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Calcium alginate entrapment of the yeast *Rhodosporidium toruloides* for the kinetic resolution of 1,2-epoxyoctane

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Abstract

Resting cells of the yeast *Rhodosporidium toruloides* (UOFS Y-0471) were immobilised in calcium alginate beads for the enantioselective kinetic resolution of racemic-1,2-epoxyoctane. The initial activity exhibited by immobilised cells was almost 50% lower than that of the free counterpart but was extremely stable when compared to the free cells. The concentration of the immobilised biomass had no effect on apparent enzyme activity but did lead to a decrease in single cell activity. An increase in both the alginate and $CaCl_2$ concentrations used for bead preparation led to a decrease in enzyme stability. An increase in the alginate concentration led to an increase in bead diameter. The stoichiometric equation for cross-linking of alginate was only obeyed when $CaCl_2$ concentrations higher than 0.4 M were utilised for bead preparation.

Introduction

While enzymatic reactions form the basis of the metabolism of all living organisms, they provide a tremendous opportunities for industry to carry out efficient and economical biocatalytic conversions, since they catalyse chemical reactions with great specificity and at high reaction rates (Van Beilen & Li 2002). One of the major advances in optimising biotechnological processes lies in immobilisation technology (Konstantin *et al.* 2000) which can be defined as any process that restricts substances or cells inside a given structure and limits their free diffusion or movement (Huebner & Buccholz 1999).

Immobilisation offers advantages such as ease of handling, easy cell separation from the liquid medium (Laca *et al.* 1998), the possibility of repeated and continuous use of biocatalysts as well as an increase in enzyme stability (Rasor 1999) and longevity in contrast to that of free cells (Cruz *et al.* 2001). These factors lead to a subsequent increase in bioprocess efficiency (Tanaka & Kawamoto 1999).

Entrapment of cells in calcium alginate is the most widely used immobilisation technique in the biocatalytic production of chemicals (Smidsrod & Skjak-Braek 1990), and has been implemented in biocatalytic processes utilising whole cells (Becerra *et al.* 2001), as well as free enzymes (Tanriseven & Dogan 2002). Alginate is cheap and readily available, has a high affinity for water, and has the ability to form gels under mild conditions, which makes it suitable for most biological cells (Buque *et al.* 2002). Alginate also has a further advantage of being non-toxic and non-pathogenic, which makes it attractive for applications in the food and pharmaceutical industry (Tanaka & Kawamoto 1999).

The yeast *Rhodosporidium toruloides* enantioselectively hydrolyses the kinetic resolution of (*R*,*S*)-1,2-epoxyoctane resulting in optically pure (*S*)-epoxide and the corresponding *vicinal* (*R*)-diol (Botes *et al.* 1999). This well established reaction was used as

model to determine the effect of immobilisation on epoxide hydrolase activity and stability within yeast. A further aim was to determine the relationship between the formulation parameters of the immobilisation matrix, the effect of these parameters on the physical characteristics of the matrix, and the subsequent effect on enzyme activity and stability.

Materials and methods

General

Rhodosporidium toruloides (UOFS Y-0471) was from the Yeast Culture Collection of the University of the Free State, Bloemfontein, South Africa. Alginate, from brown algae, as well as a medium viscosity carboxymethylcellulose (CMC) sodium salt was from Fluka BioChemika, South Africa. Pure $CaCl_2$ was from Merck, South Africa. (*R*,*S*)-1,2-Epoxyoctane and (*R*,*S*)-1,2-octanediol were from Aldrich, South Africa. Double distilled, deionised water was used. All solutions were prepared with 20 mM Tris/HCl buffer (pH 7.5).

Reactions were monitored and products were analysed by GC (ThermoFinnigan Focus equipped with FID) utilising a β-Dex 120 fused silica cyclodextrin capillary column (Supelco) and H_2 as carrier gas. The diol was analysed isothermally at 130 °C. Retention times were 15.64 min and 16.06 min for (*S*)- and (*R*)-1,2-octanediol, respectively.

Cultivation and preparation of yeast cells

R. toruloides was grown for 48 h from pre-cultures at 27 °C in 1 l shake-flasks containing 200 ml of YM media (2% malt extract, 1% peptone, and 0.5% yeast extract w/v) supplemented with 1.5% glucose (w/v) and vitamins (0.2% v/v). At the late growth phase (24–48 h) the cells were harvested by centrifugation (3000 *g*, 10 min, 4 °C) and washed with phosphate buffer (50 mM, pH 7.5). The washed cells were resuspended (20% v/v) in phosphate buffer containing 20% (v/v) glycerol and stored below −15 °C. Yeast cells were washed with Tris/HCl buffer (20 mM, pH 7.5) before immobilisation.

Preparation of calcium alginate beads

Calcium alginate beads were prepared by extrusion using a simple one step process (Fraser & Bickerstaff 1997) at room temperature (25 °C). Sodium alginate solutions were prepared by the stepwise addition of the alginate powder while rapidly stirring the buffer to avoid the formation of a lumpy precipitate. For the preparation of the cationic solutions, 0.5% (w/v) CMC was dissolved in $CaCl_2$ solutions of varying concentrations. CMC was used as a non-polar gelling polymer to modulate the viscosity and density of cationic solutions to ensure the spherical shape of the beads. Wet biomass was suspended in the dissolved alginate solution and stirred thoroughly to ensure a homogenous distribution of the cells in the alginate solution before extrusion in the $CaCl_2$ solution.

The alginate/cell suspension was added drop-wise (using a peristaltic pump and a fine needle) to 40 ml $CaCl_2$ solution through a silicone tube. The $CaCl_2$ solution was stirred at constant speed (130 rpm) using a magnetic stirrer to avoid droplet aggregation. A dropping height of 10 cm was maintained to ensure the formation of spherical droplets. The inner diameter of the silicone tube was 3 mm. The total dropping time of the alginate/cell suspension was kept to less than 1.6% of the gelation time of the beads in the cationic solution to ensure the formation of uniform beads. Gelation time was restricted to one hour after which the $CaCl_2$/CMC solution was discarded. Subsequently the beads were washed three times and stored in 20 mM Tris/HCl buffer (pH 7.5) at 4 °C.

Immobilised whole cell enzyme activity assay

The alginate beads were weighed after gentle drying on filter paper and a 1:2 alginate bead/buffer (w/v) suspension placed in a 1.5 ml micro-centrifuge tube. The epoxide substrate was added to give 20 mM in the aqueous phase. The reaction mixtures were incubated (20 min, 30 °C) on a shaking water-bath. Liquid samples were removed from the reaction mixtures and the product (*R*,*S*-1,2-octanediol) extracted by the addition of a volume of ethyl acetate equivalent of that of the aqueous sample. Product formation was quantified by GC analysis. The experiments were done in duplicate.

Determination of immobilised whole cell enzyme activity

Twenty percent wet biomass/alginate (w/w) were immobilised in calcium alginate beads and prepared as previously described with a combination of 0.5% (w/v) alginate solution in combination with a 0.4 M $CaCl_2$ was used. The beads were preincubated at room

temperature for 24 h without substrate, and activity established at three 12 h intervals after bead preparation (0, 12 and 24 h).

Determination of free/unimmobilised whole cell enzyme activity

Frozen yeast cells were thawed, resuspended in Tris/HCl buffer (20% (w/v) cell suspension), and 500 μl was dispensed in 1.5 ml micro-centrifuge tubes. Substrate was added to give 20 mM. The cell suspensions were stored at room temperature for 24 h, and activity was established at three 12 h intervals (0, 12 and 24 h). Reaction mixtures were incubated at 30 °C for 20 min on a shaking water-bath. The addition of 500 μl ethyl acetate simultaneously stopped the reaction and extracted the formed product. The product was analysed by GC analysis.

Effect of immobilised biomass

Calcium alginate beads were prepared as described earlier. Suspensions with three different cell/alginate ratios were prepared [20; 16.6 and 14.3% cell/alginate solution (w/w)], using a 0.5% (w/v) alginate solution in combination with 0.2 M $CaCl_2$. The beads were stored at room temperature for 24 h and activity was established at three 12 h intervals after preincubation at room temperature without substrate (0, 12 and 24 h).

Effect of $CaCl_2$ and alginate concentrations

Calcium alginate beads were prepared using three different alginate concentrations (0.5, 0.75 and 1% w/v), and four $CaCl_2$ concentrations (0.1, 0.2, 0.4 and 0.5 M). Wet biomass (20% w/v in alginate) was immobilised. The activity was measured immediately after preparation (0 h) and after preincubation at room temperature (25 °C) without substrate for 24 h.

Determination of bead diameter

Three beads from every $CaCl_2$ and alginate solution combination were gently dried on filter paper and the diameter measured with a Nikon 129114-Optiphot microscope fitted with a PS No 2 measuring graticule. All measurements were done in triplicate.

Determination of the viscosity of alginate solutions

Several solutions of different alginate concentrations were prepared and viscosity determined at 25 °C with a RV WinGather V1.1 Brookfield viscosimeter equipped with a SC4-18 spindle.

Table 1. Influence of immobilisation on initial enzyme activity and stability.

Time[a] (h)	Diol (mM)		Relative activity loss (%)	
	Free cells	Immobilised cells[b]	Free cells cells[b]	Immobilised
0	12.6 ± 0.8	6.4 ± 0.3	–	–
12	0.2 ± 0	6 ± 0.1	>98	6
24	0.07 ± 0.1	3.2 ± 0.2	>98	49

[a]Preincubation time of beads at room temperature (25 °C) without substrate.
[b]Biomass: alginate ratio (m/m) = 20%.

Determination of the effect of $CaCl_2$ concentration on free cell enzyme activity

Frozen yeast cells were thawed and resuspended in Tris/HCl buffer (20% cell suspension), and 500 μl dispensed in 1.5 ml micro-centrifuge tubes. Different concentrations of $CaCl_2$ (0.04 M and 0.1 M) were added. Reaction mixtures were equilibrated at room temperature for 10 min before addition of epoxide substrate at 20 mM. Remaining enzyme activities were assayed after 50 min at 30 °C.

Determination of Ca^{2+} utilised for cross-linking

Calcium alginate beads were prepared as described earlier with four different $CaCl_2$ solutions (0.1, 0.2, 0.4 and 0.5 M) and three different alginate solutions (0.5, 0.75, 1.0% w/v). The resultant Ca^{2+} ion concentration in the cationic solution after curing was analysed by atomic absorption spectrometry.

Repeated batch biotransformation

Calcium alginate beads were prepared as described earlier using a 0.5% (w/v) alginate solution in combination with 0.2 M $CaCl_2$. Twenty percent (w/v) biomass was immobilised in alginate. Substrate (20 mM) was added to the reaction mixtures and the bioconversion carried out for 20 min. A sample was drawn from the liquid phase for analysis, the beads recovered by filtration, washed with Tris/HCl buffer (3 × 50 ml) and reincubated with racemic substrate. The experiment was done in duplicate.

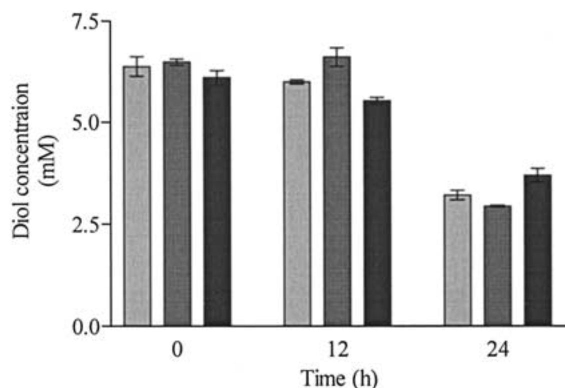

Fig. 1. The effect of biomass loading on enzyme activity and stability. Three different amounts of wet biomass were immobilised in calcium alginate beads and the resultant enzyme activity measured (diol production) at 0, 12 and 24 h preincubation times after immobilisation (▨, 20% w/v biomass; ▩, 16.6% w/v biomass and ■, 14.3% w/v biomass).

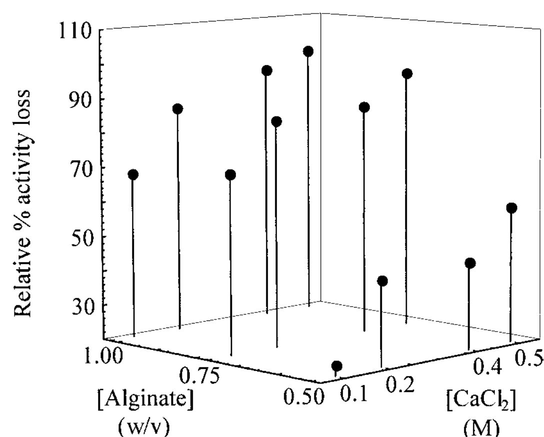

Fig. 2. The influence of $CaCl_2$ and alginate concentrations on relative % activity loss after 24 h. Twenty percent biomass was immobilised in calcium alginate beads prepared with a combination of three different alginate concentrations and four different $CaCl_2$ concentrations. Relative % activity loss can be defined as the decrease in diol production from immediately after immobilisation and after a 24 h preincubation time at room temperature for each individual alginate and $CaCl_2$ combination.

Results and discussion

Effect of immobilisation on enzyme stability

Initial enzyme activity decreased due to immobilisation (Table 1). On the other hand, the relative % activity loss, measured in terms of the relative decrease in initial diol production after 20 min (exhibited by the immobilised cells), clearly demonstrates the stabilisation effect that immobilisation has on the enzyme activity. Only 6% decrease in activity was displayed by the immobilised cells after 12 h and 49% after 24 h in comparison with the >99% decrease in activity observed for the free cells.

Effect of formulation parameters on enzyme activity, stability and bead properties

Effect of biomass loading

The variation of total enzyme activity for the different concentrations of biomasses immobilised was not statistically significant (Figure 1), but lower enzyme activity per cell with higher cell loading was exhibited. This phenomenon was also previously observed (Johansen & Flink 1986) with invertase activity, and attributed to a decrease in the stabilisation effect on the enzymes, with an increase in cell loading. Thus, breakage of the capsule structure increases the exposure of the cells to the external environment, but due to a higher cell loading the total enzyme activity does not show a significant change. The best capsule structure (spherical smooth capsules) was obtained with a cell loading of 20% (biomass/alginate ratio).

Effect of $CaCl_2$ and alginate concentrations

The cationic and anionic solution concentrations were varied to evaluate the effects and possible interactions of the three formulative parameters ($CaCl_2$ concentration, alginate concentration and the average bead diameter), on the enzymatic activity exhibited by alginate entrapped *R. toruloides*. The initial epoxide hydrolase activity (0 h) as well as stability (decrease in the initial activity after storage at 25 °C for 24 h) was assayed. No apparent difference in initial enzyme activity was observed between the different $CaCl_2$ and alginate concentration combinations (results not shown) but these variations did lead to a major difference in the relative % activity loss between the different batches of immobilised cells (Figure 2).

An increase in the $CaCl_2$ concentration for each alginate concentration, and an increase in alginate concentration for each $CaCl_2$ concentration lead to an increase in the relative % activity loss, with no major difference for the transitional $CaCl_2$ solutions (0.2 M and 0.4 M). The average bead diameter for each scenario showed a definite increase with an increase in alginate concentration (Figure 3), irrespective of the $CaCl_2$ concentration, while no major influence of $CaCl_2$ concentration on bead diameter was observed.

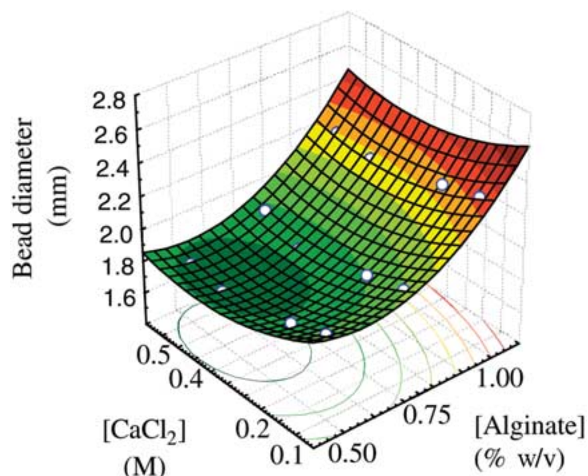

Fig. 3. The influence of $CaCl_2$ and alginate concentrations on bead diameter. Twenty percent biomass was immobilised in calcium alginate beads prepared with a combination of three different alginate concentrations and four different $CaCl_2$ concentrations. The bead diameters were measured for each alginate and $CaCl_2$ combination.

The two main factors influencing droplet size when using a simple dropping method for the preparation of microcapsules are the force of gravity pulling the solution down and the resisting interfacial tension force (Huebner & Buccholz 1999). Thus, a droplet will only fall if the gravitational force (the droplets mass under gravitational acceleration) exceeds the resisting interfacial force.

When measuring the viscosity of alginate solutions as a function of alginate concentration (Figure 4) it can clearly be seen that viscosity increases with an increase in alginate concentration. This viscosity increase leads to the increase of interfacial forces between the formed droplets and the needle tip. This interaction necessitates a larger volume of alginate solution to reach the critical droplet mass for the gravitational force to overcome the interfacial forces, explaining the larger bead diameter found with an increasing alginate concentration.

When a polyvalent metal ion comes in contact with an alginate solution, a primary membrane will be formed immediately on contact. The stoichiometry of the gelation process (Equation (1)), can be expressed by the following exchange reaction (Khairou *et al.* 2002):

$$2(\text{Na - Alg})n + M^{2+} = (M - \text{Alg}_2)n + 2\text{Na}^+, \quad (1)$$

where Na-Alg denotes the sodium alginate, M-Alg$_2$ is the metal alginate gel complex and M is the metal ion.

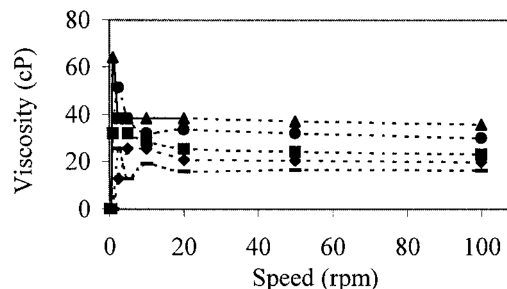

Fig. 4. The relation between alginate concentration and solution viscosity. The line (-) represent 0.45% (w/v) alginate, the diamond (♦) 0.5% (w/v) alginate, the squares (■) 0.55% (w/v) alginate, the circles (●) 0.6% (w/v) alginate, and the triangle (▲) 0.65% (w/v) alginate.

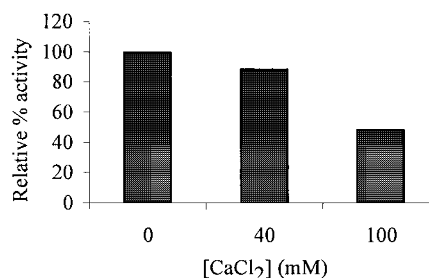

Fig. 5. The effect of $CaCl_2$ concentration on free cell enzyme activity. Different concentrations of $CaCl_2$ (0.04 M and 0.1 M), were added to 20% cell suspensions. Reaction mixtures were equilibrated at room temperature for 10 min before addition of the substrate (20 mM epoxide). Remaining enzyme activities were assayed after 50 min at 30 °C. Hundred percent value of activity is 14.2 mM diol.

A decrease in whole cell enzyme stability was observed when a higher alginate solution was employed for bead preparation (Figure 1). Due to the compatibility of alginate with living systems this decrease could not be contributed to the alginate, and had to be due to another formulation parameter. From Equation (1) it can clearly be seen that the number of Ca^{2+} ions included in the polymer structure is directly proportional to the number of alginate molecules. Thus, a higher alginate concentration should lead to a higher calcium concentration incorporated in the bead structure, and consequently in contact with the cells.

The inhibitory effect of a number of metal chlorides on the activity of an enzyme extract of *R. toruloides* was previously reported (A.L. Botes, personal communication) but it was not known if Ca^{2+} ions would have the same adverse effect on whole cells and, subsequently, on the immobilised cells. An increase in the CaCl2 concentration leads to a decrease in the free cell activity (Figure 5).

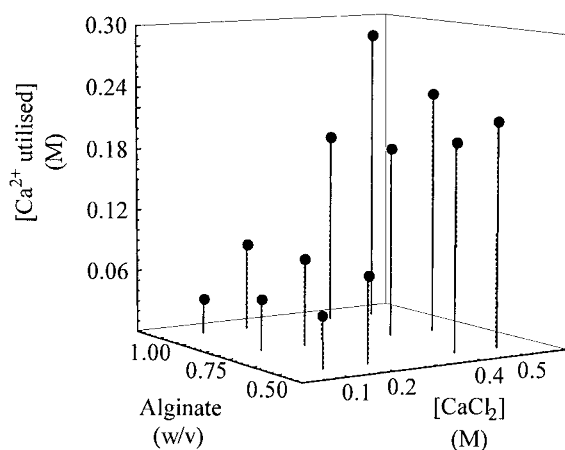

Fig. 6. The relation between Ca^{2+} utilisation and bead formulation parameters. The remaining Ca^{2+} concentration in each cationic solution was measured by atomic absorption spectometry after 20% biomass was immobilised in calcium alginate beads prepared with a combination of three different alginate concentrations and four different $CaCl_2$ concentrations.

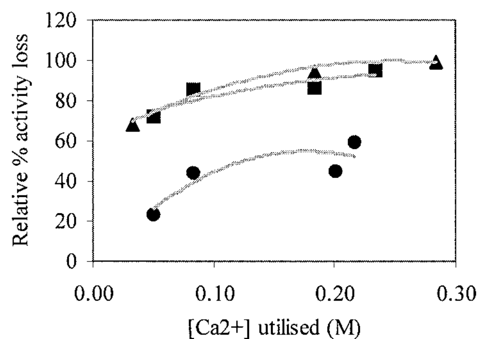

Fig. 7. Relation between utilised Ca^{2+} concentration and relative % activity loss. Twenty percent biomass was immobilised in calcium alginate beads prepared with a combination of three different alginate concentrations and four different $CaCl_2$ concentrations. The decrease in enzyme activity (diol production after 20 min) directly after immobilisation and after a 24 h preincubation at room temperature (relative % activity loss) for each alginate and $CaCl_2$ combination was plotted against the utilised Ca^{2+} concentration during bead preparation. The closed circles (●) represent 0.5% (w/v) alginate, the closed squares (■) 0.75% (w/v) alginate, and the closed triangleS (▲) 1.0% (w/v) alginate concentration combinations.

To confirm that higher calcium concentrations are incorporated into the bead structure when a higher alginate concentration is used for bead preparation, the resultant calcium concentration in the cationic solution was determined by AA analysis after curing for each $CaCl_2$ and alginate concentration combination (Figure 6).

The highest concentration of Ca^{2+} was utilised for crosslinking with a combination of 0.5 M $CaCl_2$ and 1% (w/v) alginate. At 0.5 M $CaCl_2$ an increase in the alginate concentration leads to an increase in the utilised Ca^{2+} concentration. This corresponds with the results in Figure 2, where an increased loss in relative % activity was said to be due to the increased Ca^{2+} concentration utilised for cross-linking, and therefore in contact with the enzymes situated inside whole cells.

The same tendency was however not found at the lower $CaCl_2$ concentrations (0.1, 0.2, 0.4 M). Almost equal concentrations of Ca^{2+} were utilised during cross-linking with each of the three lower $CaCl_2$ solution concentrations, irrespective of the alginate concentration.

According to the stoichiometric equation of gelformation, an increase in alginate concentration should lead to an increase in the utilised Ca^{2+} (Ouwerx *et al.* 1998). It can therefore be said that the stoichiometric equation only holds at higher Ca^{2+} concentrations. This could be attributed to the fact that the bead formation process (at lower Ca^{2+} concentrations takes place too fast kinetically for the reaction to utilise stoichiometric amounts of Ca^{2+}. Although an increase in the utilised amount of Ca^{2+} could not be observed with an increase in the alginate concentration a relation between the utilised Ca^{2+} concentration and the relative % activity loss could be observed (Figure 7). This demonstrates that the decrease in activity with higher alginate concentrations can partially be contributed to the Ca^{2+} concentration.

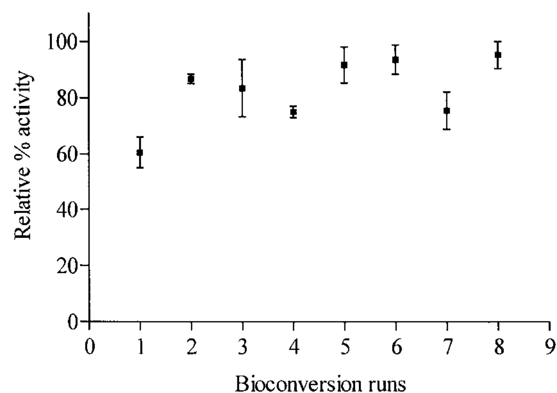

Fig. 8. Repeated batch biotransformation. Twenty percent (w/v) biomass was immobilised in alginate beads prepared with a 0.5% w/v alginate solution in combination with 0.2 M $CaCl_2$. Substrate (20 mM) was added to the reaction mixtures and the bioconversion carried out for 20 min. A sample was drawn from the liquid phase for analysis, the beads recovered by filtration, washed and reincubated with substrate. Hundred percent value of activity is 7.5 mM diol.

Repeated batch biotransformation

As previously mentioned, one of the advantages of using immobilised biocatalysts is the prospect of their continuous or repeated use (Tanaka & Kawamoto 1999). In order to evaluate the possibility for repeated use, the same beads were reused in a batch bioconversion. It was observed that immobilised *R. toruloides* in calcium alginate beads could be reused for consecutive bioconversions. Although swelling of the beads occurred after the fifth repeated batch, Figure 8 shows that no significant decrease in activity was observed after 8 consecutive repeated batch reactions.

Conclusions

The immobilisation of *R. toruloides* in calcium alginate beads clearly exhibited sufficient increase in enzyme stability to make immobilisation advantageous (in single batch reactions as well as in repeated biotransformations), despite the major decrease in initial enzyme activity. Ouwerx *et al.* (1998) found that alginate bead properties were greatly influenced by the concentration and nature of the polymer type as well as by the concentration of the cation in the maturation medium. The obtained results are in accordance with their findings, and also demonstrate the interaction between immobilisation formulation parameters, physical characteristics of the immobilisation matrix as well as the effect of the before mentioned on enzymatic stability and activity.

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