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Characterisation of tyrosinase immobilised onto spacer-arm attached glycidyl methacrylate-based reactive microbeads

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Abstract

Immobilisation of tyrosinase onto modified poly(methyl methacrylate–glycidyl methacrylate–divinyl benzene), poly(MMA–GMA–DVB), microbeads was studied. The epoxy group containing poly(MMA–MMA–DVB) microbeads were prepared by suspension polymerisation. The epoxy groups of the poly(MMA–GMA–DVB) microbeads was converted into amino groups with either ammonia or 1,6-diaminohexane (i.e., spacer-arm). Tyrosinase was then covalently immobilised on aminated and the spacer-arm-attached poly(MMA–GMA–DVB) microbeads using glutaric dialdehyde as a coupling agent. Incorporation of the spacer-arm resulted an increase in the apparent activity of the immobilised tyrosinase with respect to the enzyme immobilised on the aminated microbeads. The activity yield of the immobilised tyrosinase on the spacer-arm-attached poly(MMA–GMA–DVB) microbeads was 68%, and this was 51% for the enzyme, which was immobilised on the aminated microbeads. Both immobilised tyrosinase preparation has resistance to temperature inactivation as compared to that of the free form. The temperature profiles were broader for both immobilised preparations than that of the free enzyme. Kinetic parameters were determined for immobilised tyrosinase preparations as well as for the free enzyme. The values of the Michaels constants (K_m) for all the immobilised tyrosinase preparations were significantly larger, indicating decreased affinity by the enzyme for its substrate, whereas V_{max} values were smaller for the both immobilised tyrosinase preparations. In a 40 h continuous operation with spacer-arm-attached poly(MMA–GMA–DVB) microbeads at 30 °C, only 3% of immobilised tyrosinase activity was lost. The operational inactivation rate constant (k_{opi}) of the immobilised tyrosinase was 1.25×10^{-5} min^{-1}.

Keywords: Suspension polymerisation; Polymer microbeads; Immobilisation; Tyrosinase; Enzyme reactor

1. Introduction

The enzyme tyrosinase (E.C.1.14.18.1; monophenol monoxygenase) is a copper-dependent enzyme and widely distributed throughout the phylogenetic scale from bacteria to mammals [1,2]. Tyrosinase catalyses two different oxygen-dependent reaction via separate copper-dependent active sites: the *o*-hydroxylation of monophenols to yields *o*diphenols (cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity) [3–5]. The enzyme has been proposed for synthesis of 3,4-dihydroxyphenylalanine (L-DOPA) [6], dephenolization of industrial wastewater [7–10], as a part of an enzyme electrode for the determination of phenol and its derivatives [11,12], bioremediation of contaminated soils [13,14], and fruit juice clarification [15].

Mushroom tyrosinase was covalently immobilised on zeolite [6], entrapped in alginate, polyacrylamide and gelatine [16], and utilised for the production of L-DOPA, which is a commonly prescribed drug for the treatment of Parkinson's disease. This disease is caused by the deficiency of a neurotransmitter dopamine and L-DOPA is a precursor of dopamine. L-DOPA has been currently produced by chemical methods [17]. Recent researches has focused on microbial production from *Erwinia herbicola* and *Escherichia coli* and enzymic production from tyrosinase hydroxylase and tyrosinase. The cresolase activity of tyrosinase catalyses the synthesis of L-DOPA from L-tyrosine in the presence of molecular oxygen. The subsequent catecholase activity can be suppressed by L-ascorbate [6,17]. Another major

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application area of the immobilised tyrosinase is removal of phenol and its derivatives from wastewaters. Atlow et al. proposed the application of mushroom tyrosinase to the treatment of phenolic wastewaters [18]. In the successive reports, tyrosinase was immobilised on polymeric supports and used in enzyme reactor to transform several phenols and its derivatives in the treated wastewaters to *o*-quinones and low molecular weight polymers [19–22].

The availability of large number of support materials and methods of enzyme immobilisation leave virtually no bioactive species without a feasible route of immobilisation. It is, thus, important that the choice of support material and immobilisation method over the free bioactive agent should be well justified [23–26]. Acrylic copolymers are especially versatile as a family of carrier materials for enzyme immobilisation that can be prepared with a wide variety of properties. Among these epoxy group carrying acrylic copolymer exhibited some significant advantages as a potential carrier matrix, i.e., easy and stable covalent linkages with different groups such as amino, thiol, and phenolic ones under mild experimental conditions [27–30].

In this study, acrylic copolymer supports were synthesised in the bead form from the monomers methyl methacrylate (MMA), glycidyl methacrylate (GMA) and divinyl benzene (DVB). The epoxy groups of poly(MMA–GMA–DVB) microbeads were modified into amino group using ammonia or/and 1,6-diaminohexane (i.e., spacer-arm). Tyrosinase was then immobilised onto aminated and spacer-arm attached poly(MMA–GMA–DVB) microbead using glutaric dialdehyde as coupling agent. The Michaelis–Menten kinetics constants (K_m and V_{max}), optimum pH and temperature for the free and immobilised enzymes were investigated. Thermal deactivation of the free and immobilised enzymes at various temperatures was studied and the half-lives of the immobilised tyrosinase under these operation conditions were calculated. Finally, the immobilised enzyme system was applied to a packed-bed reactor to study the behaviour of the immobilised enzyme in a continuous system.

2. Experimental

2.1. Materials

Tyrosinase [EC 1.14.18.1; polyphenol oxidase; monophenol monooxygenase, from mushroom, 2000 U mg^{-1} solid], L-tyrosine, bovine serum albumin (BSA), 1,6-diaminohexane, glutaric dialdehyde, DVB and maleic anhydride were all obtained from the Sigma Chemical Company (St. Louis, USA). MMA, glycidyl methacrylate (methacrylic acid 2,3-epoxypropyl isopropyl ether; GMA), and α,α'-azoisobisbutyronitrile (AIBN) were obtained from Fluka Chemie, AG (Buchs, Switzerland) and the monomers distilled under reduced pressure before use. All the other analytical grade chemicals were purchased from Merck AG (Darmstadt, Germany).

2.2. Preparation of poly(methyl methacrylate-co-glycidylmethacrylate) microbeads

Poly(methyl methacrylate-co-glycidyl methacrylate) microbeads were prepared via suspension polymerisation. The aqueous continuous phase with suspension stabiliser was obtained by the following method. Styrene–maleic anhydride (0.74 g) alternating copolymer, NaOH (0.30 g), Na_2SO_4 (4.0 g) and distilled water (300 ml) were transferred in to a three-necked reactor (1.0 l) and stirred at 70 °C for 1 h until a clear solution was obtained. The reaction product (i.e., sodium salt of styrene–maleic anhydride copolymer) was cooled to room temperature and used as suspension stabiliser. The reactor was then equipped with a mechanical stirrer, nitrogen inlet and reflux condenser. The organic phase contained MMA (26.5 ml; 0.25 mol), glycidyl methacrylate (26.4 ml; 0.2 mol) and DVB (7.1 ml; 0.05 mol; cross-linker) were mixed together with 1.0 g of AIBN in 60 ml of toluene. The reactor was placed in a water bath, heated to 65 °C and stirred at 375 ± 25 rpm under a nitrogen atmosphere. The polymerisation mixture was placed into a dropping funnel and was introduced drop wise into the reactor in about 30 min. The polymerisation reaction was maintained at 65 °C for 6 h. After the reaction, the beads were separated by decanting simply and washed with distilled water and methanol. The product was dried under vacuum for 24 h at room temperature. The total polymerisation yield was 58.6. The beads were sieved and 105–210 μm size of fraction (28.7 g) was used in further reactions.

2.3. Immobilisation of tyrosinase onto poly(MMA–GMA–DVB) microbeads

The epoxy groups carrying poly(MMA–GMA–DVB) microbeads were aminated with 0.5 M ammonia or 1,6-diaminohexane solution (i.e., spacer-arm) at 65 °C in a reactor containing 25 g microbeads and stirred magnetically for 5 h. After the reaction, the aminated and/or spacer-arm attached poly(MMA–GMA–DVB) microbeads were washed with distilled water.

The aminated and/or spacer-arm attached poly(MMA–GMA–DVB) microbead (5 g) were equilibrated in phosphate buffer (10 ml, 50 mM, pH 7.0) for 18 h, and transferred to the same fresh medium containing glutaric dialdehyde (20 ml, 0.5%, v/v). The activation reaction was carried out at 25 °C for 12 h, while continuously stirring the medium. After the reaction period, the excess glutaric dialdehyde was removed by washing sequentially the microbeads with distilled water, acetic acid solution (100 mM, 100 ml) and phosphate buffer (100 mM, pH 7.0). The resulting modified poly(MMA–GMA–DVB) microbeads were dried in a vacuum oven at 40 °C.

Immobilisation of tyrosinase on the aminated and spacer-arm attached poly(MMA–GMA–DVB) microbeads was carried out at 22 °C in a shaking water bath for 6 h. Physically bound enzyme was removed first by washing the supports with saline solution (20 ml, 1.0 M) and then phosphate buffer (50 mM, pH 7.0). It was stored at 4 °C in same fresh buffer until use.

The amount of immobilised tyrosinase on the poly(MMA–GMA–DVB) microbeads was determined by measuring the initial and final concentrations of protein within the immobilisation medium, using Coomassie Brilliant Blue as the method described by Bradford [31]. A calibration curve constructed with BSA solution of known concentration (0.05–0.50 mg ml^{-1}) was used in the calculation of protein in the enzyme and wash solutions.

2.4. Activity assays of free and immobilised tyrosinase

Tyrosinase is a copper dependent enzyme that catalyses two different reaction using molecular oxygen; the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity). These quinones are reactive and can undergo subsequent conversions to form other intermediates such as hydroxylated biphenyls. These coloured polymeric pigments can be monitored spectrophotometrically, a measure of these coloured compounds produced in the presence of tyrosinase can be used as an indication of the monophenol levels present. The polyphenol oxidase reaction is:

$$\text{L-tyrosine (monophenolic substrate)} + O_2 \xrightarrow{\text{tyrosinase}} o\text{-dihydroxyphenol} \quad (1)$$

$$o\text{-dihydroxyphenol} + O_2 \xrightarrow{\text{tyrosinase}} o\text{-benzoquinone} + H_2O \quad (2)$$

2.4.1. Free enzyme

The reaction was carried out in a quartz cuvette (3 ml) at 30 °C and change in absorbance ($A_{280\ nm}$) were measured using a Shimadzu Model 1601 spectrophotometer (Tokyo, Japan) equipped with temperature control cell holder unit. 2.9 ml of L-tyrosine solution (2.0 mM L-tyrosine in phosphate buffer (50 mM, pH 6.5)) was saturated with pure oxygen at 30 °C for 2 min. The reaction was started by adding 0.1 ml of enzyme solution (1 mg tyrosinase ml^{-1}) and the increase in absorbance at 280 nm was measured.

2.4.2. Immobilised enzyme

For the determination of immobilised tyrosinase activity, 0.2 g enzyme beads were introduced to L-tyrosine solution (2.0 mM, 5 ml). It was then incubated in a shaking water-bath and the reaction medium was purged with pure oxygen. At different time interval, the increase in absorbance was followed as above.

2.5. Determination of the kinetic constants

The kinetic constants, K_m and V_{max} values of the free and the immobilised tyrosinase were determined by measuring initial rates of the reaction with L-tyrosine (1.0–6.0 mM) in phosphate buffer (50 mM, pH 6.5) at 30 °C. K_m and V_{max} values were calculated from Line weaver–Burk plots. The kinetic constants of the free and immobilised enzyme were calculated from the initial rate of the reaction data obtained during enzymatic oxidation of L-tyrosine. The enzymic reactions were followed for 10 min for the free enzyme and 15 min for the immobilised enzyme.

One unit of enzyme activity is defined as an increase in absorbance, at 280 nm, of 0.001 min^{-1} at pH 6.5 and at 30 °C in a reaction mixture containing L-tyrosine.

2.6. Dependence of enzyme activity on pH and temperature

The effect of pH on the activity of the free and the immobilised enzyme was carried out over the pH range 4.0–8.0 and at 30 °C. The concentration of the L-tyrosine solution was 2.0 mM and was prepared in 50 mM acetate buffer in the pH range 4.0–5.5, and 50 mM phosphate buffer in the pH range 6.0–8.0.

The effect of temperature on enzyme productivity was studied in the range 20–50 °C with a L-tyrosine concentration of 2.0 mM in 50 mM phosphate buffer pH 6.5. The results for pH and temperature are presented in a normalised form with the highest value of each set being assigned the value of 100% activity.

2.7. Packed bed reactor and operation

The reactor (length 12 cm, diameter 1.2 cm, total volume 13.5 ml), was made from Pyrex® glass. The enzyme microbeads were equilibrated in phosphate buffer (50 mM, pH 6.5) at 4 °C for 1 h. Ten grams enzyme microbeads were loaded into the reactor yielding a void volume of about 3 ml.

In order to determine the effect of substrate concentration on reactor productivity, L-tyrosine solution (1.0–6.0 mM) in the phosphate buffer was introduced to the reactor at a rate of 20 ml h^{-1} with a peristaltic pump (Cole Parmer, Model 7521-00, USA) through the lower inlet part. The solution leaving the reactor was assayed for tyrosinase activity at the end of each hour.

The effect of flow rate on reactor performance was studied by varying the flow rate in the range 20–60 ml h^{-1} at 30 °C for 2 h, while keeping the concentration of L-tyrosine at 2.0 mM in phosphate buffer (50 mM, pH 6.5).

In order to determine operational stability of immobilised tyrosinase, the reactor was loaded with immobilised tyrosinase and operated at 30 °C for 40 h. The feed solution was contained L-tyrosine (2.0 mM) in phosphate buffer (50 mM,

pH 6.5) with a flow rate of 20 ml h^{-1}. Enzyme activity in the solution leaving the reactor was measured as described above.

2.8. Thermal stability measurements of free and immobilised enzymes

Thermal stabilities of the free and immobilised tyrosinase preparations were carried out by measuring the residual activity of the enzyme exposed to three different temperatures (50, 55 and 60 °C) in phosphate buffer (50 mM, pH 6.5) for 120 min. A sample was removed 15 min time interval and assayed for enzymatic activity. The first order inactivation rate constant, k_i, was calculated from the following equation:

$$\ln A = \ln A_0 - k_i t \quad (3)$$

where A_0 and A are the initial activity and the activity after time t (min.).

The results were converted to relative activities (percentage of the maximum activity obtained in that series). The residual activity was defined as the fraction of total activity recovered after covalent attachment on the poly(MMA–GMA–DVB) microbeads compared with the same quantity of free enzyme.

2.9. Storage stability

The remaining activity of free and immobilised enzymes after storage in phosphate buffer (50 mM, pH 6.5) at 4 °C was measured in a batch mode with the experimental conditions given above.

2.10. Characterisation of poly(GMA–MMA–DVB) microbeads

2.10.1. Determination of the water content

The modified poly(GMA–MMA–DVB) microbeads were allowed to soak in distilled water for 24 h, swollen microbeads (∼1 g) were weighed after removing the excess water, dried in vacuum oven at 60 °C for 24 h until constant weight. The water content of the poly(GMA–MMA–DVB) microbeads were calculated as follows:

$$\text{water content (\%, w/w)} = \left[\frac{W_s - W_d}{W_d}\right] \times 100 \quad (4)$$

where W_s and W_d are the weights of swollen and dry microbeads, respectively.

2.10.2. Scanning electron microscopy

Scanning electron micrographs of the dried poly(GMA–MMA–DVB) microbeads were obtained using a JEOL, JMS 5600 scanning electron microscope, after coating with gold under reduced pressure.

2.10.3. FTIR spectra

The FTIR spectra of the poly(GMA–MMA–DVB) microbead were obtained using an FTIR spectrophotometer (Shimadzu, FTIR 8000 Series, Japan). Poly(GMA–MMA–DVB) microbeads (0.1 g) and KBr (0.1 g) were thoroughly mixed and the mixture was pressed to form a tablet, and the spectrum was recorded.

2.10.4. Determination of the epoxy groups content

The available epoxy groups content of the poly(MMA–GMA–DVB) beads was determined by pyridine-HCl method as described previously [32].

2.10.5. Surface area measurement

The surface area of the poly(GMA–MMA–DVB) microbead sample was measured with a surface area apparatus (BET method).

2.10.6. Elemental analysis

The amount of attached 1,6-diaminohexane or amino group on to poly(MMA–GMA–DVB) microbead was determined from elemental analysis device by considering nitrogen stoichiometry.

3. Results and discussion

3.1. Properties of poly(GMA–MMA–DVB) microbeads

In the present study, a porous epoxide groups-containing poly(GMA–MMA–DVB) microbead was prepared from HEMA, GMA and DVB via suspension polymerisation in the presence of an initiator (AIBN). The chemical structure of the poly(GMA–MMA–DVB) microbeads is presented in Fig. 1. The amount of available epoxy groups was determined titration of pyridine-HCl solution with 0.1 M NaOH and was found to be 3.44 ± 0.05 mmol g^{-1} microbeads.

Epoxy groups in the poly(GMA–MMA–DVB) structure were converted to amino groups by reacting with ammonia or 1,6-diaminohexane (to form a six-carbon spacer-arm). Elemental analyses of the spacer-arm attached poly(GMA–MMA–DVB) microbeads were performed, and the amounts of the incorporated amino groups were found to be 1.22 and 0.84 mmol g^{-1} for aminated and spacer-arm attached poly(GMA–MMA–DVB) microbead from the nitrogen stoichiometry.

FTIR spectra of plain and 1,6-diaminohexane attached poly(GMA–MMA–DVB) microbeads are presented in Fig. 2. The broad band in the 3300–3500 cm^{-1} ranges indicates –OH stretching vibrations in the structure of the modified microbeads. Among the characteristic vibrations of both GMA and MMA are the methylene vibration at ∼2930 cm^{-1} and the methyl vibration at 2960 cm^{-1}. The vibration at 1740 cm^{-1} represents the ester configuration of both GMA and MMA. The aromatic ring stretching vibration band at 1575 cm^{-1} was arising from DVB molecules

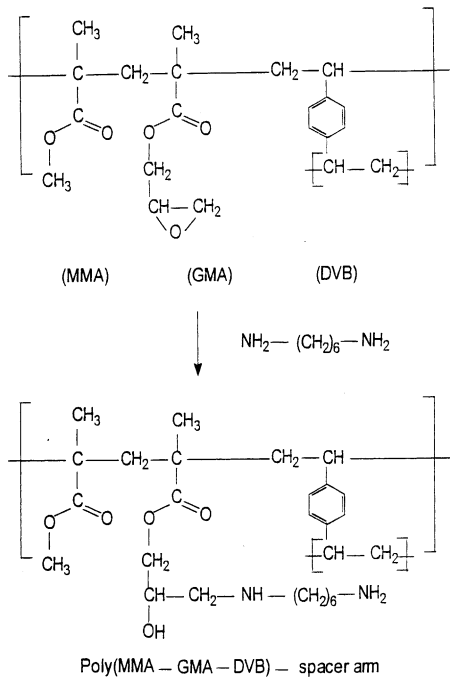

Fig. 1. Chemical structure of poly(GMA–MMA–DVB) microbead.

in the terpolymer. The epoxide group gives the band at 910 cm^{-1}. The FTIR spectra of the 1,6-diaminohexane attached poly(GMA–MMA–DVB) microbeads have some adsorption bands different from those of the plain microbeads. The most important adsorption bands at 1550 cm^{-1} representing N–H bending, is due to diaminohexane bonded to the poly(GMA–MMA–DVB) microbead.

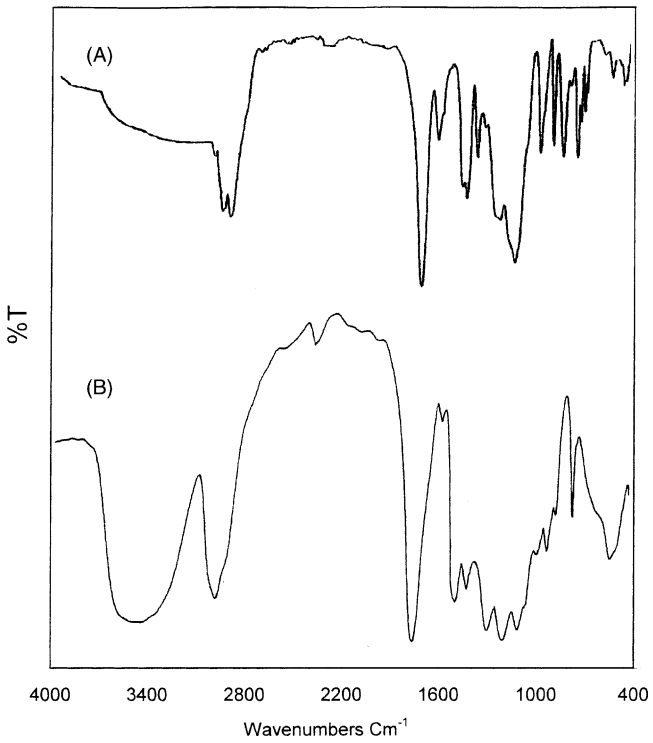

Fig. 2. FT-IR spectra of poly(GMA–MMA–DVB) microbeads: (A) plain of the microbead; (B) spacer-arm attached microbead.

The water content is very important when the use of a matrix in enzyme immobilisation is contemplated. The water content of the aminated and spacer-arm attached poly(GMA–MMA–DVB) microbeads were increased 53 and 47% with respect to unmodified microbeads, respectively. These are moderate swelling degrees and suitable to use as column packing material for application of immobilised enzyme in a continuous flow system.

Scanning electron microscopy (SEM) micrographs presented in Fig. 3A and B shows the surface structure of a poly(GMA–MMA–DVB) microbeads. The SEM micrograph shows that the microbeads have a porous surface structure (Fig. 3B). The surface area of the 105–210 μm fractions of poly(GMA–MMA–DVB) microbeads was measured as 0.674 $m^2\,g^{-1}$. The surface properties of the poly(GMA–MMA–DVB) microbeads would favour higher immobilisation capacity for the enzyme due to increase in the surface area.

3.2. Immobilisation of tyrosinase onto poly(GMA–MMA–DVB) microbeads

The activation of the amino groups of the aminated and diaminohexane attached poly(GMA–MMA–DVB) microbeads were achieved by the reaction with glutaric dialdehyde under neutral conditions. Tyrosinase was then covalently immobilised via amino groups to the activated support. In this coupling reaction, shift-base formation between amine and aldehyde groups could be considered. Glutaric dialdehyde can readily react with an amino group, therefore, the aldehyde group content should be close to the content of the amino group on the microbead. The –SH groups of tyrosinase can also react with glutaric dialdehyde after amine groups have been used. It is important to notice that $–NH_2$ groups are more susceptible than –SH groups to react with glutaric dialdehyde.

Spacer-arm constituted by aliphatic chains of six-carbon atoms has been used to move away-immobilised tyrosinase from the support. The attachment of six-carbon atoms hydrophobic spacer-arm on the poly(GMA–MMA–DVB) microbead surface could prevent undesirable side interaction between large enzyme molecules and support. In this way, more areas of the immobilised tyrosinase could become accessible to its substrate L-tyrosine. As seen in Table 1, the attachment of spacer-arm on the poly(GMA–MMA–DVB) microbead significantly increased the immobilised tyrosinase activity. The immobilisation of tyrosinase via glutaric dialdehyde coupling onto spacer-arm attached poly(GMA–MMA–DVB) microbead resulted in a higher enzyme loading (2.47 mg g^{-1}) than that of tyrosinase immobilised onto aminated poly(GMA–MMA–DVB) microbeads (1.73 mg l^{-1}). These results indicated that attachment of the spacer-arm on the poly(GMA–MMA–DVB) microbeads surface might reduce steric interference with the substrate

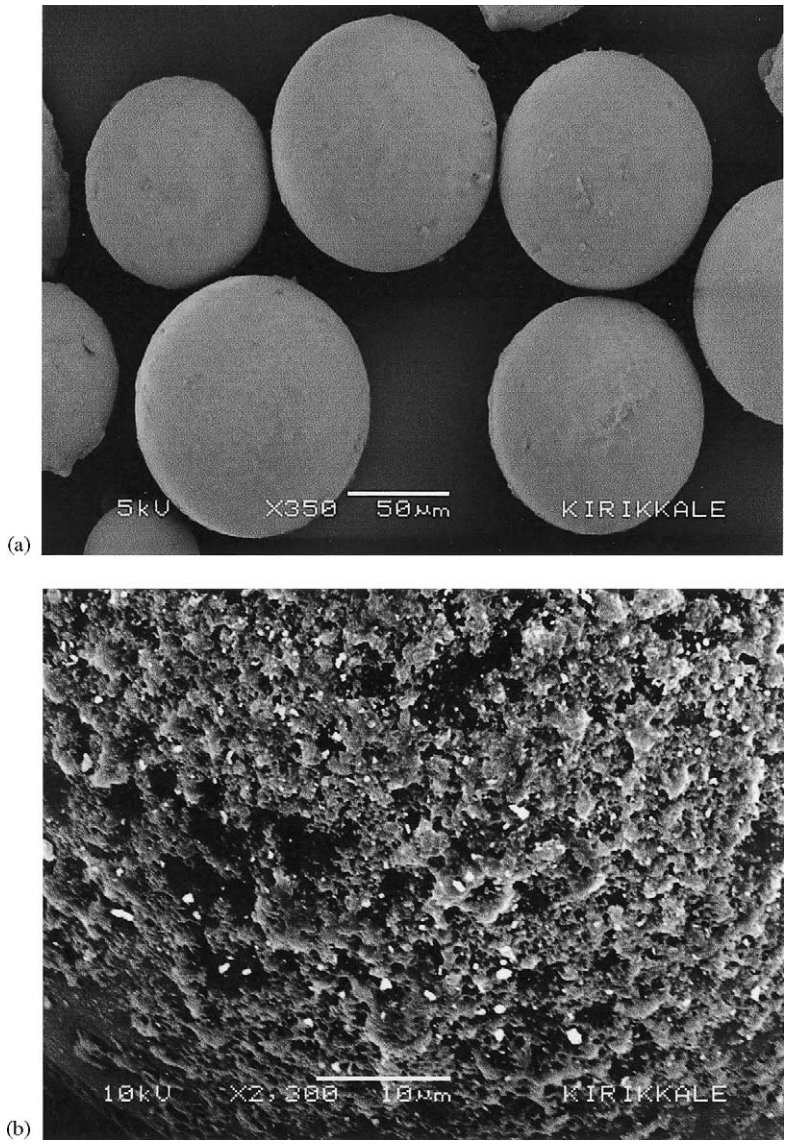

Fig. 3. SEM micrographs of poly(GMA–MMA–DVB) microbeads: (A) magnification 350×; (B) magnification 2300×.

binding process for the substrate. On the other hand, the coupling agent glutaric dialdehyde has a five-carbon length and it should be acted as an extra spacer-arm in this immobilisation method. This result indicated that an optimum spacer length should be existed for the immobilised tyrosinase toward the oxidation of substrate L-tyrosine molecules. As seen in Table 1, the relative activity of the immobilised enzyme on the aminated microbeads via glutaric dialdehyde coupling was 51%, it was significantly lower than that of the spacer-arm attached counterpart (68%). The low relative activity obtained the former probably reflect the presence of steric hindrance caused by the immobilisation and/or binding difficulty of the substrate molecule on the active site of the enzyme.

No enzyme leakage was observed during washing of the immobilised enzyme preparations or during continuous or

Table 1
Properties of the free and immobilised tyrosinase on the poly(GMA–MMA–DVB)

Form of enzyme	Activity (U mg^{-1} enzyme)	Recovered activity (%)	Enzyme loading (mg enzyme g^{-1} microbead)	Activity (U g^{-1} microbead)
Free enzyme	1856	100	–	–
Immobilised on aminated-microbead	947	51	1.73	1638
Immobilised on spacer-arm-attached microbead	1262	68	2.47	3117

batch operation mode. All these indicate that the applied immobilisation processes were irreversible under the conditions used.

Several publications have appeared describing the investigation of new supports for tyrosinase immobilisation. For example, tyrosinase covalently immobilised on carboxymethylcellulose hydrogel beads. The amount of the immobilised enzyme to 1.0 g of beads was 603 μg and immobilised enzyme retained about 44% its initial activity [22]. Munjal and Sawhney used alginate, polyacrylamide and gelatine gels for entrapment of tyrosinase. The maximum activity immobilisation yield of 88% was obtained in gelatine followed by 67 and 57% in Cu–alginate and polyacrylamide gels, respectively [16]. Chitosan-coated polysulphone capillary membranes were used for the immobilisation of tyrosinase and the specific activity of the immobilised enzyme was 8.6 mg U [7]. Wada et al. reported that the retained activity of covalently immobilised tyrosinase on the weakly acidic cationic exchange resin was 16.3% [9]. Boshoff et al. reported that about 34% activity was retained when tyrosinase immobilised on inorganic support [33]. Tyrosinase was immobilised on chitosan beads and flakes, the immobilisation capacity of beads was around 14 times greater than the chitosan flakes [34]. The tyrosinase was covalently immobilised onto aminated and spacer-arm attached poly(GMA–MMA–DVB) microbeads. The spacer-arm attached poly(GMA–MMA–DVB) microbeads resulted good loading of tyrosinase (2.47 mg g^{-1} microbead) that retains high specific activity (68%) than that of the aminated one. All immobilisation studies published in the literature have been performed under different conditions. Therefore, it is almost impossible to compare immobilisation results. However, the activity retention (68%) obtained with this immobilisation method appears to be quite promising.

3.3. Kinetic constants

Kinetic parameters, K_m and V_{max} values of the free and the immobilised tyrosinase were determined using L-tyrosine as a substrate. In order to avoid of the effect of second substrate oxygen on the enzyme activity the concentration of oxygen was kept saturated in the assay medium, and L-tyrosine concentration was varied between 1.0 and 6.0 mM. In the free and immobilised enzyme systems, a Michaelis–Menten kinetic behaviour was observed. The effect of immobilisation on the Michaelis constant (K_m) and the maximum activity (V_{max}) of the tyrosinase are presented in Table 2. The calculated value of kinetic constant K_m for L-tyrosine of the enzyme immobilised on the aminated and spacer-arm attached poly(GMA–MMA–DVB) were 1.44 and 0.97 mM, respectively, which were higher than that of the free tyrosinase (0.58 mM). The maximum velocity, V_{max} decreased significantly upon immobilisation of tyrosinase. The V_{max} value of the free tyrosinase was found to be 2065 U mg^{-1} protein whereas the V_{max} values of the enzyme immobilised onto aminated and spacer-arm attached poly(GMA–MMA–DVB) were estimated from the data as 1053 and 1404 U mg^{-1} protein, respectively. As expected, the K_m and V_{max} values were significantly affected after covalent binding of tyrosinase on poly(GMA–MMA–DVB) microbeads. In general, the K_m of an immobilised enzyme is different from that of the free enzyme due to diffusional limitations, steric effects and ionic strength [35]. The change in the affinity of the enzyme to its substrate is also caused by structural changes in the enzyme introduced by the immobilisation procedure and by lower accessibility of the substrate to the active site of the immobilised enzyme [36].

Table 2
K_m and V_{max} values of free and immobilised tyrosinase

Form of enzyme	K_m for L-tyrosine (mM)	V_{max} for L-tyrosine (U mg^{-1} enzyme)
Free enzyme	0.58	2065
Immobilised on aminated-microbead	1.44	1055
Immobilised on spacer-arm-attached microbead	0.97	1404

3.4. Effect of pH and temperature on the catalytic activity

The effect of pH on the activity of the free and immobilised tyrosinase preparations for L-tyrosine oxidation was examined in the pH range 4.0–8.0 at 30 °C and the results are presented in Fig. 4. The pH value for optimum activity for the free tyrosinase was found to be at 6.5, which was similar to that reported previously [22]. On the other hand, the optimum pH for both immobilised tyrosinase preparations is found to have shifted to pH 7.0. The optimum is defined as the pH where a known amount of free or immobilised tyrosinase achieved maximum oxidation of L-tyrosine to *o*-benzoquinone. This observed displacement toward to the neutral region for the immobilised tyrosinase

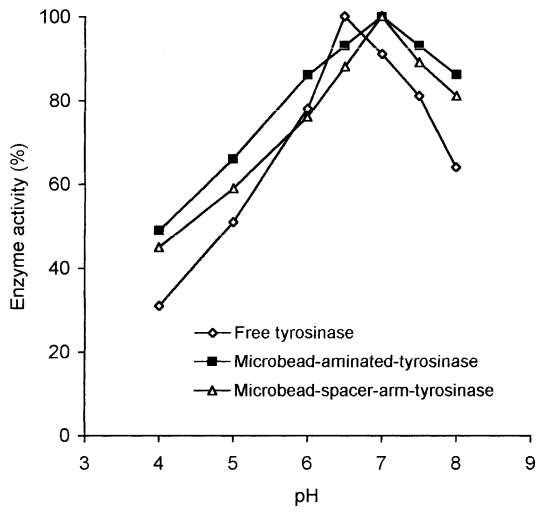

Fig. 4. pH profiles of the free and immobilised tyrosinase preparations.

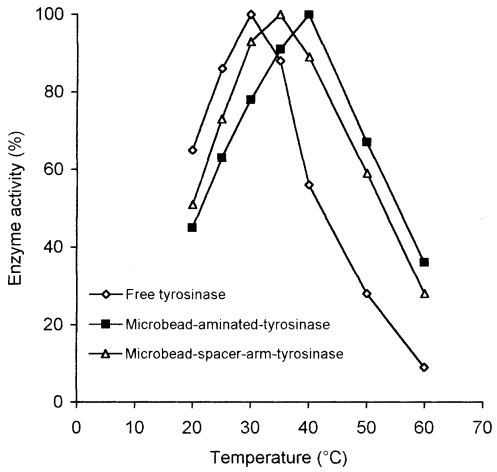

Fig. 5. Temperature profiles of the free and immobilised tyrosinase preparations.

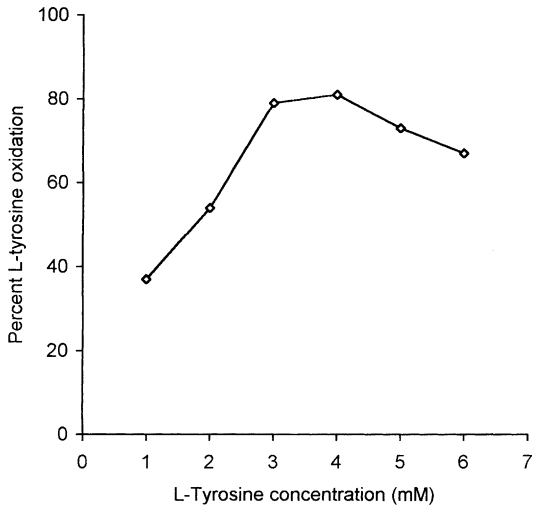

Fig. 6. Effect of substrate concentration on the L-tyrosine oxidation rate in the enzyme reactor.

preparation is because pH conditions in the pore space of the polymeric matrix are different from those in the rest of the solution. Furthermore, the pH profiles of the immobilised tyrosinase preparations are broader than that of the free enzyme, which means that the immobilisation methods preserved the enzyme activity in a wider pH range. These results could probably be attributed to the stabilisation of tyrosinase molecules resulting from multipoint attachment of the enzyme molecules on the surface of the poly(GMA–MMA–DVB) microbead.

The activities obtained in a temperature range of 20–60 °C were expressed as percentage of the maximum activity (Fig. 5). The activity of the free tyrosinase is strongly dependent on temperature, with the optimum temperature being observed at about 30 °C. The optimum reaction temperature for the spacer-arm attached immobilised preparation was at 35 °C, and this was 40 °C for the aminated immobilised preparation. The temperature profiles of both immobilised enzymes were broader than that of the free one. The increase in optimum temperature was caused by the changing physical and chemical properties of the enzyme. The covalent bond formation via amino groups of the immobilised tyrosinase might also reduce the conformational flexibility and may result in higher activation energy for the molecule to reorganise the proper conformation for the binding to substrate. One of the main reasons for enzyme immobilisation is the anticipated increase in its stability to various deactivating force due to restricted conformational mobility of the molecules following immobilisation [37].

3.5. Enzyme reactor productivity

The effect of L-tyrosine concentration on reactor productivity was determined using different L-tyrosine concentration in the feed solution. A linear increase in oxidation rate was observed up to a L-tyrosine concentration of 3.0 mM (Fig. 6). After a plateau between 3.0 and 4.0 mM, a decrease in the L-tyrosine oxidation rate was obtained. This decrease in the oxidation rate could be due to insufficient contact duration of L-tyrosine.

Fig. 7 shows the effect of residence time on oxidation of L-tyrosine by immobilised tyrosinase. The results were converted to relative activities (percentage of the maximum oxidation obtained in this series). When the residence time is increased, that the efficiency of L-tyrosine oxidation is also increased (Fig. 7). This rate is not so linear and further increase in residence time, does not yield a higher degree of L-tyrosine oxidation. Eventually, at a contact duration of about 4.5 min a plateau is reached (obtained with a flow rate 40 ml h^{-1} and 2.0 mM L-tyrosine). As previously reported, this could be due either to substrate depletion in the reaction medium or possible interference with the enzyme activity at lowest substrate concentration by the product diffusion limitation from porous surface of the microbeads [38].

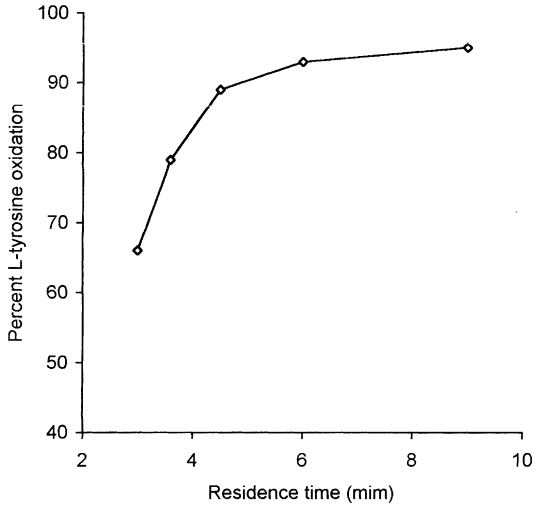

Fig. 7. Effect of residence time on the L-tyrosine oxidation rate in the enzyme reactor.

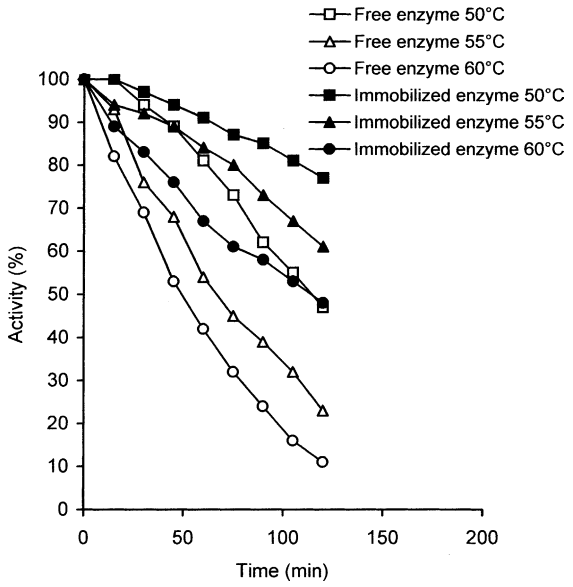

Fig. 8. Effect of temperature on the stability of the free and immobilised tyrosinase preparations.

It is important for economical use of an enzyme, as a means for the mass production of the desired product, that the enzyme reaction is continuous. One of the problems in continuous enzyme reactions is the operational stability of the enzyme immobilised on the support. The operational stability of covalently immobilised tyrosinase in the packed bed reactor was monitored at 30 °C for 40 h. During the initial 24 h, continuous operation the immobilised tyrosinase preserved all of its initial activity. After this period, a small decrease in enzyme activity is observed with time. After 40 h, the immobilised enzyme lost about 3% of its initial activity, this would be possibly resulting from the inactivation of tyrosinase upon use. The operational inactivation rate constant of the immobilised tyrosinase at 30 °C with 2.0 mM L-tyrosine was calculated as $k_{opi} = 1.25 \times 10^{-5}\,min^{-1}$.

3.6. Thermal stability

The thermal stability of an immobilised enzyme is one of the most important criteria of their application. In general, the activity of the immobilised enzyme, especially in a covalently bound system, is more resistant than that of the soluble form against heat and denaturing agents. Thermal stability experiments were carried out with free and the poly(GMA–MMA–DVB) spacer-arm-enzyme preparation, which were incubated in the absence of substrate at various temperatures (Fig. 8). At 50 °C, the free and the spacer-arm attached immobilised preparation retained their activity to a level of 47 and 77% during a 120 min incubation period. At 55 °C, the free and the immobilised enzymes retained their activity about to a level of 23 and 61%, respectively. Immobilised tyrosinase was inactivated at a much slower rate than that of the native form. The half-live values and thermal inactivation rate constants (k_i) of the free and immobilised enzyme were presented in Table 3. These results suggest that the thermostability of immobilised tyrosinase increased considerably because of covalent immobilisation onto spacer-arm attached poly(GMA–MMA–DVB) microbead.

Table 3
Inactivation rate constant (k_i) and half-life ($t_{1/2}$) values of the free and immobilised tyrosinase on the spacer-arm-attached microbeads for different temperature

Temperature (°C)	Free enzyme		Immobilised enzyme	
	k_i ($\times 10^3\,min^{-1}$)	$t_{1/2}$ (min)	k_i ($\times 10^3\,min^{-1}$)	$t_{1/2}$ (min)
50	6.29	113	2.18	261
55	12.5	78	4.11	154
60	18.4	67	6.12	125

3.7. Storage stability

One of the most important parameters to be considered in enzyme immobilisation is storage stability. The stabilities of the free and the immobilised tyrosinase preparations were determined after the preparations were stored in phosphate buffer (50 mM, pH 6.5) at 4 °C for a predetermined period. Under the same storage conditions, the activities of the immobilised tyrosinase preparations decreased slower than that of the free tyrosinase (Fig. 9). The free enzyme lost all its activity within 4 weeks. The immobilised tyrosinase preserved about 36% of its initial activity during a two months storage period. The covalent immobilisation definitely holds the enzyme in a stable position in comparison to the free counterpart [39]. On the other hand, hydrophobic group containing hydrophilic support should provide a stabilisation effect, minimising possible distortion effects imposed from aqueous medium on the active site of the immobilised enzyme [22,40,41]. Thus, the hydrophobic group

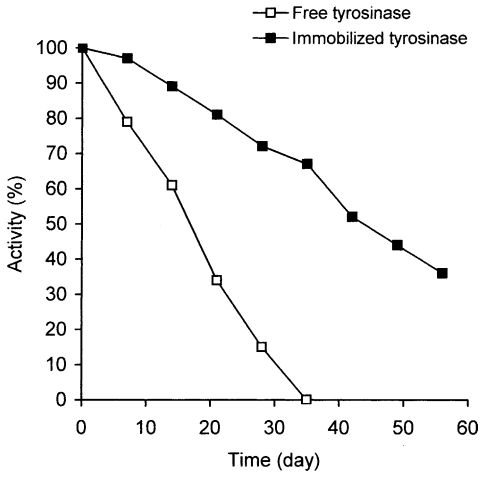

Fig. 9. Storage stabilities of the free and immobilised tyrosinase preparations.

carrying hydrophilic support and the immobilisation method provide higher shelf life compared to that of its free counterpart.

4. Conclusion

In this study, poly(GMA–MMA–DVB) microbeads was prepared via suspension polymerisation and the epoxy groups of microbeads was modified into amino groups using ammonia or 1,6-diaminohexane (as a spacer-arm). The enzyme, tyrosinase, was then covalently immobilised onto aminated and spacer-arm attached poly(GMA–MMA–DVB) microbeads via glutaric dialdehyde coupling. The attachment of the spacer-arm on the microbead surface resulted a significant increase in the immobilised enzyme activity. The Michaelis–Menten kinetic constants K_m and V_{max} of the free and immobilised tyrosinase preparations were also determined. All the immobilised tyrosinase preparation retained much of their activity in wider ranges of temperature and pH than that of the free form. A high operational stability obtained with the immobilised tyrosinase indicates that the immobilised tyrosinase could successfully be used in a continuous system for various biotechnological applications. In addition, the poly(GMA–MMA–DVB) microbeads proposed in this work showed promising potential for applications to enzyme immobilisation.

References

[1] Burton SG. Biocatalysis with polyphenoloxidase: a review. Catal Today 1994;22:459–87.

[2] Ikehata K, Nicell JA. Characterisation of tyrosinase for the treatment of aqueous phenols. Bioresour Technol 2000;74:191–9.

[3] Funaki T, Takanohashi Y, Fukazawa H, Kuruma I. Estimation of kinetic parameters in the inactivation of an enzyme by a suicide substrate. Biochim Biophys Acta 1991;1078:43–6.

[4] Robb DA. Tyrosinase. In: Lontie R, editor. Copper proteins and copper enzyme, vol. II. Boca Raton, FL: CRC Press; 1984. p. 208–40.

[5] Duran N, Rosa MA, D'Annibale A, Gianfreda L. Application of laccases and tyrosinases (phenoloxidases) immobilised on different supports: a review. Enzyme Microb Technol 2002;31:907–31.

[6] Seetharam G, Saville BA. L-DOPA production from tyrosinase immobilised on zeolite. Enzyme Microb Technol 2002;31:747–53.

[7] Edwards W, Leukes WD, Rose PD, Burton SG. Immobilisation of polyphenol oxidase on chitosan-coated polysulphone capillary membranes for improved phenolic effluent bioremadiation. Enzyme Microb Technol 1999;25:769–73.

[8] Sun W-Q, Payne GF. Tyrosinase-containing chitosan gels: a combined catalyst and sorbent for selective phenol removal. Biotechnol Bioeng 1996;51:79–86.

[9] Wada S., Ichikawa, Tatsumi K. Removal of phenols and wastewater by a combination treatment with tyrosinase and a coagulant. Biotechnol Bioeng 1995;45:304–9.

[10] Davis S, Burs RG. Decolorization of phenolic effluents by soluble and immobilised phenoloxidases. Appl Microbial Biotechnol 1990;32:721–6.

[11] Freire RS, Duran N, Kubota LT. Effects of fungal laccase immobilisation procedures for the development of a biosensor for phenol compounds. Talanta 2001;54:681–6.

[12] Yaropolov AI, Kharybin AN, Emneus J, Marko-Varga G, Gorton L. Flow injection analysis of phenols at a graphite electrode modified with co-immobilised laccase and tyrosinase. Anal Chim Acta 1995;308:137–44.

[13] Vakurov AV, Gladilin AK, Levashov AV, Khmelnitsky YL. Dye enzyme polymer complexes: stable organosoluble biocatalysts for non-aqueous enzymology. Biotechnol Lett 1994;16:175–8.

[14] Crecchio C, Ruggiero P, Pizzigallo MDR, Curci M. Potential utilisation of phenoloxidases immobilised in organic gels for decontamination of polluted sites. Dev Plant Soil Sci 1997;71:545–52.

[15] DeStefano G, Piacquadio P, Sciancalepore V. Metal-chelate regenarable carries in food processing. Biotechnol Technol 1996;10:857–60.

[16] Munjal N, Sawhney SK. Stability and properties of mushroom tyrosinase entrapped in alginate, polyacrylamide and gelatine gels. Enzyme Microb Technol 2002;30:613–9.

[17] Berkow R, Beers MH, Fletcher AJ. Movement disorders. In: Keryan AG, William Schind ST, editors. The Merck manual of medical information. White House Station, NJ: Merck Research Laboratories; 1997. p. 244–6.

[18] Atlow ST, Bonadonna-Aparo L, Klibanov AM. Dephenolization of industrial wastewaters catalysed by polyphenol oxidase. Biotechnol Bioeng 1984;26:599–603.

[19] Husain Q, Jan U. Detoxification of phenols and aromatic amines from polluted wastewater by using phenoloxidases. J Sci Ind Res 2000;59:286–93.

[20] D'Annibale A, Stazi SR, Vinciguerra V, Di Mattia E, Sermanni GG. Characterisation of immobilised laccase from *Lentinula edodes* and its use in olive mill wastewater treatment. Process Biochem 1999;34:697–706.

[21] Burton SG, Boshoff A, Edwards W, Rose PD. Biotransformation of phenols using immobilised polphenoloxidases. J Mol Catal B: Enzym 1998;5:411–26.

[22] Arıca MY. Immobilisation of polyphenoloxidase on carboxymethylcellulose hydrogel beads: preparation and characterisation. Polym Int 2000;49:775–81.

[23] Chen D-H, Liao M-H. Preparation and characterisation of YADH-bound magnetic nanoparticles. J Mol Catal B: Enzym 2002;16:283–91.

[24] Akgöl S, Kacar Y, Denizli A, Arıca MY. Hydrolysis of sucrose by invertase immobilised onto novel magnetic polyvinylalcohol microspheres. Food Chem 2001;74:281–8.

[25] Yodoya S, Takagi T, Kurotani M, Hayashi T, Furuta M, Oka M, Hayashi T. Immobilisation of bromelain onto porous copoly(g-methyl-L-glutamate/L-leucine) beads. Eur Polym J 2003;39:173–80.

[26] Akgöl S, Yalcınkaya Y, Bayramoglu G, Denizli A, Arıca MY. Reversible immobilisation of urease onto Procion Brown MX-5BR-Ni(II) attached poyamide hollow-fibre membranes. Process Biochem 2002;38:675–83.

[27] Bayramoglu G, Kacar Y, Denizli A, Arıca MY. Covalent immobilisation of lipase onto hydrophobic group incorporated poly(hydroxyethylmethacrylate) based membrane matrix. J Food Eng 2002;52:367–74.

[28] Schulz B, Riedel A, Abel PU. Influence of polymerisation parameters and entrapment in poly(hydroxyethyl methacrylate) on activity and stability of GOD. J Mol Catal B: Enzym 1999;7:85–91.

[29] Arıca MY. Epoxy-derived pHEMA microbead for use bioactive macromolecules immobilisation: covalently bound tyrosinase in a continuous model system. J Appl Polym Sci 2000;77:2000–8.

[30] Mateo C, Abian O, Fernandez-Lafuente R, Guisan JM. Increase in conformational stability of enzymes immobilised on epoxy-activated supports by favouring additional multipoint covalent attachment. Enzyme Microb Technol 2000;26:509–15.

[31] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[32] Sidney S. Quantitative organic analysis. 3rd ed. New York: Wiley; 1967.
[33] Balulescu M, Iordachescu D, Herdan JM. Immobilised enzyme. II. Immobilisation of polyphenoloxidase on inorganic support. Prog Catal 1998;7:97–100.
[34] Wu F-C, Tseng R-L, Juang R-S. Enhanced ability of highly swollen chitosan beads for colour removal and tyrosinase immobilisation. J Hazard Mater 2001;B81:167–77.
[35] Arıca MY, Senel S, Alaeddinoglu NG, Patır S, Denizli A. Invertase immobilised on spacer-arm attached poly(hydroxyethylmethacrylate) membrane: Preparation and properties. J Appl Polym Sci 2000;75:1685–92.
[36] Palmieri G, Giardina P, Desiderio B, Marzullo L, Giamberrini M, Sannia G. A new enzyme immobilisation procedure using copper alginate gel: application to a fungal phenoloxidase. Enzyme Microb Technol 1994;16:151–8.
[37] Estrada P, Sanchez-Muniz R, Acebal C, Arche R, Castillon MP. Characterisation and optimisation of immobilised polyphenoloxidase in low-water organic solvents. Biotechnol Appl Biochem 1991;14:12–20.
[38] Sengubta S, Modak JM. Optimisation of fed-batch bioreactor for immobilised enzyme process. Chem Eng Sci 2001;56:3315–25.
[39] Canofeni S, Di Sario S, Mela J, Pilloton R. Comparison of immobilisation procedures for development of an electrochemical PPO-based biosensor for on line monitoring of a depuration process. Anal Lett 1994;27:1659–69.
[40] Jia H, Zhu G, Vugrinovich B, Kataphinan W, Reneker DH, Wang P. Enzyme-carrying polymeric nanofibers prepared via electrospinning for use as unique biocatalysts. Biotechnol Prog 2002;18:1027–32.
[41] Godjevargova T, Konsulov V, Dimow A. Preparation of an ultrafiltration membrane from the copolymer of acrylonitrile-glyciylmethacrylate utilised for immobilisation of glucose oxidase. J Membr Sci 1999;152:235–40.