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Tyrosol to hydroxytyrosol biotransformation by immobilised cell extracts of *Pseudomonas putida* F6

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Abstract

Cell extracts of *Pseudomonas putida* F6 expressing tyrosinase activity were immobilised in a calcium alginate matrix. Immobilised cell extracts consumed 21% more tyrosol substrate (2.5 mM) than non-immobilised cell extracts of *P. putida* F6. However, approximately the same level of hydroxytyrosol accumulation was observed. A number of approaches were attempted for complete removal of tyrosol substrate and maximum hydroxytyrosol accumulation. These approaches included addition of ascorbic acid, washing and re-use of immobilised cell extracts, sequential addition of fresh immobilised cell extracts to the biotransformation, and alteration of the starting concentration of tyrosol. Complete substrate removal was observed in the presence of both 1.0 and 2.5 mM tyrosol with equimolar concentrations of ascorbic acid. The sequential addition of immobilised cell extracts was required to ensure complete removal of 2.5 mM substrate. However, only 6% of the 2.5 mM substrate accumulated as product (hydroxytyrosol). The biotransformation of 1 mM tyrosol resulted in the accumulation of 0.8 mM hydroxytyrosol in a single biotransformation with immobilised cell extracts. Ninety-six percent of the hydroxytyrosol was subsequently recovered from the biotransformation reaction using an Amberlite XAD-4® resin. Immobilised cell extracts retained 58% of their activity after 9 days of storage on filter paper at 4 °C while cell free extracts retained only 22% of their activity under the same storage conditions.

Keywords: *Pseudomonas putida* F6; Tyrosol; Hydroxytyrosol

1. Introduction

Tyrosinase (EC 1.14.18.1) is a copper enzyme, which catalyses two reactions; the hydroxylation of monophenols to catechols (monophenolase activity) and the oxidation of catechols to quinones (diphenolase activity), which are subsequently polymerised to red, brown or black pigments [1–6]. The addition of reducing agents such as ascorbic acid to this reaction has been shown to result in the accumulation of the catechol [4,7].

Mushroom tyrosinase activity has attracted interest for use in the synthesis of value-added products such as L-dopa, the anti-Parkinson's disease drug [7,8]. In addition, recent reports have suggested its application in the production of the anti-oxidant and anti-microbial agent hydroxytyrosol (2-(3,4-dihydroxyphenyl) ethanol) from tyrosol (2-(4-hydroxyphenyl) ethanol) [4,9–11]. Although hydroxytyrosol has been produced from the transformation of tyrosol by whole cells of *Pseudomonas aeruginosa* [12], the enzyme system responsible for this biotransformation has not been elucidated [12].

In previous work, *Pseudomonas putida* F6 was isolated from soil and shown to possess a tyrosinase enzyme capable of transforming *p*-hydroxyphenylacetic acid (phenol) to 3,4-dihydroxyphenylacetic acid (catechol) [13] as well as a range of halogenated phenols to the corresponding catechols [14]. This catalytic ability, like that of mushroom tyrosinase, does not require the exogenous addition of co-factors [13]. The immobilisation of enzymes can lead to stability and longevity allowing for reuse of a valuable biocatalyst. The ability of tyrosinase to produce catechols makes it a worthy target for immobilisation as catechols are desirable pharmaceutical synthons. Thus, the aim of this study was to examine the ability of immobilised cell extracts of *P. putida* F6 to transform tyrosol to hydroxytyrosol.

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A bacterial tyrosinase may offer a cheaper and more accessible source of tyrosinase activity than mushroom tyrosinase and to-date no attempt has been made to immobilise tyrosinase activity from a bacterial source. Substrate removal, product accumulation and product retrieval from the biotransformation medium by the immobilised system were optimised.

2. Materials and methods

2.1. Organism and growth conditions

P. putida F6 was isolated from soil on a minimal medium with *p*-hydroxyphenylacetic acid as the sole source of carbon and energy as previously described [13]. The culture medium used for growth was E2 medium [15], supplemented with a vitamin mix, trace elements and magnesium sulphate solution (1 mM).

2.2. Protein determination

Protein concentration in cell extracts was determined using the method of Lowry et al. [16] with bovine serum albumin as the standard. The protein content of the immobilised enzyme was calculated by subtracting the amount of unbound protein from the protein originally added.

2.3. Growth and harvesting of cells

P. putida F6 was grown in batch culture with phenylacetic acid (5 mM) as the sole source of carbon and energy. Cells were induced with *p*-hydroxyphenylacetic acid for 2 h and harvested at an optical density at 540 nm of 0.9–1.0. Cells were placed on ice, centrifuged at 15,000 × *g* for 10 min at 4 °C and washed in ice-cold phosphate buffer (50 mM, pH 7.0). Cell suspensions for disruption were resuspended in phosphate buffer (50 mM, pH 7) containing 10% (w/v) glycerol and 1.0 mM dithiothreitol (DTT). These cells were passed twice through a precooled French pressure cell and centrifuged at 36,700 × *g* for 30 min. The resulting cell extract was collected and stored on ice prior to analysis. Cell extracts were stored at −20 °C for a maximum of 1 week without loss of activity.

2.4. Determination of enzyme activity

Cell extracts of *P. putida* F6 were incubated in air saturated universal buffer (50 mM, pH 7.0) at 30 °C with stirring and supplied with substrate at an initial concentration of 1.0 mM. Samples (900 μl) were withdrawn from the reaction over time, acidified with 100 μl of 1 M hydrochloric acid, stored on ice for 60 min, centrifuged at 20,000 × *g* for 20 min at 4 °C and subsequently filtered through 0.2 μm Spartan®-3 nylon filters (Agilent, UK). All assay samples were analysed by HPLC using a C-18 Hypersil ODS 5μ column (125 mm × 3 mm) (Hypersil, Runcorn, UK) and a Hewlett Packard HP1100 instrument equipped with an Agilent 1100 Series diode array detector. The samples were isocratically eluted using a phosphoric acid (0.1%, v/v) and methanol mix in a ratio of 15:85 (v/v), respectively, at a flow rate of 0.5 ml/min. The substrate (tyrosol) and product (hydroxytyrosol) were identified by comparison to the retention time and wavelength spectrum of authentic standards. A unit of activity was defined as the amount of enzyme/cell extract catalysing the transformation of 1 μmol substrate/min at 30 °C. For the assays in this study 500 μl of cell extract (13.3 units/ml) were immobilised in 4 g of beads giving a total activity of 1.6 units activity/g of beads.

2.5. Immobilisation of cell extracts

2.5.1. Preparation of calcium alginate beads

Enzyme immobilisation was carried out using calcium alginate beads. Five hundred microliters of cell extract was gently mixed with 5 ml of sodium alginate paste (4%, w/v). This mixture was added dropwise into an ice-cold calcium chloride solution (2%, w/v). The resulting beads, weighing 4 g were recovered from solution by passage through a sieve with a pore size smaller than the beads ensuring no beads passed through the sieve. The filtrate was retained, dialysed against water for 3 h to remove any calcium chloride present and the protein level measured to determine the level of protein immobilised. Non-immobilised cell extracts were also added to calcium chloride to a known concentration, dialysed and assayed in the same manner and used for comparative purposes, to ensure that no protein was lost during dialysis.

2.5.2. Substrate depletion assays using immobilised cell extracts

To prevent possible degradation of the beads by phosphate buffer, all assays were carried out in air saturated Tris–HCl buffer (50 mM, pH 8.6) at 30 °C [24]. Several methods for tyrosol biotransformation were attempted to maximise substrate utilisation and product formation. These methods are described in the following sections.

2.5.2.1. Continuous sampling biotransformation. 3.3 units of tyrosinase activity as a cell extract of *P. putida* F6 or 2 g of calcium alginate beads containing immobilised cell extract of *P. putida* F6 were incubated in 20 ml of air saturated Tris–HCl buffer (50 mM, pH 8.6) with tyrosol supplied at an initial concentration of either 1.0 or 2.5 mM. The reaction was monitored over time for substrate depletion and product formation.

2.5.2.2. Biotransformation of tyrosol using recycled immobilised cell extract in calcium alginate beads. Five hundred microliters of a cell extract of *P. putida* F6 was immobilised in 4 g calcium alginate beads giving a total activity of 6.6 units of tyrosinase activity. This was divided into 2 g batches. The first batch (Batch A) containing 3.3 units of bacterial tyrosinase activity was incubated in 20 ml of air saturated Tris–HCl buffer (50 mM, pH 8.6) containing tyrosol (2.5 mM) and ascorbic acid (2.5 mM) at 30 °C for 30 min. The calcium alginate beads were subsequently removed from the biotransformation medium and replaced by the second batch of beads (Batch B). Batch A was washed in Tris–HCl buffer (50 mM, pH 8.6) and stored at room temperature awaiting re-addition to the biotransformation medium. Batch B was removed from the biotransformation reaction after 30 min and replaced by washed Batch A. Batch B was washed in Tris–HCl buffer (50 mM, pH 8.6) and stored at room temperature awaiting re-addition to the biotransformation medium. A total of 7 by 30 min biotransformations of tyrosol was performed.

2.5.2.3. Biotransformation of tyrosol through multiple additions of fresh immobilised cell extract in calcium alginate beads. Two grams of calcium alginate beads containing 3.3 units of bacterial tyrosinase activity were incubated in 20 ml of air saturated Tris–HCl buffer (50 mM, pH 8.6) containing tyrosol (2.5 mM) and ascorbic acid (2.5 mM) at 30 °C for 30 min. Following a 30 min incubation the beads were removed by filtration. Two grams of fresh calcium alginate beads were added to the biotransformation medium. A total of 20 g of calcium alginate beads (33.0 units of tyrosinase activity) were added to the biotransformation medium through 10 single additions.

2.6. Separation of biotransformation product from the reaction medium using Amberlite XAD-4® resin

Amberlite XAD-4® resin was packed into a glass column of 4 mm diameter × 100 cm in length and the resin washed three times with 10 ml methanol–HCl (pH 2.5). Reaction medium (20 ml) from incubations was sampled and analysed by HPLC to ensure that all substrate had been transformed. This reaction medium (15 ml) was passed three times through the resin and the eluate sampled each time. The resin was washed twice with 10 ml methanol–HCl (pH 2.5) and the eluate collected and sampled after each wash again, all samples were analysed by HPLC.

2.7. Chemicals

Tyrosol was supplied by Fluka Chemika (Dublin, Ireland) and hydroxytyrosol was supplied by Alexis Corporation (Nottingham, UK). All other chemicals were supplied by Sigma–Aldrich Ltd., Dublin, Ireland.

3. Results and discussion

3.1. Immobilisation of cell extracts of P. putida F6

Crude cell extracts of *P. putida* F6 expressing tyrosinase activity were immobilised in a calcium alginate matrix with an efficiency of approximately 95%. This compares favourably with the immobilisation of tyrosinase from other sources such as mushroom where the enzyme was immobilised on supports such as nylon 6,6 with a efficiency of 67% [7] and on sodium aluminosilicate and calcium aluminosilicate with efficiencies of 82 and 85%, respectively [23]. The enzyme activity on tyrosol as substrate was measured and compared to that of non-immobilised cell extracts under the same conditions (Section 3.2).

3.2. Biotransformation of tyrosol by immobilised and non-immobilised cell extracts of P. putida F6

Cell extracts were immobilised in calcium alginate beads to give an activity of 1.6 units/g of beads. Addition of 3.3 units of activity (2 g of beads) to the reaction medium containing 2.5 mM tyrosol effected the removal of 2.41 mM of the substrate over 2 h. Incubation of 3.3 units of non-immobilised enzyme resulted in the removal of 21% less tyrosol in the same time period (Fig. 1). However, the level of hydroxytyrosol detected was approximately two-fold higher when non-immobilised cell extracts were used (Fig. 1). This may be due to product through conversion or uptake by the calcium alginate beads, as assays where hydroxytyrosol was incubated with beads, which did not contain cell extracts showed a 10% removal of hydroxytyrosol.

3.3. Biotransformation of tyrosol using immobilised cell extracts in the presence of ascorbic acid

While the inclusion of ascorbic acid at high concentrations is known to cause inhibition of mushroom tyrosinase activity [17,18] the inclusion of ascorbic acid in biotransformations with tyrosinase has also been reported to result in the increased accumulation of catechol [4,7,14,19]. Ascorbic acid is believed to reduce quinones formed by tyrosinase activity back to catechol [4].

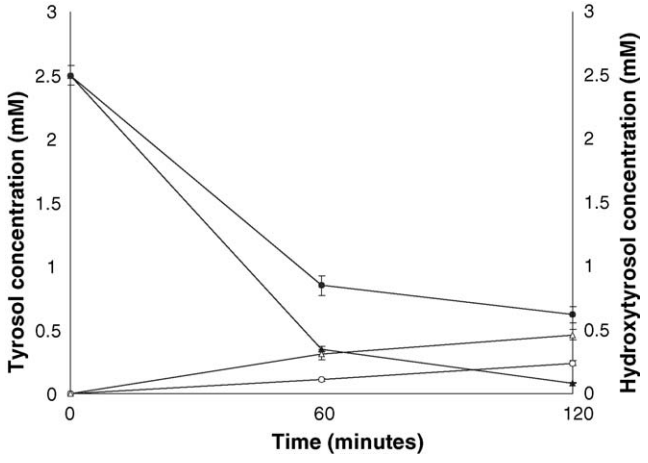

Fig. 1. Biotransformation of tyrosol to hydroxytyrosol by immobilised cell extracts (▲, tyrosol; △, hydroxytyrosol) and non-immobilised cell extracts (●, tyrosol; ○, hydroxytyrosol) of *P. putida* F6. All data are the average of at least three independent determinations.

3.3.1. Biotransformation of tyrosol using recycled calcium alginate beads

Incubation of immobilised cell extracts in a biotransformation medium with tyrosol and ascorbic acid resulted in a complete inhibition of substrate removal within 3 h (data not shown). To overcome this problem, two batches of immobilised enzyme (3.3 units of tyrosinase activity) were alternately added to the biotransformation medium (Section 2.5.2.2) for a total of 7 by 30 min biotransformations in the presence of ascorbic acid. This system resulted in the removal of 1.07 mM tyrosol from a starting concentration of 2.5 mM (Fig. 2). When the recycled biotransformation system was employed, the product (hydroxytyrosol) accumulated to a maximum concentration of 0.81 mM (Fig. 2). However, the rate of tyrosol utilisation by immobilised cell extracts of *P. putida* F6 in the presence of ascorbic acid (Fig. 2) was significantly lower than in the absence of ascorbic acid (Fig. 1). The decrease in the rate of substrate utilisation could be explained by product accumulation, as was observed for 4-fluorophenol transformation by non-immobilised cell extracts of *P. putida* F6 [14]. Greater than 57% of the tyrosol substrate still remained in the biotransformation medium. This will pose problems for simple extraction of the product from the reaction medium, as solid phase extraction methods currently

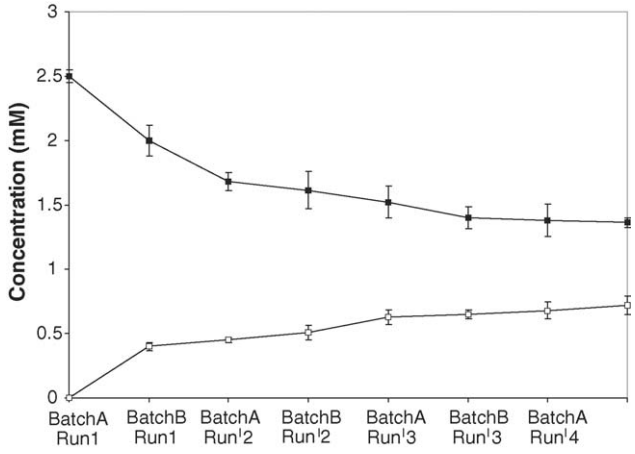

Fig. 2. The effect of reusing beads on tyrosol removal (■) and hydroxytyrosol formation (□) by immobilised cell extracts of *P. putida* F6. 6.6 units of tyrosinase activity from cell extracts of *P. putida* F6 were immobilised in 4 g of calcium alginate. The calcium alginate beads were divided into two Batches A and B. Both Batches A and B contained 3.3 units of tyrosinase activity in 2 g of beads. Batch A was added to the biotransformation medium for 30 min, subsequently removed and replaced by Batch B. Batch A was washed in Tris–HCl (50 mM, pH 8.6) and stored at room temperature while Batch B was in the biotransformation medium. Batch B was then removed, replaced by Batch A and washed in Tris–HCl (50 mM, pH 8.6). An alternating addition and removal of beads occurred over a 210-min period. All data are the average of at least three independent determinations.

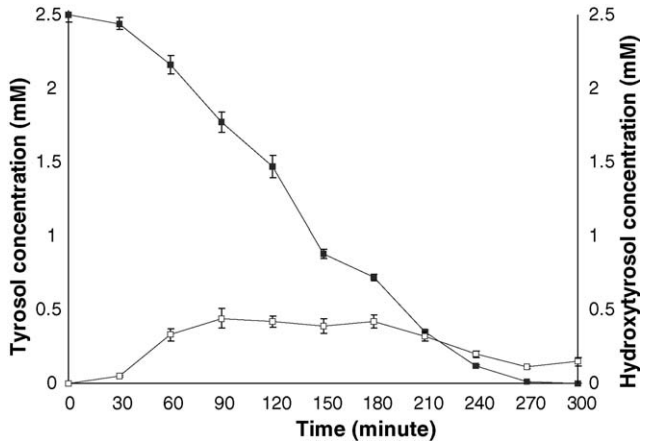

Fig. 3. Effect of sequential addition of fresh beads (2 g) on tyrosol removal (■) and hydroxytyrosol formation (□) by immobilised cell extracts of *P. putida* F6 (3.3 units of tyrosinase activity). All data are the average of at least three independent determinations.

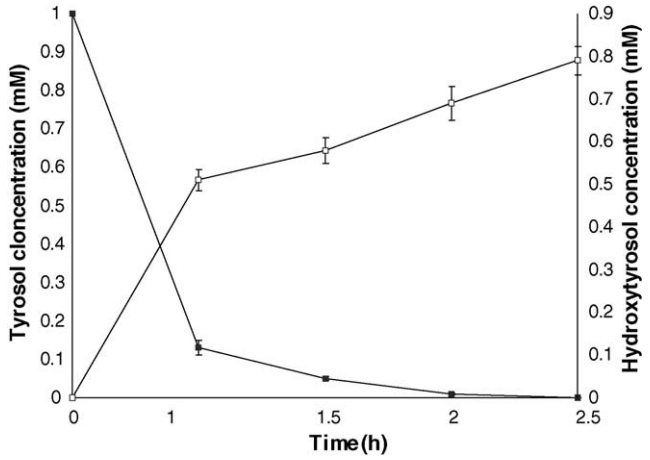

Fig. 4. Removal of 1 mM tyrosol by 2 g (3.3 units) of immobilised cell extracts of *P. putida* F6. (■) Tyrosol; (□) hydroxytyrosol. All data are the average of at least three independent determinations.

available do not distinguish between phenols (tyrosol) and catechols (hydroxytyrosol).

A further modification of the system was developed involving the sequential addition and removal of 10 sets of beads to the biotransformation medium.

3.3.2. Biotransformation of tyrosol using multiple additions of fresh immobilised cell extract in calcium alginate beads

The addition of 10 sets of fresh beads (3.3 units/2 g) resulted in the complete removal of 2.5 mM tyrosol in the presence of 2.5 mM ascorbic acid. However, the product only accumulated to a concentration of 0.15 mM (Fig. 3). This is thought to be due to the affinity of the enzyme for both catechols and phenols. Therefore, when catechol accumulates in the medium it can also be utilised as a substrate for fresh enzyme added to the reaction. Control experiments where beads which had no enzyme encapsulated in them were incubated with hydroxytyrosol under the exact same conditions, also showed 10% removal of product over the entire course of the reaction (data not shown). This would also contribute to the decrease in product yield observed.

The biotransformation was allowed to proceed in the absence of ascorbic acid and an attempt was made to reduce the quinone formed back to catechol post-reaction by adding the ascorbic acid at the end of the reaction. However, the addition of ascorbic acid post-reaction did not facilitate any significant increase in the accumulation of product when compared to reactions where no ascorbic acid was added post-reaction (data not shown). The failure of ascorbic acid to increase product accumulation may be due to polymerisation of quinones making them unavailable for reduction to catechols. Indeed polymerisation of quinones due to tyrosinase activity is well documented [2,3].

3.3.3. Biotransformation of tyrosol (1 mM) by immobilised cell extracts of P. putida F6

Due to the problems associated with the transformation of 2.5 mM tyrosol, the concentration of substrate was decreased to 1 mM. The lower concentration of substrate was removed completely by a single batch of immobilised cell extracts of *P. putida* F6 (3.3 units/mM) in the presence of 1 mM ascorbic acid (Fig. 4). The resulting hydroxytyrosol product was seen to accumulate to a maximum concentration of 0.77 mM (Fig. 4). This is a significant improvement on product yield when compared to that observed with the higher substrate concentration. The use of a single batch of beads is advantageous in terms of the cost effectiveness of the overall process as well as providing considerably less surface area for product uptake from the reaction medium.

3.4. Hydroxytyrosol recovery from an aqueous biotransformation medium using Amberlite XAD-4® resin

Due to the ability of Amberlite XAD-4® resin to bind phenols as well as catechols it was necessary to ensure that all the phenolic substrate was transformed prior to attempting product recovery. Failure to achieve this would result in a mixture of both substrate and product in the methanol–HCl wash necessitating further purification procedures. Following complete transformation of 1 mM 2-(4-hydroxyphenyl) ethanol by immobilised cell extracts in the presence of 1 mM ascorbic acid (Fig. 4), the desired product (hydroxytyrosol) was recovered by passing the reaction medium through a column packed with the adsorbent resin Amberlite XAD-4®. Amberlite XAD-4® has been used previously in the biotransformation of two-substituted phenols to three-substituted catechols using whole cells of *Pseudomonas azelaica* HBP-1 [20,21].

As no hydroxytyrosol was detected in the eluent (biotransformation medium) that had passed through the Amberlite XAD-4® resin it was assumed that all of the product had bound to the Amberlite XAD-4® resin (data not shown). The resin was subsequently washed with methanol–HCl to elute the bound hydroxytyrosol. Samples from the first and second wash contained 93 and 3.3% of the hydroxytyrosol, respectively. The third wash did not contain any hydroxytyrosol indicating that no more hydroxytyrosol had been removed from the resin after the second wash (Table 1). This solid phase extraction represents an overall product yield of 96.5%.

Table 1
Recovery of hydroxytyrosol from an aqueous reaction medium using Amberlite XAD-4® adsorbent resin

Eluent from	Product concentration (mM)
Biotransformation medium before addition to resin	0.8
Biotransformation medium after addition to resin	0
Methanol–HCl wash (1)	0.745
Methanol–HCl wash (2)	0.026
Methanol–HCl wash (3)	0

3.5. Storage of immobilised cell extracts

When immobilised and non-immobilised cell extracts of *P. putida* F6 were stored under differing conditions at 4 °C an overall loss of activity was observed in both preparations over a 9-day period. However, the immobilised form was 35% more stable and removed more substrate than the free enzyme at the end of the 9 days of storage (Fig. 5). Storage of the beads dry on filter paper at 4 °C resulted in maximum retention of activity, whereby the rate of substrate removal decreased by 18%, compared to non-immobilised cell extracts also stored at 4 °C, where the rate of substrate removal was 51% of the fresh cell extracts over the same time period. Beads stored at 4 °C in Tris–HCl buffer lost all activity after 5–7 days storage (Fig. 5), while freezing the beads resulted in complete degradation of the matrix.

Mushroom tyrosinase entrapped in 2% calcium alginate gel exhibited increased relative activity over a period of 8 days, which some authors attribute to the entrapped enzyme leaching out of the highly porous beads [24]. However, immobilised cell extracts in this study were prepared using a 4% (w/v) sodium alginate paste leading to a decreased pore size. In general immobilisation confers enhanced stability on the tyrosinase enzyme, as was observed with mushroom tyrosinase immobilised on calcium aluminosilicate where the enzyme was recycled for a total of 12 batches over 5 days covering 40 h operation [23] and on magnetite where the enzyme exhibited only 5% loss of activity after 15 days storage at ambient temperature [22].

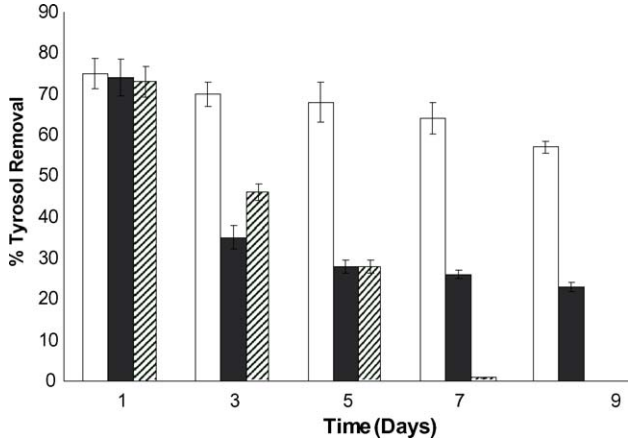

Fig. 5. Effect of storage conditions on tyrosol removal cell extracts of *P. putida* F6. (□) Immobilised cell extracts of *P. putida* F6 in calcium alginate beads stored dry on filter paper at 4 °C; (■) non-immobilised cell extracts stored at 4 °C; (▨) immobilised cell extracts of *P. putida* F6 in calcium alginate beads stored at 4 °C in Tris–HCl buffer. All data are the average of at least three independent determinations.

4. Conclusion

Optimisation of tyrosol transformation by an immobilised bacterial tyrosinase activity resulted in complete substrate removal and an overall yield of 77% of hydroxytyrosol in the presence of ascorbic acid. Recovery of hydroxytyrosol from an aqueous medium was achieved using a simple solid phase extraction. While the efficiency of tyrosol to hydroxytyrosol conversion by the immobilised system is high the system will have to be improved to accumulate higher levels of hydroxytyrosol. Indeed the immobilised tyrosinase system will have to be improved to be as effective as the whole cell transformation reported by Allouche et al. where up to 96% of the tyrosol (29 mM) was converted to hydroxytyrosol [12].

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