Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 49 (2007) 69–74
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Immobilization of α-amylase from mung beans (*Vigna radiata*) on Amberlite MB 150 and chitosan beads: A comparative study

Pallavi Tripathi, Arpana Kumari, Parthasarthi Rath, Arvind M. Kayastha *

School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi 221005, India

Received 27 July 2007; received in revised form 27 August 2007; accepted 30 August 2007
Available online 4 September 2007

Abstract

α-Amylase from mung beans (*Vigna radiata*) was immobilized on two different matrices, Amberlite MB 150 and chitosan beads. Maximum immobilization obtained was 72% and 69% in case of Amberlite and chitosan beads, respectively. The pH optima of soluble α-amylase were 5.6, whereas that for immobilized amylase on chitosan and Amberlite was 7.0. Soluble amylase and Amberlite immobilized amylase showed maximum activity at 65 °C, whereas chitosan immobilized amylase showed maximum activity at 75 °C. α-Amylase immobilized on Amberlite showed apparent K_m of 2.77 mg/ml, whereas α-amylase immobilized on chitosan showed an apparent K_m of 5 mg/ml. The Amberlite-amylase and chitosan-amylase showed a residual activity of 43% and 27%, respectively, after 10 uses. The loss of activity for free amylase after 100 days of storage at 4 °C was 70%, whereas that for Amberlite- and chitosan-amylases, under the same experimental conditions, the losses were 45% and 55%, respectively. The easy availability of mung bean α-amylase, the ease of its immobilization on low-cost matrices and good stability upon immobilization in the present study makes it a suitable product for further use in industrial applications.

Keywords: Amberlite; Chitosan; α-Amylase; Immobilization; Stability

1. Introduction

Biotechnology is currently considered as a useful alternative to conventional process technology in industrial and analytical fields. Over the last few decades, intense research in the area of enzyme technology has provided many approaches that facilitate their practical applications. Among them, the newer technological developments in the field of immobilized biocatalysts can offer the possibility of a wider and more economical exploitation of biocatalysts in industry, waste treatment, and medicine and in the development of bioprocess monitoring devices like the biosensor. The main advantages of immobilized enzymes are that they are easily separated from reaction mixture, providing the ability to control reaction times and minimize the enzymes lost in the product and can be re-used for many reaction cycles, lowering the total production cost of enzyme mediated reactions.

Among starch hydrolyzing enzymes that are produced on industrial scale, α-amylases (EC 3.2.1.1) are of considerable commercial interest. α-Amylases randomly hydrolyze α-1,4 glycosidic linkages in starch or its hydrolysis products. The sale of amylolytic enzymes accounted for almost US$ 225 million world wide [1]. Today, a large number of microbial amylases are available commercially and these have almost completely replaced chemical hydrolysis of starch in starch processing industry [2–4]. Industrial processes for starch hydrolysis to glucose rely on inorganic acids or enzyme catalysis. The use of enzymes is preferred as it offers a number of advantages including improved yields and favorable economics. Enzymatic hydrolysis allows greater control over amylolysis, the specificity of the reaction, and the stability of generated products [3,5].

In the present communication, we have immobilized α-amylase from mung beans (*Vigna radiata*) on two different matrices, viz., Amberlite and chitosan, and compared their properties with the soluble enzyme. It is noteworthy that there are no reports on immobilization of plant α-amylases. There are a number of reports on bacterial α-amylases immobilization on different matrices and they have been well characterized [6–11]. Aksoy et al. [12] had reported immobilization of porcine

* Corresponding author. Tel.: +91 542 2368331; fax: +91 542 2368693.
E-mail addresses: kayasthabhu@gmail.com, kayastha@fastmail.ca (A.M. Kayastha).

1381-1177/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2007.08.011

α-amylase on poly(methyl methacrylate-acrylic acid) microspheres.

Chitosan is a polysaccharide made up of 2-amino-2-deoxy-D-glucose units, which are joined by β-1,4-linkages is obtained by deacetylation with drastic alkaline treatment of chitin, a principle component of the exoskeletons of crustaceans and insects. Chitosan has been previously used for immobilization of some enzymes and DNA [13–16]. It is well known as an ideal support for enzyme immobilization because of its features like hydrophilicity, biocompatibility, biodegradability and antibacterial properties [17–19]. Furthermore, chitosan appears to be economically attractive because chitin is second most abundant biopolymer available in nature after cellulose [20]. Enzyme immobilization on chitosan can be achieved by means of the glutaraldehyde reaction between the free amino groups of chitosan and the enzyme molecule to form covalent linkages.

Amberlite MB 150 is an ionically equilibrated mixed bed resin. It is a mixture of strongly acidic cationic resin and a strongly basic anionic resin. In general, Amberlite MB 150 resin is suitable for many industrial water treatment applications and is an excellent choice for portable exchange deionization. This resin provides high capacity with reliable production of the highest quality water and rapid rinse. Amberlite has been successfully utilized for the immobilization of enzymes like, lipase [21] and urease [22].

2. Materials and methods

2.1. Enzymes and chemicals

α-Amylase from mung beans (*V. radiata*) was purified by the procedure recently described in Ref. [23]. Amberlite MB 150, Crab shell Chitosan, DNS, Glutaraldehyde were obtained from Sigma Chemical Co. (St. Louis, MO, USA), sodium acetate was procured from Sisco Research Labs, Mumbai. All other chemicals were of Analytical grade. Milli-Q (Millipore, USA) water was used all throughout.

2.2. Immobilization of α-amylase on Amberlite

Amount of Amberlite, percentage of glutaraldehyde used for matrix activation and time period of incubation of matrix with glutaraldehyde was optimized. Since Amberlite is a mixed bed exchanger, pH also plays an important role in immobilization optimization. Therefore, Amberlite was first incubated overnight with buffers of different pH and maximum immobilization was further checked. Desired amount of pre-treated Amberlite was incubated with different glutaraldehyde concentration and kept for (30 min to 4 h) and then washed with distilled water to remove excess glutaraldehyde. Activated Amberlite was treated with different protein concentration (1–6 mg/ml). This matrix was left overnight at room temperature. Next day the solution was decanted and Amberlite-amylase was washed with the same buffer to remove any unbound enzyme. Washing was kept for activity measurement and protein estimation. Amberlite-amylase was finally stored in semi-dry condition, without any buffer at 4 °C.

Percentage immobilization was calculated by the following formula:

$$\%\ \text{immobilization} = \frac{\text{specific activity of immobilized enzyme}}{\text{specific activity of soluble enzyme}} \times 100$$

where specific activity of immobilized enzyme = specific activity of soluble enzyme − specific activity of unbound enzyme.

2.3. Preparation of chitosan beads

Different concentrations of chitosan were tried to obtain beads of firm quality. Chitosan (1.5%) was dissolved in 1.5% acetic acid by heating at 60 °C with continuous stirring unless chitosan is dissolved. This solution was taken in a syringe and allowed to fall in 100 ml solution of 1 M potassium hydroxide. The beads of uniform shape and size were obtained. Beads were then washed with double distilled water and stored in sodium acetate buffer at 4 °C, until used.

2.4. Immobilization of α-amylase on chitosan beads

Activation of chitosan beads was performed by treating them with desired glutaraldehyde concentration and allowed to stand at room temperature overnight. Following the activation, beads were thoroughly washed with sodium acetate buffer to make it free from glutaraldehyde. Beads were then treated with desired protein concentration and left overnight at room temperature and 4 °C to check the best immobilization. Next day the solution was decanted and beads were washed with the same buffer to remove any unbound enzyme. Beads were finally stored in 50 mM sodium acetate buffer, pH 5.5 at 4 °C.

2.5. α-Amylase assay

α-Amylase activity, both soluble and immobilized, was assayed by DNS method [24]. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar/min. Two beads were taken for routine assay of the activity of immobilized enzyme on chitosan, beads were removed after 10 min incubation with 1 ml starch (1%) and DNS was added for color development. For Amberlite immobilized enzyme, matrix was incubated with 1 ml starch (1%) for 10 min and the supernatant was transferred to another test tube. DNS was added for color development and absorbance was observed at 540 nm. Amberlite was washed with buffer and kept at 4 °C for reuse.

2.6. Protein assay

Protein was assayed by the method of Lowry et al. [25]. The amount of protein immobilized was estimated by subtracting the amount of protein determined in supernatant after immobilization from the total amount of protein used for immobilization.

2.7. Storage stability of immobilized preparation

For storage stability studies immobilized amylase chitosan beads and Amberlite were kept at 4 °C. The activity of immobilized amylase was determined on different days by the method described above. After each assay, chitosan beads and Amberlite were washed with buffer and stored at 4 °C for further use.

2.8. Steady-state kinetics

For the steady-state kinetics, the α-amylase concentration was 5 mg/ml for chitosan beads and 6 mg/ml for Amberlite. The optimum pH for the immobilized α-amylase was determined by varying the pH of assay buffers from 3.6 to 9. Buffer used were 0.1 M sodium acetate buffer (pH 3.6–5.6), 0.1 M phosphate buffer (pH 6.0–7.6), 0.1 M Tris–HCl buffer (pH 7.2–9.0). The enzymatic activity was determined in each buffer by the method described for enzyme assay. Optimal temperature was studied by varying the temperature of immobilized α-amylase in assay buffer; the activity of immobilized amylase was assayed at an increasing temperature ranging from 25 to 95 ± 1 °C. K_m was determined by the Lineweaver–Burk plot by varying the substrate [starch] from 0.5 to 10 mg/ml.

3. Results and discussion

3.1. Optimum conditions for α-amylase immobilization on Amberlite

Amberlite (100 mg) at pH 5.0 gave maximum immobilization (72%, Table 1) at 2.5% glutaraldehyde concentration and time of activation was optimized to be 2 h (data not shown). Since Amberlite is a mixed bed exchanger, pH plays an important role in binding of enzyme with the matrix along with glutaraldehyde. Below and above pH 5.0 a decrease in immobilization was observed. It is clear from Table 2 that maximum immobilization is obtained at a protein concentration 5 mg/100 mg of Amberlite. The volume optimized was 100 μl and incubation time period of enzyme with matrix was best at 12 h. Amberlite MB 150 appears transparent. After activation with glutaraldehyde and coupling with enzyme, it appears brownish in color (Fig. 1).

Table 1
Conditions tested for optimal immobilization are applicable for 100 mg of Amberlite activated with 2.5% glutaraldehyde for 2 h

Conditions	Volume of enzyme loaded (μl)	Protein concentration (mg/ml)	Incubation (h)	Immobilization (%)
Protein concentration (mg/ml)				
1	100	–	12	50
2	100	–	12	57
3	100	–	12	62
4	100	–	12	69
5	100	–	12	72
6	100	–	12	71
Volume of enzyme loaded (μl)				
20	–	5	12	51
30	–	5	12	56
40	–	5	12	61
50	–	5	12	65
100	–	5	12	72
150	–	5	12	67
Incubation (h)				
6	100	5	–	53
12	100	5	–	72
24	100	5	–	67
48	100	5	–	60

Table 2
Conditions tested for optimal immobilization are applicable for one bead of chitosan activated with 2% glutaraldehyde for 3 h

Conditions	Volume of enzyme loaded (μl)	Protein concentration (mg/ml)	Incubation (h)	Immobilization (%)
Protein concentration (mg/ml)				
0.5	50	–	24	48
1	50	–	24	55
2	50	–	24	60
3	50	–	24	65
4	50	–	24	69
5	50	–	24	69
Volume of enzyme loaded (μl)				
10	–	4	24	45
20	–	4	24	52
30	–	4	24	59
40	–	4	24	63
50	–	4	24	69
100	–	4	24	45
Incubation (h)				
6	50	4	–	52
12	50	4	–	65
24	50	4	–	69
48	50	4	–	62

3.2. Optimum conditions for α-amylase immobilization on chitosan beads

Chitosan concentrations (1.5%) resulted in optimally rigid bead formation; concentration above this made chitosan very hard and impractical to cast any beads. Glutaraldehyde concentration was standardized to be 2% and time of activation of beads with glutaraldehyde was 3 h (data not shown). Table 2 shows the different conditions, which were standardized for maximum immobilization (69%) of α-amylase on chitosan beads. Data showed maximum immobilization at a protein concentration of 4 mg per bead. Above 4 mg protein there was not much increase in percent immobilization showing that all the free aldehyde groups of glutaraldehyde were saturated with the enzyme.

3.3. Steady-state kinetics

The behavior of an enzyme molecule may be modified by its immediate microenvironment. An enzyme in solution can have an altered pH optimum upon immobilization on a solid matrix. Depending upon the surface and residual charges on the solid

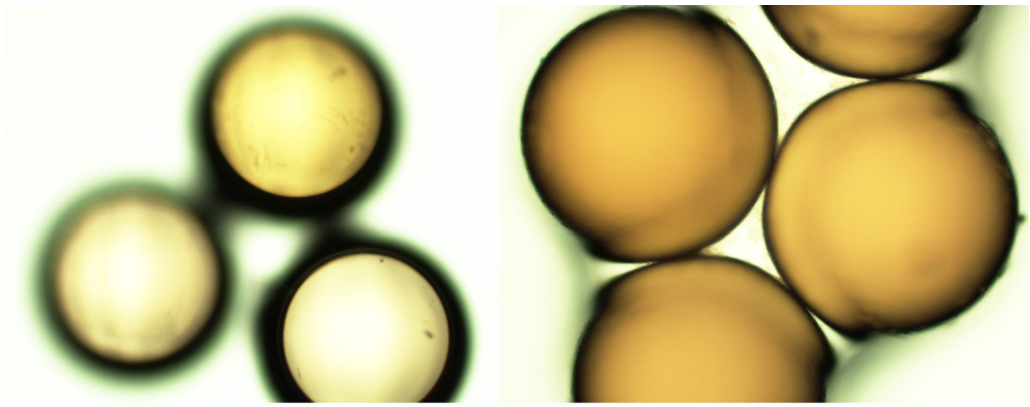

Fig. 1. Microscopic images of (A) plain Amberlite MB 150 (B) α-amylase coupled to glutaraldehyde activated Amberlite MB 150 using Nikon light microscope.

matrix and the nature of the enzyme bound, the pH value in the immediate vicinity of the enzyme molecule may change and thus cause a shift in the pH optimum of the enzyme activity [26].

The effect of pH on the activity of free and immobilized amylase is shown in Fig. 2. The pH optima of soluble and immobilized amylase on chitosan and Amberlite were 5.6 and 7.0, respectively. There was a shift of 2.4 U towards the basic side resulting from immobilization of α-amylase on both chitosan and Amberlite. Similarly, pigeon pea urease immobilized on chitosan beads and jack bean urease immobilized on a fixed bed reactor showed a shift towards basic side [16]. No shift was observed when urease was immobilized on Amberlite [22]; similar observations were reported by Boyd and Mortland [27] and Chellapandian and Krishnan [28]. No change in optimum pH was reported in case of commercial α-amylase (*Bacillus* sp.) immobilized onto pHEMA microspheres and to a composite temperature sensitive membrane, respectively [9,29]. A shift towards acidic region has been observed when α-amylase was immobilized on poly(methylacrylate-acrylic acid) microspheres [12] and on zirconium dynamic membrane [10].

Results of the effect of temperature on immobilized and soluble amylase are shown in Fig. 3. Soluble amylase and Amberlite immobilized amylase showed maximum activity at 65 °C, whereas chitosan immobilized amylase showed maximum activity at 75 °C. Similarly, commercial α-amylase immobilized to a composite temperature sensitive membrane was more stable at high temperature as compared to soluble enzyme [29]. α-Amylase from *Bacillus circulans* immobilized in calcium alginate beads showed an increase in operating temperature [30]. For α-amylase, shifts towards both higher and lower temperatures were reported [9,10]. The increase in the optimum temperature may arise from changing the conformational integrity of the enzyme structure by covalent bond formation via amino groups. The covalent bond formation might also reduce the conformational flexibility of the enzyme molecule and may impart higher activation energy for the molecule to reorganize to the appropriate conformation for catalysis to occur [31].

α-Amylase immobilized on Amberlite (Fig. 4) showed an apparent K_m of 2.77 mg/ml, which is slightly higher than that of soluble α-amylase (1.66 mg/ml) and V_{max} of 0.76 U/mg, which is slightly lower that that of soluble enzyme (1.63 U/mg), whereas α-amylase immobilized on chitosan showed an apparent K_m of 5 mg/ml, which is approximately four times higher than that of soluble α-amylase and V_{max} of 0.83 U/mg, which

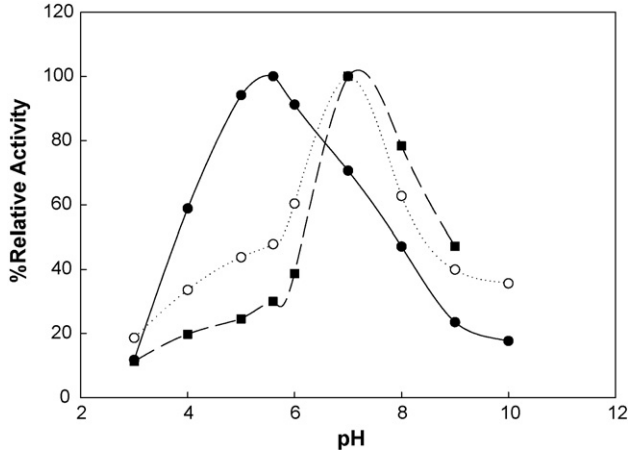

Fig. 2. Effect of pH on soluble (●), Amberlite-amylase (■) and chitosan-amylase (◯).

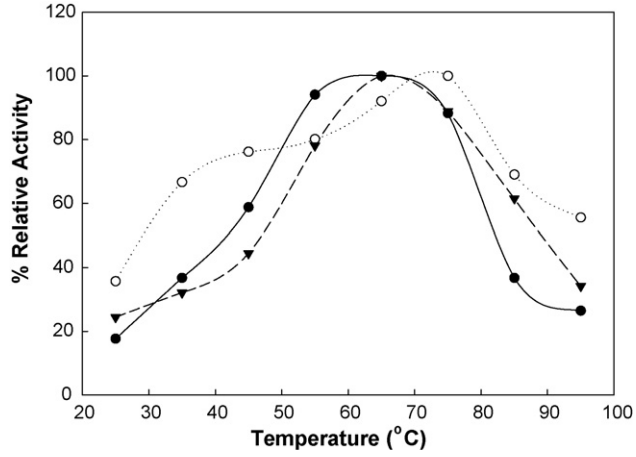

Fig. 3. Effect of temperature on soluble (●), Amberlite-amylase (▼) and chitosan-amylase (◯).

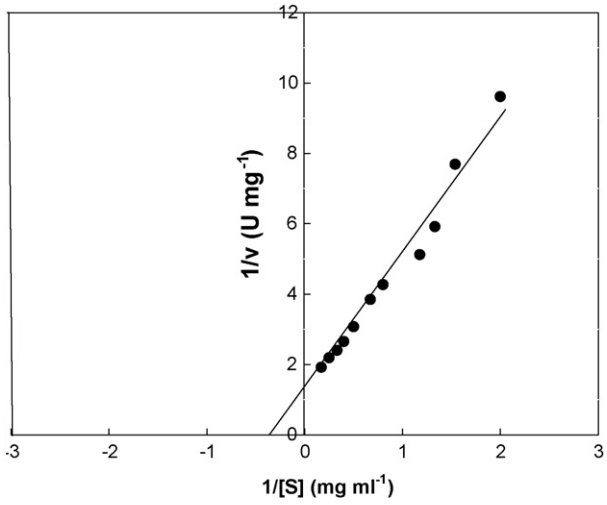

Fig. 4. Determination of K_m for Amberlite-amylase by Lineweaver–Burk plot method.

is slightly lower than that of soluble α-amylase (Fig. 5). The fourfold increase in K_m can be explained as a result of diffusional limitation of the substrate or due to an interaction between the substrate and the support or the conformational changes of the enzyme resulting in a lower possibility to form a substrate–enzyme complex. Chen et al. [29] also reported an increase in K_m, which clearly indicates an apparent low affinity of the enzyme towards its substrate compared to the soluble enzyme. It is postulated that an unstirred layer of solvent surrounds suspended water insoluble particles. This unstirred layer is known as "Nernst layer", and with water insoluble enzymes, i.e., immobilized enzymes; a concentration gradient of substrate is established across the layer. Consequently, saturation of an enzyme attached to a water insoluble particle will occur at a higher substrate concentration than normally required for the saturation of the freely soluble enzyme, so this leads to an increase in the K_m value [32–34]. A similar change was also observed in case of pigeon pea urease immobilized on chitosan beads [16].

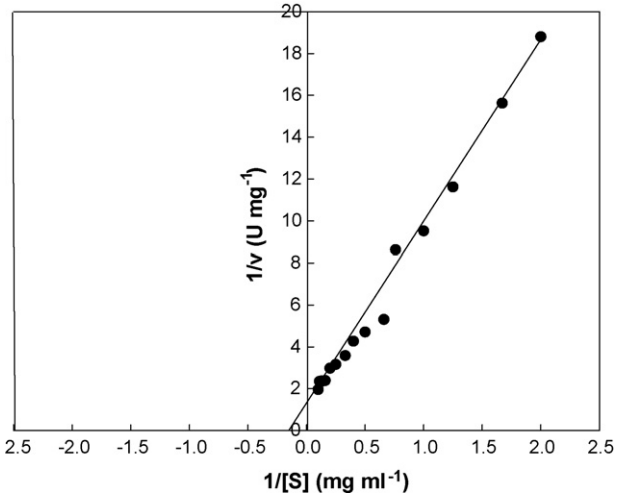

Fig. 5. Determination of K_m for chitosan-amylase by Lineweaver–Burk plot method.

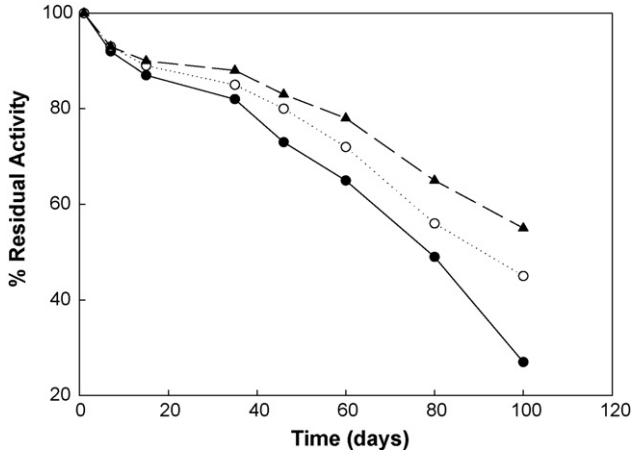

Fig. 6. Storage stability of soluble (●), Amberlite-amylase (▲) and chitosan-amylase (◯) for 100 days at 4 °C.

3.4. Storage stability and reusability

The stability of amylase was enhanced upon immobilization. Amberlite immobilized enzyme was stored at 4 °C, without any buffer. There was no change in activity whether the matrix is stored with or without buffer. It may be noted that amylase can also be immobilized on Amberlite without cross-linking by glutaraldehyde, because of ionic interaction among the enzyme and exchanger. However, the shelf-life of this matrix was much less as compared to the matrix treated with glutaraldehyde (data not shown). Chitosan beads stored at 4 °C showed practically no leaching of enzyme over a period of 2 weeks. The loss of activity, for free amylase after 100 days of storage at 4 °C was 70% and in comparison to Amberlite and chitosan-amylase the losses were 45% and 55%, respectively, during the same period (Fig. 6). Also storing the matrices at 4 °C improved the shelf-life as compared to room temperature (27 °C).

The Amberlite-amylase showed a residual activity of 43% and chitosan-amylase showed residual activity of 27%, after 10 uses (Fig. 7). The activity loss upon reuse could be due to weakening in the strength of binding between the matrix and

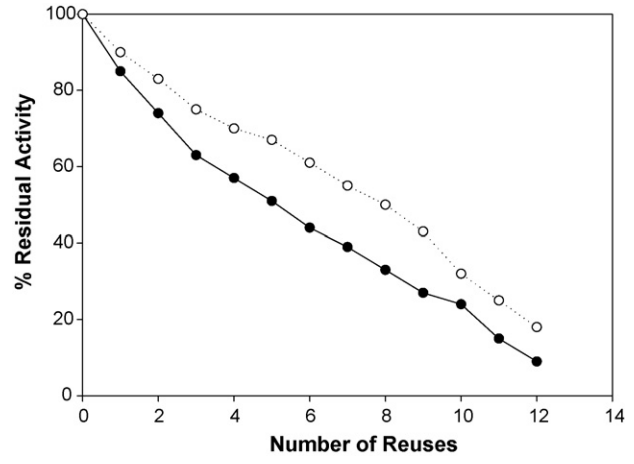

Fig. 7. Reusability (10 uses) of Amberlite-amylase (◯) and chitosan-amylase (●) at 4 °C.

enzyme on repeated use and hence the enzyme might leach out from the matrix and therefore yield a loss in activity. Furthermore, frequent encountering of substrate into the same active site might distort it and this distortion would dwindle the catalytic efficiency either partially or fully.

4. Conclusions

Amberlite is more promising matrix as compared to chitosan for immobilization of α-amylase. Also, it is easier to handle and there is less chance of leaching of enzyme as the Amberlite-amylase complex is quite stable in semi-dry condition.

Acknowledgements

Financial assistance of the Council of Scientific and Industrial Research, New Delhi (Junior Research Fellowship to PT) is thankfully acknowledged. We thank Prof. Amitabh Krishna and Ms. Padmasana, Zoology Department of our University for helping us with microscopy.

References

[1] G. Walsh (Ed.), Biochemistry and Biotechnology, John Wiley & Sons Co. Ltd., New York, 2002, p. 393.
[2] M. Vihinen, P. Mantsala, Crit. Rev. Biochem. Mol. Biol. 24 (1989) 329.
[3] A. Pandey, P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh, R. Mohan, Biotechnol. Appl. Biochem. 31 (2000) 135.
[4] M.J.E.C. van der Maarel, B. van der Veen, J.C.M. Uitedehaag, H. Leemhuis, L. Dijkhuizen, J. Biotechnol. 94 (2002) 137.
[5] G. Antranikian, in: G. Winkelmann (Ed.), Microbial Degradation of Natural Products, VCH, Weinheim, Germany, 1992, p. 27.
[6] T. Handa, A. Hirose, T. Akino, K. Watanabe, H. Tsuchiya, Biotechnol. Bioeng. 25 (1983) 2957.
[7] R. Ulbrich, A. Schellenberger, W. Damerav, Biotechnol. Bioeng. 2 (1986) 511.
[8] D.S. Cord, K. Vanhoof, J. Hu, G. Maesmans, M. Hendrickx, P. Tabback, Biotechnol. Bioeng. 40 (1992) 396.
[9] M.Y. Arica, V. Hasirci, N.G. Alaeddinoglu, Biomaterials 16 (1995) 761.
[10] C.J. Tien, B.H. Chaing, Process Biochem. 35 (1999) 377.
[11] S. Varanit, N. Chaokasem, S. Shobsngob, Sci. Asia 28 (2002) 247.
[12] S. Aksoy, H. Tumturk, N. Hasirci, J. Biotechnol. 60 (1998) 37.
[13] H. Tanaka, M. Matsumara, I.A. Veliky, Biotechnol. Bioeng. 26 (1984) 53.
[14] C.R. Carrara, A.C. Rubiolo, Biotechnol. Prog. 10 (1994) 220.
[15] T. Alexakis, D.K. Boadi, D. Quong, A. Groboillot, I. O'Neill, D. Poncelet, R.J. Neufeld, Appl. Biochem. Biotechnol. 50 (1995) 93.
[16] A.M. Kayastha, P.K. Srivastava, Appl. Biochem. Biotechnol. 96 (2001) 41.
[17] G.F. Payne, W.Q. Sun, A. Sohrabi, Biotechnol. Bioeng. 40 (1992) 1011.
[18] G.F. Payne, W.Q. Sun, Appl. Environ. Microbiol. 60 (1994) 397.
[19] R.S. Juang, F.C. Wu, R.L. Tseng, R.L. Biores, Technology 80 (2001) 187.
[20] S.E. Bailey, T.J. Olin, R.M. Bricka, D.D. Adrian, Water Res. 33 (1999) 2469.
[21] I.C. Omar, R. Suguna, Asia Pacific J. Mol. Biol. Biotechnol. 1 (1993) 103.
[22] A. Anita, C.A. Sastry, M.A. Hashim, Bioprocess Eng. 17 (1997) 355.
[23] P. Tripathi, L.L. Leggio, J. Mansfeld, R.U. Hofmann, A.M. Kayastha, Phytochemistry 68 (2007) 1623.
[24] P. Bernfeld (Ed.), Methods Enzymol, vol. 1, Academic Press Inc., New York, 1955, p. 149.
[25] H. Lowry O, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[26] K. Mosbach, Sci. Am. 224 (1971) 26.
[27] S.A. Boyd, M.M. Mortland, J. Soil Sci. Soc. Am. 49 (1985) 617.
[28] M. Chellapandian, M.R.V. Krishnan, Process Biochem. 33 (1998) 595.
[29] J.P. Chen, Y.M. Sun, D.H. Chu, Biotechnol. Prog. 14 (1998) 473.
[30] G. Dey, B. Singh, R. Banerjee, Br. Arch. Biol. Technol. 46 (2003) 167.
[31] P.M. Lee, K.H. Lee, S.Y. Siaw, J. Chem. Technol. Biotechnol. 58 (1993) 65.
[32] K.R.C. Reddy, P.K. Srivastava, P.M. Dey, A.M. Kayastha, Biotechnol. Appl. Biochem. 39 (2004) 323.
[33] P.K. Srivastava, A.M. Kayastha, Srinivasan, Biotechnol. Appl. Biochem. 34 (2001) 55.
[34] K.R.C. Reddy, A.M. Kayastha, J. Mol. Catal. B: Enzym. 38 (2006) 104.