Catalytic properties of catechol 1,2-dioxygenase from Acinetobacter radioresistens S13 immobilized on nanosponges

UNIVERSITÀ DEGLI STUDI DI TORINO

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Abstract

Catechol 1,2-dioxygenases are iron containing enzymes able to convert catechol in *cis,cis*-muconate, a precursor of the industrially important compound adipic acid. Catechol 1,2-dioxygenase from *Acinetobacter radioresistens* S13 was immobilised on β-cyclodextrins cross-linked with carbonate groups (nanosponges) with a yield of 29 mg enzyme/g of support. This support was chosen for its low cost and its ability to offer different types of interactions with the enzyme. The activity profiles at different pH and temperatures showed a shift of the optimal pH from 8.5 to 9.5 and optimal temperature from 30°C to 50°C for the free and immobilized proteins respectively. The K_M resulted increased from 2.0 ± 0.3 μM for the free form to 16.6 ± 4.8 μM for the immobilised enzyme, whereas the k_{cat} values were found to be 32 ± 2 s^{-1} and 27 ± 3 s^{-1} for the free and immobilized forms respectively. The immobilization process also increased the thermostability of the enzyme with 60% residual activity after 90 minutes at 40°C for the immobilized protein versus 20% for the free enzyme. A residual activity of 75% was found after 15 minutes at 60°C for the immobilized enzyme while the free form showed a total loss of activity under the same conditions. The activity toward other substrates, such as 3- and 4-methylcatechol and 4-chlorocatechol, was retained by the immobilised enzyme. A small scale bioreactor was constructed and was able to convert catechol into *cis-cis* muconic acid with high efficiency for 70 days.

Introduction.

The exploitation of enzymes as biocatalysts for the "green" production of industrially important compounds is an interesting challenge for biotechnology. One such process is the enzymatic cleavage of catechol to lead to *cis-cis* muconic acid that in turn is hydrogenated to produce adipic acid, commonly used for the benzene-free synthesis of nylon-6,6.[1] The worldwide industrial production of adipic acid is estimated to be 2.3 million metric tons per year and 80% of it was used for nylon-6,6 synthesis.[2] The global market for adipic acid has been predicted to reach 6.3 billion pounds by 2012 (report by Global Industry Analysts, Inc.) as it is used as basic feedstock also in the production of engineering plastics, polyurethane elastomer, pharmaceuticals and derivatives for clothes. Furthermore, adipic acid is also used for the synthesis of biodegradable polymers with applications in many processes among which green polyurethane coatings[3] and tissue engineering.[4] For this reasons our laboratory has turned its attention to catechol 1,2-dioxygenases from *Acinetobacter radioresistens*, an iron containing enzyme involved in the degradation of aromatic compounds, including phenols. It catalyses the intradiol cleavage of catechol resulting in the formation of *cis-cis* muconic acid.[5] Catechol is a common intermediate in the degradation pathways of many monocyclic aromatic compounds including benzene and toluene[6,7] and polycyclic aromatic molecules such as pyrene[8] and naphthalene.[9,10]

In order to construct a bioreactor able to produce muconic acid with high efficiency and operational stability, the enzyme catechol 1,2-dioxygenase was selected as it does not require any external cofactor for catalysis. In particular, in this work the isoenzyme B of catechol 1,2-dioxygenase from *Acinetobacter radioresistens* S13[11,12] was immobilised on nanosponges formed by β-cyclodextrins linked by carbonate groups to give a solid support.[13] To our knowledge this is the first work where direct

enzyme immobilization has been performed on modified cyclodextrins with the aim to create a bioreactor. Recently, cyclodextrin derivatives have been used to construct supramolecular assemblies to increase the number of adamantine-modified protein molecules at the surface of gold electrodes,[14-16] but without the advantage of a sponge-like solid support suitable for the construction of a bioreactor, as described in this paper.

One of the major current goals in the field of biocatalysis is to develop low-cost systems using suitable enzymes that are permanently immobilized on a solid support and are able to perform different cycles of catalysis. Both catechol 1,2-dioxygenase and nanosponges were selected here as they respond to these requirements.[13,17] In particular, nanosponges were chosen for enzyme immobilization on the basis of its physico-chemical properties. On one hand, the hydrophobic cavities of the β-cyclodextrins are known to accommodate solvent exposed aromatic side chains of the protein.[18] On the other hand, hydroxyl groups are displayed on their exterior and carbonates are present in the linkers between different cyclodextrin units, giving rise to a complex network of polar groups able to interact via hydrogen bonds and electrostatic interactions with polar side chains of the protein. These protein-support interactions between the nanosponges and the enzyme do not present the disadvantages of a covalent immobilization, where the enzyme is forced in a single conformation that may not warrant the most efficient catalysis. The sum of all the weak interactions taking place between the enzyme and nanosponges is expected to lead to a tight binding of the protein to the support allowing different cycles of catalysis whilst immobilized. In addition, the support offers the advantage that is produced via a cheap synthetic procedure, key to its applicability in large scale industrial processes.

Catechol 1,2-dioxygenase immobilised on nanosponges was characterised in terms of stability at different pH and temperatures, storage and kinetic properties. The stability of the bioreactor was tested over time for different reaction cycle.

Experimental

Materials

Catechol 1,2-dioxygenase was expressed and purified as previously described[19] whereas cyclodextrin-based nanosponges were synthesized as reported by Cavalli et al.[17]

Chemicals including catechol and substituted catechols were purchased from Sigma-Aldrich.

Determination of the protein concentration and activity assay

Protein concentration was extimated at 280 nm by using an extinction coefficient ε_{280} of 53560 $M^{-1}cm^{-1}$.[12]

The concentration of active protein was determined from its specific activity and was evaluated for each batch of protein used.

The enzyme activity was assayed by recording the increase of absorbance at 260 nm due to the formation of *cis-cis* muconic acid.[11] The slope of the initial linear increase in absorbance at 260 nm was considered. Assays were carried out at 30°C in a 1 cm path-length cell. The reaction mixture contained the enzyme in nanomolar quantity, 200 μM catechol in a 50 mM Hepes pH 8.0 buffer. The substrate catechol was freshly

prepared in a 50 mM Hepes pH 8.0 buffer solution. The product was quantified by using an extinction coefficient at 260 nm (ε_{260}) of 17600 M^{-1} cm^{-1}.

Immobilization of catechol dioxygenase

The dried nanosponges were hydrated and washed five times with 100% ethanol and ten times with 50 mM Hepes pH 8.0. The resuspended nanosponges (1 g) were gently mixed with a known enzyme concentration (0.2 mg) in a final volume of 1 mL at 22°C. The supernatant was then removed and nanoponges washed with 50 mM Hepes pH 8.0 until no catechol 1,2-dioxygenase activity was detected in the washing solutions.

The yield of immobilisation was determined from the difference between the initial quantity of protein loaded onto the support and the quantity found in the washing solutions.

Determination of the maximal binding capacity of nanosponges

The maximal amount of enzyme retained by the support was determined by incubating 1.7 mg of protein with 0.05 g of nanosponges at 22°C for 4 hours and by calculating the residual protein content in the washing solutions.

Effect of pH and temperature on the enzyme activity

The effect of pH on the activity of catechol 1,2-dioxygenase in the free and immobilised forms was investigated in the pH range 5.5-10 at 30°C.

The enzyme was incubated for 3 minutes in different buffers with the desired pH and the reaction was then started with the addition of 200 μM of catechol. The buffer solutions used were: Mes (for pH ranging from 5.5 to 7), Hepes (for pH ranging from

7 to 8.5) and Ches (for pH ranging from 8.5 to 10). The buffer concentration was chosen appropriately to maintain a constant ionic strength.

The activity at different temperatures (from 10 to 60°C) was monitored by incubating the enzyme for 3 minutes in a 50 mM Hepes pH 8.0 buffer in the spectrophotometer cell, equipped with a Peltier 89090A temperature control system (Agilent Technologies). The reaction was then started with the addition of 200 μM of catechol and the activity measured.

The activation energies (E_a) were calculated by using the Arrhenius equation:

$$\ln k = - E_a/RT + \ln A$$

where k is the rate constant, T is the temperature (Kelvin), R the gas constant and A the pre-exponential factor.

Fittings of the experimental data were performed by using Sigma Plot 8.0 software.

Thermostability and storage stability

The thermal stability was investigated by incubating the enzyme in a 50 mM Hepes buffer at 2 different temperatures (40°C and 60°C). Aliquots were removed at regular intervals and assayed for their residual activity.

Storage stability was investigated by monitoring the residual activity of both free and immobilised enzymes after storing them at 4°C and 25°C.

Determination of the catalytic parameters

The catalytic parameters (K_M and k_{cat}) of the free and immobilised enzyme were calculated by measuring the initial linear rates of the reaction after the addition of

different concentrations of the substrate catechol, ranging from 1 to 100 μM at 30°C. Five independent measurements were carried out for each substrate concentration. The experimental data were fitted to the Michaelis-Menten curve by using Sigma Plot 8.0 software.

Substrate specifity

The ability of immobilised catechol 1,2-dioxygenase to recognise 3-methyl-, 4-methylcatechol and 4-chlorocatechol was measured spectrophotometrically by the increase in absorbance at 260 nm due to the formation of the reaction product. The relative activity was calculated considering the activity toward catechol as 100%. The substrate concentration was 200 μM, known to be saturating and not inhibiting for the enzyme.

Reusability

A solution containing 1 g of nanosponges and 0.3 mg of catechol 1,2-dioxygenase was used as stationary phase to pack a column (5 cm height, 1 cm diameter, GE Healthcare). The column was stored at 4°C. At regular intervals (1 day) 1 mM catechol (1 ml) was loaded into the column and, after 1 hour, the column was washed with 50 mM Hepes pH 8.0. The absorbance spectrum of the washing solutions was recorded and the product *cis-cis* muconic acid was quantified by using an extinction coefficient (ε_{260}) of 17600 M^{-1} cm^{-1}.[11]

Results

Immobilization of catechol 1,2-dioxygenase on nanosponges

The purified catechol 1,2-dioxygenase was immobilized by adsorption on nanosponges and the yield of immobilization was evaluated by incubating at different times 0.3 mg of enzyme with 1 gram of solid support at 4°C and 22° C. As shown in figure 1, 96% of the enzyme was immobilized onto the support after 4 hours at 22°C; a yield of 50% was reached after 10 minutes of incubation, indicating a high affinity of the enzyme for the support.

FIGURE 1.

The maximal binding capacity of nanosponges was then evaluated by mixing 1.69 mg of protein with 0.05 g of nanosponges for 4 hours at 22°C in a 50 mM Hepes pH 8.0 buffer. The nanosponges were extensively washed with the buffer solution and the unbound protein was quantified. The presence of catechol 1,2-dioxygenase activity in the first two washing solutions demonstrated that the maximal binding capacity of the support is 28.9 mg of protein per 1 g of nanosponges.

Effect of pH and temperature on enzyme activity

The activity of free and immobilised catechol 1,2-dioxygenase was measured at different pH values (from 5.5 to 10). As shown in figure 2, the activity profiles for the free and immobilized proteins were different and the pH value of optimum activity was shifted from 8.5 to 9.5 for the free and the immobilised enzyme respectively (table 1). Furthermore, the immobilised enzyme showed 40% of activity at pH 6.5 whereas the free form was inactive.

FIGURE 2.

The effect of temperature on enzyme activity was investigated in a range 10-60°C. The free enzyme exhibited the optimum activity in the range 30-40° C with a complete loss of activity at 60°C, while the immobilised enzyme showed the highest activity at 50°C and retained 70% of its activity at 60°C (figure 3A).

Arrhenius plots were then constructed and a linear relationship was found up to 30°C (303 K) and 60°C (333 K) for the free and immobilised enzymes respectively (figure 3B). As the temperature was further increased, a drop in enzyme activity was observed indicating protein denaturation. However, a biphasic behaviour was observed for the immobilised protein with a discontinuity at 25°C.

FIGURE 3

The experimental data were then fitted with linear regressions to calculate the activation energies (E_a) from the Arrhenius equation (figure 3B) and the results are shown in table 1. The activation energies were similar for the free and immobilised catechol dioxygenase below 25°C (45 ± 5 kJ/mol and 49 ± 2 kJ/mol respectively), whereas a lower E_a (19 ± 3 kJ/mol) was found for the immobilised enzyme above 25°C.

Thermostability and storage stability

The stability of the free and immobilised enzymes over time was measured at 40°C and 60°C. Figure 4A shows a complete loss of activity after 90 minutes at 40°C in the case of the free enzyme, whereas after the same time the immobilised form retained 50% of the maximal activity and became totally inactivated only after 200 minutes. Furthermore, the activity profile of the immobilised protein shows a biphasic behaviour that might reflect the presence of two enzymatic conformations differently stabilized by the immobilization process.

Figure 4B shows the thermal stability profile at 60°C. In this case a complete loss in activity was observed after 15 minutes in the case of the free enzyme whereas the immobilised form retained more than 70% of the activity after the same time.

The storage stability was also measured at 4°C and 25°C. The immobilised enzyme was demonstrated to retain 50% of the initial activity after 10 days at 4°C, whereas the activity of the free form was lower than 10% of the initial activity.

FIGURE 4

Similar results were found by storing the protein at 25°C. The free catechol dioxygenase retained 10% of the starting activity after 4 days whereas for the immobilised form the residual activity was 50%. Complete inactivation was observed after 5 days for the free enzyme and after 11 days for the immobilised protein. Also in this case a biphasic behaviour was observed for the immobilised enzyme, but not for the free form.

Determination of the catalytic parameters and substrate specifity

In order to calculate the apparent values of Michaelis-Menten parameters (K_M and k_{cat}) the activity of the free and immobilised enzyme were measured at different substrate concentrations. The reaction was followed at 260 nm and the initial linear rate considered. A typical hyperbolic trend was observed for both free and immobilized protein (figure 5). Fittings of the experimental data to Michaelis-Menten curve resulted in a K_M of 2.0 ± 0.3 and 16.6 ± 4.8 μM and a k_{cat} of 32 ± 2 and 27 ± 3 s^{-1} for the free and immobilized enzyme respectively. As reported in table 1, an increase in K_M (from 2.0 ± 0.3 to 16.6 ± 4.8 μM) was found for the immobilised protein whereas the k_{cat} values were very similar for the free and immobilised forms.

FIGURE 5 AND TABLE 1.

The ability of the immobilised enzyme to turn over substituted 3-methyl-, 4-methyl- and 4-chlorocatechol was found to be similar for the first two compounds and 3 fold higher toward 4-chlorocatechol when compared to the free form (table 2).

TABLE 2

Reusability

In order to assess the applicability of the enzyme-support system in a bioreactor, 1 gram of nanosponges carrying 0.3 mg of enzyme was used to pack a column and the ability of the immobilised enzyme to support different cycles of reaction was tested as a function of time. For each cycle a solution of 1 mM catechol was injected through the column and after 1 hour the column was washed with buffer and the amount of *cis,cis*-muconic acid was quantified by UV spectroscopy at 260 nm. As shown in figure 6, the system converted 83% of the catechol to muconic acid in 1 hour after 10 cycles and 40% after 60 cycles. The presence of catechol dioxygenase activity was assayed in the solution coming from the column and no activity was found.

FIGURE 6

Discussion

The enzyme catechol 1,2-dioxygenase was successfully immobilized by adsorption on nanosponges, a support that offers the opportunity of exploitation in protein immobilization field for the development of a biocatalyst suitable for industrial scale production of muconic acid. The binding of the protein is explained by different types of interactions. In fact, literature NMR data about the interaction between proteins and cyclodextrins in solution,[18] showed that the hydrophobic cavity of cyclodextrins can accommodate the aromatic side chains of solvent exposed residues such as

tryptophans, tyrosines and phenylalanines. Furthermore, the polar groups (hydroxyls and carbonates) of the support can interact with polar residues of the protein and form hydrogen bonds. Although the structure of catechol 1,2-dioxygenase from *Acinetobacter radioresistens* is not available, it is most probable that all these interactions can occur with aromatic and polar residues present in the sequence of the protein.[19] Sequence alignment of the enzyme used in this work with that of catechol 1,2-dioxygenase from *Acinetobacter* sp. ADP1 revealed 71% of sequence homology between the two proteins. The analysis of the known crystal structure of catechol 1,2-dioxygenase from *Acinetobacter* sp. ADP1 shows the presence of tryptophans, tyrosines and phenilalanines spread on the protein surface. Most of them result to be conserved in the sequence of our protein and could potentially interact with nanosponges.

It is well known that immobilized enzymes can show a higher stability than the free counterparts. For this reason, we investigated the activity of the enzyme over a wide range of pH values and temperatures and we found a shift of the maximal activity to a higher pH value for the immobilized enzyme in comparison to the free counterpart. An opposite behaviour was previously observed for catechol 1,2-dioxygenase from *Nocardia* sp. NCIB 10503 immobilized on cyanogens bromide-activated agarose, where the optimal pH was found in the range 8-10 for the free enzyme and at 7.5 for the immobilized form.[21]

Also in the case of the temperature profile (figure 3), a shift in the optimal temperature from 30°C for the free to 50°C for the immobilised enzyme was observed. This kind of behaviour has already been reported for both intradiol catechol dioxygenase entrapped on another support (calcium alginate hydrogels)[22] and extradiol catechol dioxygenase covalently immobilized by multi-point attachment on

highly activated glioxyl agarose beads.[23] A shift in the optimal temperature can be explained by the increase in conformational stability given to the protein by the support. For the free catechol 1,2-dioxygenase used in this work, it was previously demonstrated that structural changes of the enzyme and loss of catalytic iron from the active site are linearly proportional.[12] As such, the interaction with the support could decrease the mobility of the protein allowing a tighter binding of the catalytic iron to the active site of the enzyme.

The experimental data obtained from the temperature profiles were used to construct Arrhenius plots that revealed a biphasic behavior for the immobilized protein with a decrease in the activation energy starting from 25°C. Biphasic Arrhenius plots can derive from different effects including protein denaturation at high temperatures, mass transfer limitations, presence of two different conformations of the enzyme stabilized in different temperature ranges and a change in the rate determining step of the reaction across the two temperature ranges.[24-25] Enzyme denaturation at high temperatures was tested by measuring the residual activity during time at 40°C and 60°C and the immobilized enzyme resulted more stable than the free counterpart with a complete loss of activity after 90 minutes at 60°C. These results clearly indicated that the biphasic Arrhenius plot obtained for the immobilized enzyme could not be simply explained by protein denaturation at high temperatures.

As stated before, mass transfers limitations can also give rise to biphasic Arrhenius plots and a decrease of the activation energy after immobilization has already been observed for other enzymes[26] and correlated to intraparticle diffusion of the substrate,[27] consistent with the presence of a pore diffusion effect.[28] In the case of immobilized catechol 1,2-dioxygenase, the activation energy calculated below 25°C was comparable to that of the free enzyme (table 1) whereas it was significatively

decreased above 25°C. A possible effect of the support used for the immobilization was excluded because it is known that nanosponges are chemically and physically stable up to 120°C (Trotta F., unpublished work). In order to test if mass transfer limitations could affect our system, we compared the catalytic parameters of the free and immobilised enzyme. Even if an increase in K_M value for the immobilised enzyme was observed, the k_{cat} was not affected excluding the influence of pore diffusion effects[29] and of a change in the rate determining step of the reaction across the two temperature ranges, known to be possible causes of a biphasic Arrhenius plot. These data, together with the biphasic behaviour found in the thermostability profiles (figures 4A) strongly suggest that two different conformations of the enzyme are present on the support and that a conformational transition could take place at 25°C. The support would stabilize two different forms of the enzyme depending on the conditions used and giving conformational constraints that would explain also the change in K_M observed and the increase in activity of the immobilised form toward 4-chlorocatechol.

The enzyme-support system obtained from the immobilization of catechol dioxygenase on nanosponges was also tested for its reusability and showed the ability to convert 50% of the substrate catechol after 50 cycles without enzyme detachment from the support. These results demonstrate not only the reusability of the system for different cycles, but also that the enzyme is tightly bound to the support despite the non-covalent immobilization method used.

Conclusions

In conclusion, this work shows the feasibility of an enzyme based bioreactor for the production of muconic acid. The enzyme catechol 1,2-dioxygenase, which does not

require an external cofactor for catalysis, was successfully immobilized by adsorption on a support formed by chemically modified cyclodextrins. The stability of the enzyme was improved at high temperatures and over a wide range of pH and the small bioreactor engineered was found to be stable over a period of 70 days without enzyme detachment. The bioreactor presents all the features required to be upgraded to a continuous-flow system that could maximize the production of muconic acid. Such engineered system would be a useful tool to obtain an expensive compound such as muconic acid from the much cheaper catechol for the production of the industrially important adipic acid.

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TABLES

Table 1. Enzymatic parameters calculated for free and immobilised catechol 1,2-dioxygenase.

	Free catechol 1,2-dioxygenase	Immobilised catechol 1,2-dioxygenase
K_M (μM)	2.0 ± 0.3	16.6 ± 4.8
k_{cat} (s^{-1})	32 ± 2	27 ± 3
pH for maximal activity	8.5	9.5
Temperature for maximal activity (°C)	30	50
E_a (kJ / mol)	45 ± 5	E_{a1} = 49 ± 2 E_{a2} = 19 ± 3

Table 2. Substrate specificity of catechol 1,2-dioxygenase in free and immobilised forms.

Substrate	Relative activity % (free catechol 1,2-dioxygenase)	Relative activity % (immobilised catechol 1,2-dioxygenase)
Catechol	100	100
3-methylcatechol	4.1 ± 1.6	9.9 ± 5.2
4-methylcatechol	37.5 ± 6.3	44.0 ± 9.0
4-chlorocathecol	5.2 ± 2.7	15.6 ± 3.9

Figure Legends

Figure 1. Time-course of immobilization of catechol 1,2-dioxygenase on nanosponges at 22°C. The quantity of protein immobilized is expressed in % where a yield of 100% represents the complete immobilization of the enzyme initially loaded (0.2 mg) onto the support.

Figure 2. pH profile of catechol 1,2-dioxygenase activity in the free (white squares) and immobilized (black circles) form. The enzyme was incubated for 3 minutes in different buffers with the desired pH and the reaction was then started with the addition of 200 μM of catechol. The activity was measured as increase of absorbance at 260 nm, due to the formation of *cis,cis*-muconic acid. The initial linear increase was considered over a time of 2 minutes. The data points represent the average of 5 independent measurements.

Figure 3. A) Temperature profile of catechol 1,2-dioxygenase activity in the free (white squares) and immobilized (black circles) form. B) Arrhenius plot for the free (white squares) and immobilized (black circles) catechol 1,2-dioxygenase. The enzyme was incubated at the desired temperature for 3 minutes in a 50 mM Hepes pH 8.0 buffer . The activity was measured as increase of absorbance at 260 nm, due to the formation of *cis,cis*-muconic acid. The initial linear increase was considered over a time of 2 minutes. The data points represent the average of 5 independent measurements.

Figure 4. Thermostability at A) 40°C and B) 60°C of free (white squares) and immobilized (black circles) catechol 1,2-dioxygenase. The enzyme was incubated at the desired temperature in a 50 mM Hepes pH 8.0 buffer and aliquots were removed at regular intervals and assayed for their residual activity. The data points represent the average of 5 independent measurements.

Figure 5. Michaelis-Menten plot for the immobilized catechol 1,2-dioxygenase. The activity was monitored as increase in absorbance at 260 nm. The initial linear rate was considered at the different substrate concentration. The data points represent the average of 5 independent measurements.

Figure 6. Reusability of catechol 1,2-dioxygenase immobilized on nanosponges. The percentage of product formed after 1 hour at room temperature from a 1 mM solution of the substrate catechol is shown as function of time (days). One cycle of reaction a day was performed.

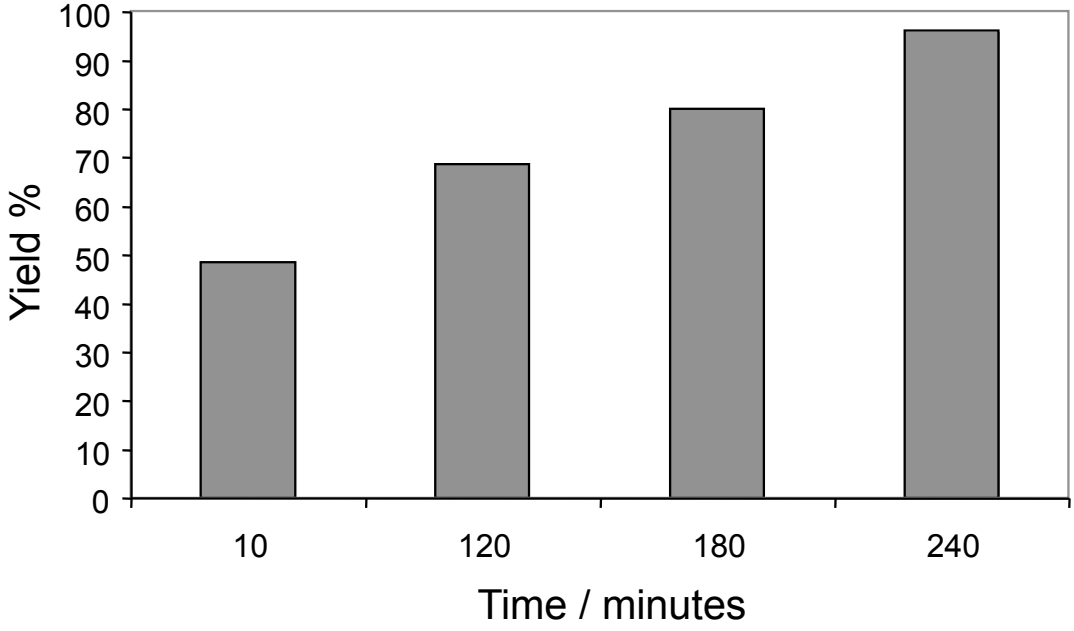


FIGURE 1

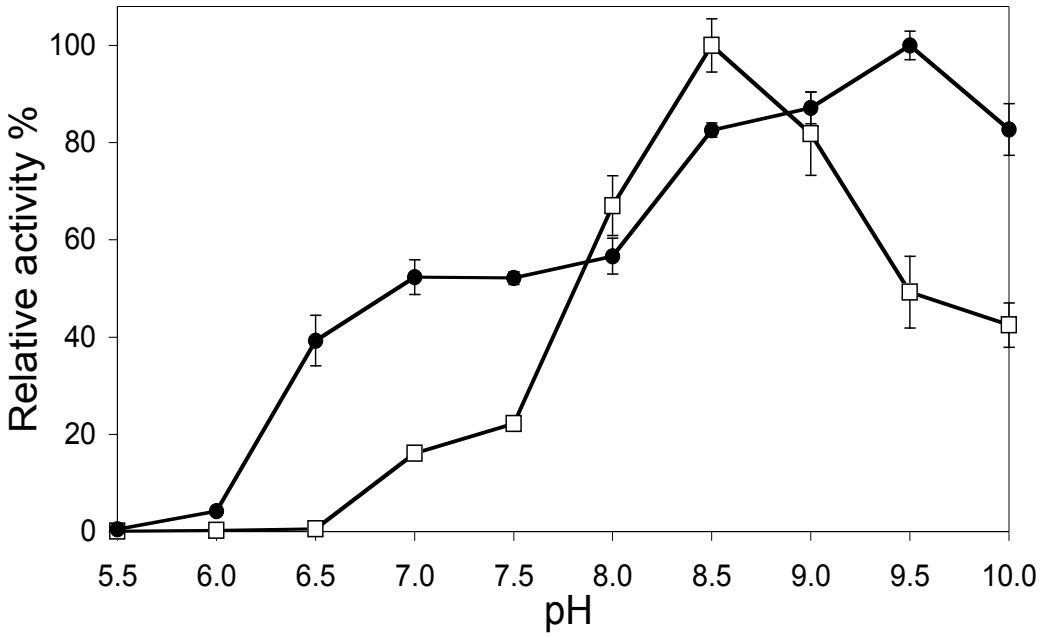
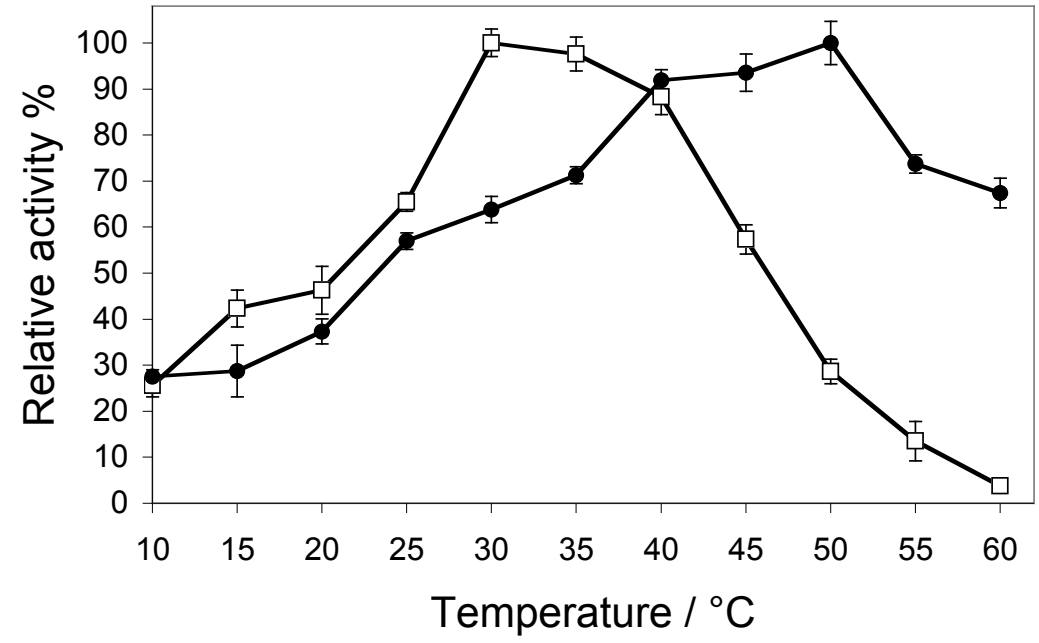

Relative activity %
100
80
60
40
20
0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

FIGURE 2


Relative activity %
100
90
80
70
60
50
40
30
20
10
0
10
15
20
25
30
35
40
45
50
55
60
Temperature / °C

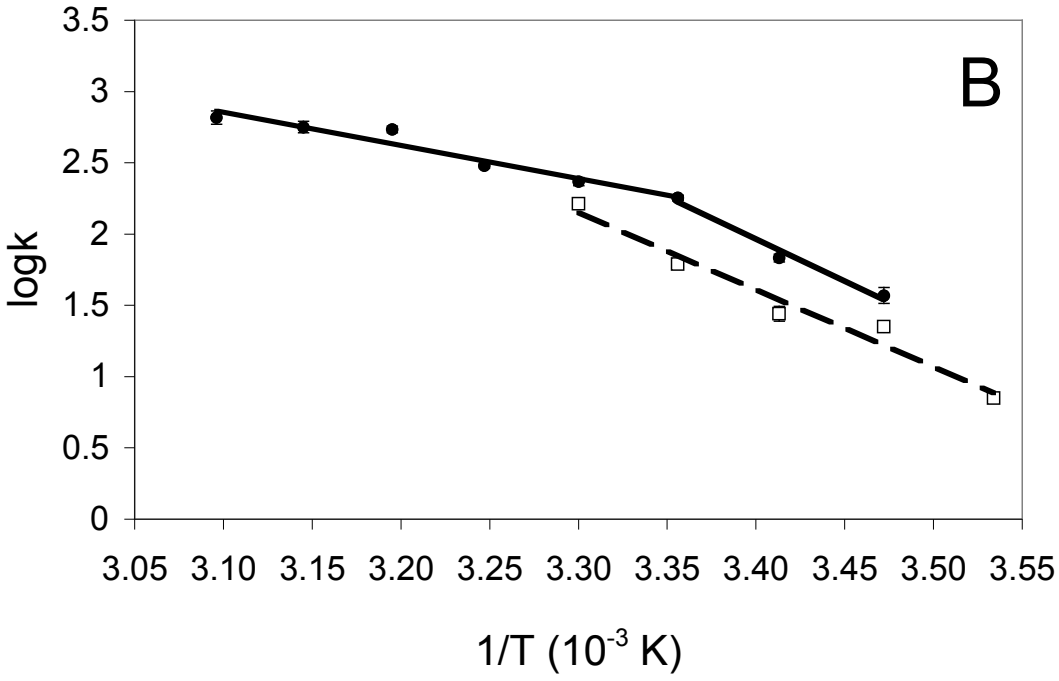
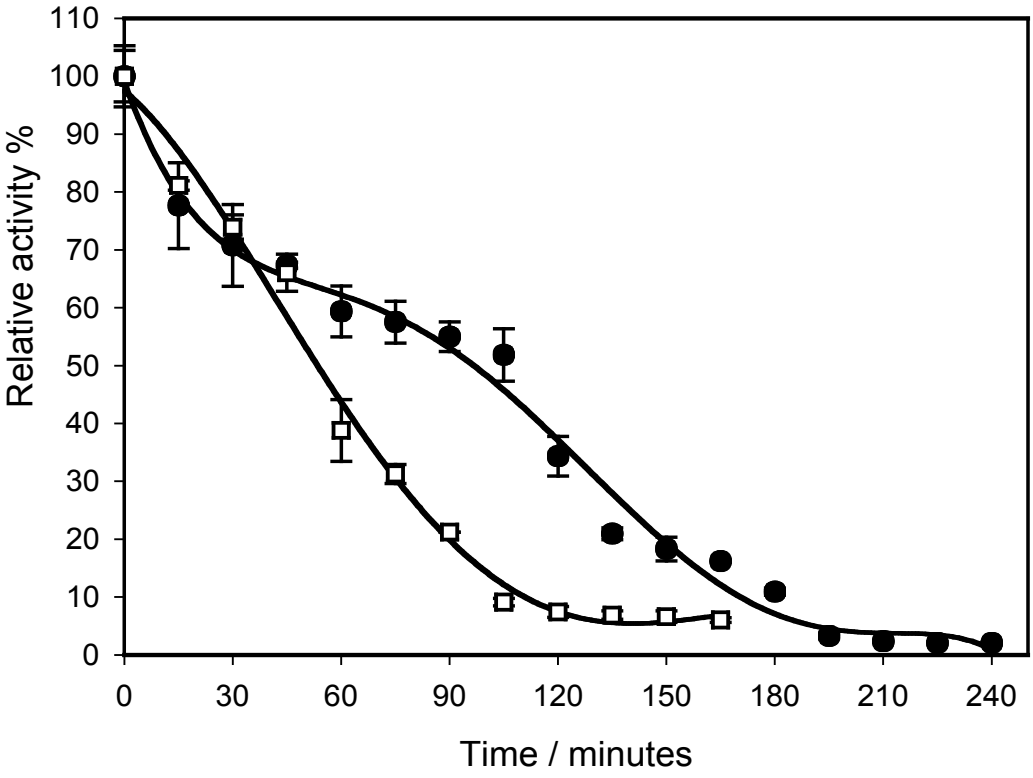

B
logk
3.5
3
2.5
2
1.5
1
0.5
0
3.05
3.10
3.15
3.20
3.25
3.30
3.35
3.40
3.45
3.50
3.55
1/T (10-3 K)

FIGURE 3


Relative activity %
110
100
90
80
70
60
50
40
30
20
10
0
0
30
60
90
120
150
180
210
240
Time / minutes

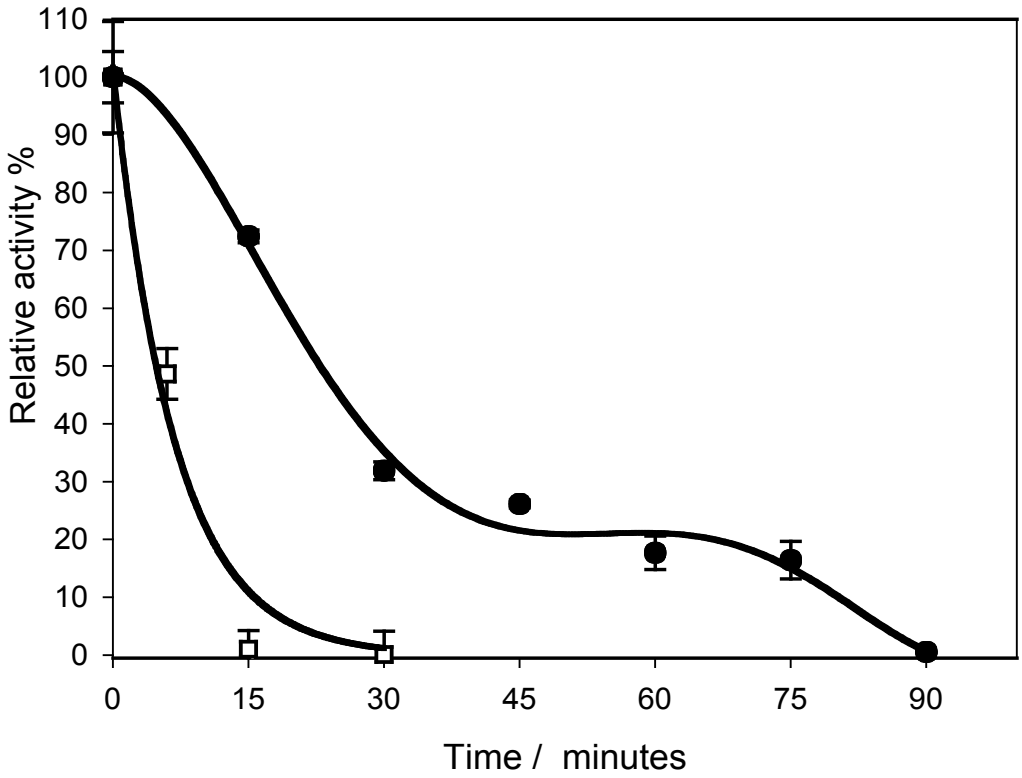

Relative activity %
110
100
90
80
70
60
50
40
30
20
10
0
0
15
30
45
60
75
90
Time / minutes

FIGURE 4

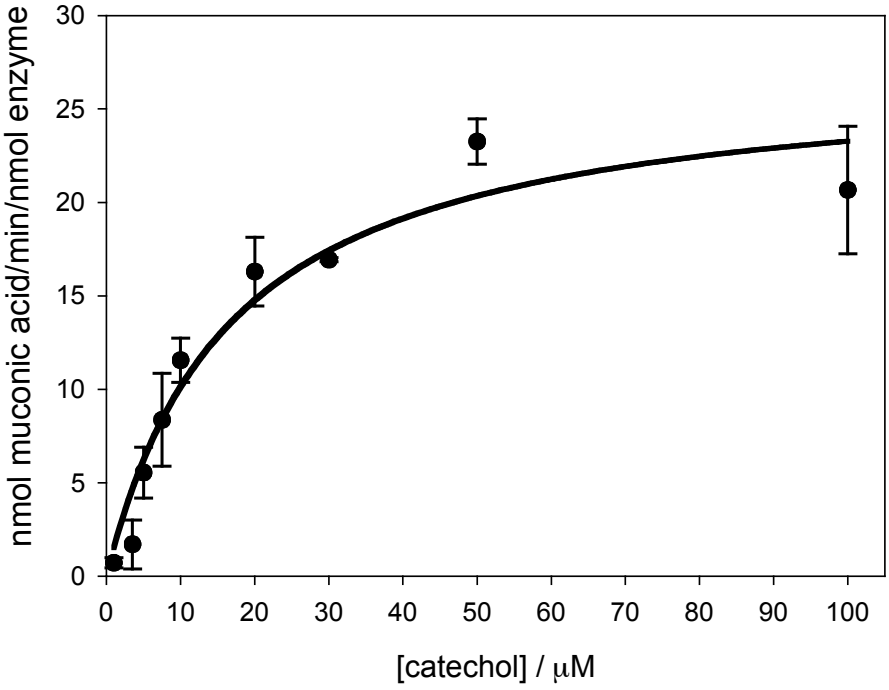

nmol muconic acid/min/nmol enzyme
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
90
100
[catechol] / μM

FIGURE 5

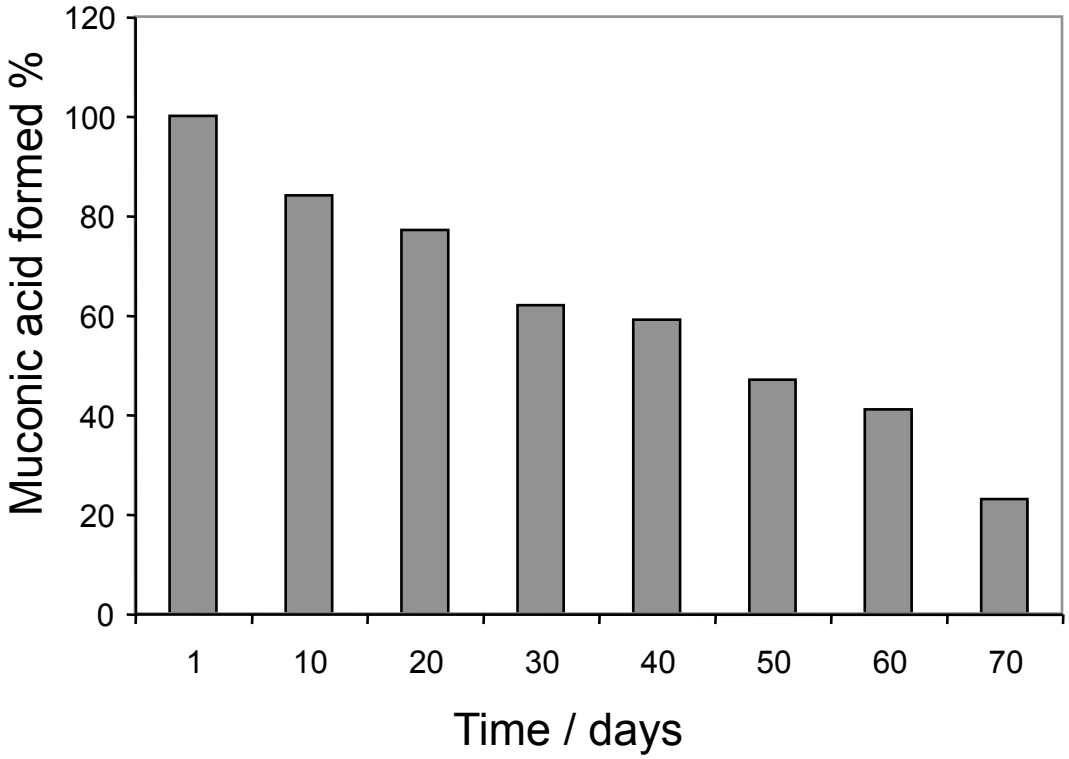

Muconic acid formed %
120
100
80
60
40
20
0
1
10
20
30
40
50
60
70
Time / days

FIGURE 6