced, decreases the amount of 1-dehydro derivative and at the same time increases the accumulation of by-products. There appears to exist a critical concentration of dissolved oxygen for the conversion, as can be seen in Fig. 7. Low levels of dis-

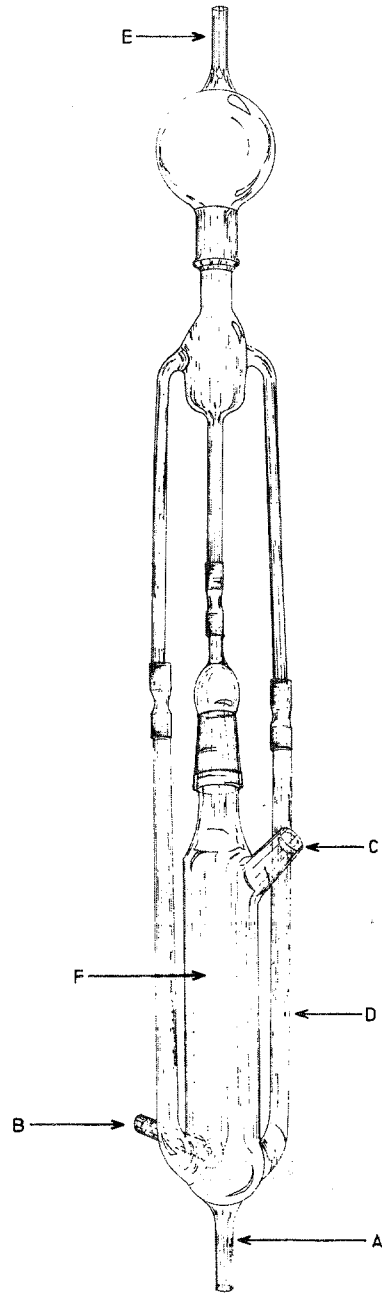

Fig. 4. Air-lift type reactor used for conversion of RCS by immobilized *A. simplex* cells. (Volume, 300 ml; working volume, 270 ml). **A.** Air flow inlet. **B.** feed port. **C.** pH or/and D.O. electrodes. **D.** external loops. **E.** air flow outlet. **F.** reactor.

solved oxygen increase the concentration of by-products. *A. simplex* is an aerobic microorganism and oxygen supply is a critical point in technical processes with aerobic microorganisms because of the low solubility of oxygen in aqueous solutions. If the cells are immobilized, the problems concerning oxygen supply are more severe,

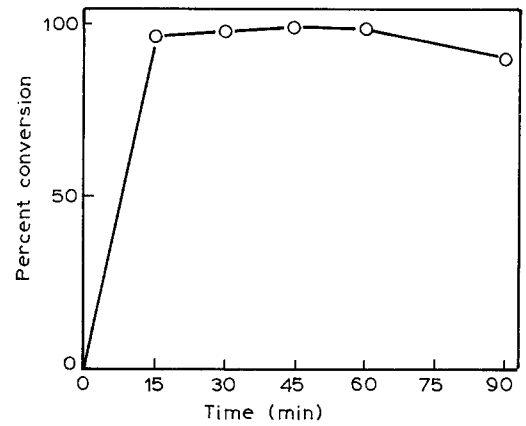

Fig. 5. Effect of the incubation time on the bioconversion of RCS in air-lift type reactor. Immobilized cells 70 ml; RCS 50 mg; menadione 0.03 mM pH 8.0; room temperature.

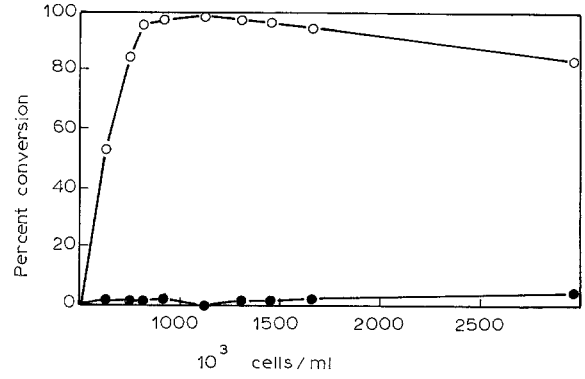

Fig. 6. Effect of the cells concentration on the bioconversion of RCS. Reaction mixture: immobilized cells 70 ml; menadione 0.03 mM, RCS 50 mg; pH 8.0; room temperature; incubation time 15 min. ○, dehydrogenated RCS; ●, by-products.

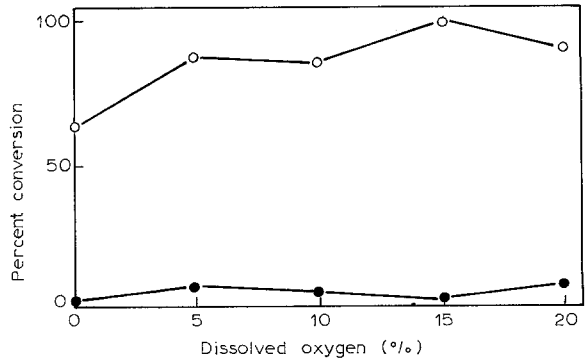

Fig. 7. Bioconversion of RCS by immobilized *A. simplex* as a function of the concentration of dissolved oxygen. Reaction mixture: immobilized cells 70 ml; menadione 0.03 mM; RCS 50 mg; pH 8.0; room temperature, incubation time 15 min. ○, dehydrogenated RCS; ●, by-products.

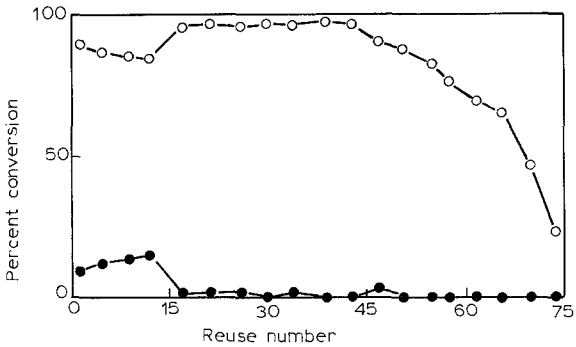

Fig. 8. Repeated batchwise bioconversion of RCS to Δ′ RCS by immobilized *A. simplex*. Reaction mixture: immobilized cells 70 ml; menadione 0.03 mM; RCS 50 mg; pH 8.0; room temperature; incubation time 15 min; oxygen dissolved 15%. ○, dehydrogenated RCS; ●, by-products.

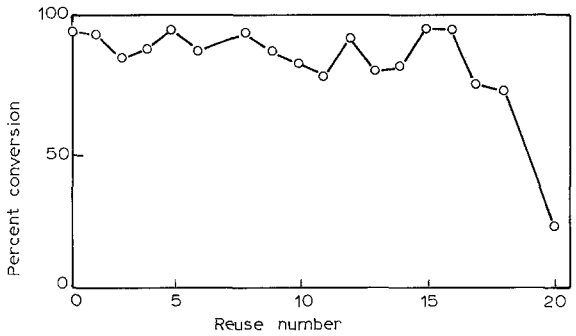

Fig. 9. Repeated batchwise bioconversion of hydrocortisone to prednisolone by immobilized *A. simplex*. Reaction mixture: immobilized cells 70 ml; menadione 0.03 mM; hydrocortisone 50 mg; pH 8.0; room temperature; incubation time 30 min; oxygen dissolved 15%.

since oxygen must diffuse through the matrix in order to reach the cells [2]. The low dissolved oxygen concentration tends to cause the cells to accumulate reduced pyridine nucleotides. Further metabolism or side reactions catalysed by enzymes dependent on pyridine nucleotides can occur [23].

The reactor was operated for 30 days in a repeated batch experiment using RCS as substrate. Figure 8 shows the results obtained. Three main phases can be seen; initially there was a significant accumulation of by-products, followed by a phase in which the dehydrogenated derivative is the major product and finally after about batch 45 the dehydrogenating activity declines. However, 69 batches were tested before the system attained 50% activity. Using HC as substrate (Fig. 9) no by-products were found but the stability of the immobilized cells was different when compared to when RCS was used.

Probably the most important aspect in the commercial exploitation of biocatalysts is the stability. The term 'maintenance stability' was introduced by Kloosterman and Lilly [7] to describe the stability of the 1-dehydrogenation system of immobilized *A. simplex* under operational conditions. The immobilized system, *A. simplex* -calcium polygalacturonate described here, shows interesting results. The immobilization procedure is simple and preserves a viable cell population. A wide pH range for dehydrogenation and a high conversion of steroid even at low temperatures make the immobilized cells better than the free bacteria for introducing the double bond into the steroid. The airlift type reactor used for scaling up the system in repeated batch experiments can also be used for continuous dehydrogenation. The reactor has been applied to convert RCS to HC with immobilized spores (and germinated in situ) of *Cunninghamella blakesleeana* (unpublished results). Short times of incubation are needed to convert the steroid. The half-life of about 30 days and the data mentioned above seem to be good when compared to other immobilized steroid bioconverting systems reviewed by Ohlson et al. [16].

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