Lactose hydrolysis by LactozymT^{M} immobilized on cellulose beads in batch and fluidized bed modes

Ipsita Roy, Munishwar N. Gupta*

Chemistry Department, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110016, India

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Abstract

LactozymT^{M} is a commercially available preparation of b-galactosidase from *Kluyveromyces fragilis.* It has valid generally recognized as safe (GRAS) status for whey hydrolysis and production of low lactose milk. Immobilized b-galactosidase from *K. fragilis* has been less studied. In this work, LactozymT^{M} was immobilized on cellulose beads via epichlorohydrin coupling chemistry. The optimized preparation was characterized in terms of its kinetic parameters. The fluidized bed hydrolyzed whey lactose (> 90% conversion) in 5 h as compared to 48 h taken by the same enzyme in continuous batch mode. The immobilized enzyme could be reused three times without any change in the performance of the fluidized bed reactor. The fluidized bed could also hydrolyze milk lactose up to 60% within 5 h. The above data show that this enzyme from a GRAS status source can also be used to develop a process for lactose hydrolysis for whey utilization as well as production of low lactose milk.

Keywords: Fluidized bed reactor; b-Galactosidase; *Kluyveromyces fragilis;* Lactose hydrolysis; LactozymTM; Low lactose milk; Whey hydrolysis

1. Introduction

Lactose hydrolysis by lactase (b-galactosidase) has two main biotechnological applications [1,2]. The first is in the utilization of whey because both glucose and galactose, the hydrolysates, have greater fermentation potential [3]. Production of low lactose milk (and dairy products made from it) for consumption by lactose-intolerant persons is the second major application [4]. In view of these twin applications, there is a vast amount of literature on the subject. b-Galactosidases have been studied from a variety of sources; the enzyme from *E. coli* is perhaps the best studied [2]. As applications involve food systems, it is more meaningful to work with enzymes from generally recognized as safe (GRAS). Currently, GRAS status is valid for *Aspergillus niger*, *Aspergillus oryzae*, *Kluyveromyces lactis* and *Kluyveromyces fragilis* only. Industrially, both soluble and immobilized lactases are used for lactose hydrolysis. LactozymT^{M} is a free enzyme preparation from *K. fragilis*, available from Novo Nordisk A/S, Denmark. There have been a few early attempts to immobilize b-galactosidase from *K fragilis*, mostly on membranes [1]. Recently, Ladero et al. [5] carried out the kinetic modeling of lactose hydrolysis by the enzyme immobilized on silica-alumina matrix and Szczodrak [6] has studied the hydrolysis of lactose in whey permeate by the enzyme immobilized on silanized porous glass modified by glutaraldehyde.

Fluidized beds offer an attractive design for enzyme reactors especially when dealing with unclarified substrate fluids [7,8]. Thus, it is not surprising that the first pilot-plant for whey hydrolysis used a fluidized bed reactor consisting of *A. niger* lactase adsorbed on porous alumina and crosslinked with glutaraldehyde [9]. It was thought worthwhile to evaluate the use of LactozymT^{M} in immobilized form. Furthermore, the performance of the immobilized enzyme was compared in the batch and fluidized bed modes. The process is described for both whey hydrolysis and the production of low lactose milk.

2. Materials and method

2.1. Materials

b-Galactosidase (from *K. fragilis)* is sold as a commercial product called Lactozym 3000L (Novozymes, Denmark) and was obtained from Arun and Co., Mumbai, India. The enzyme is supplied as a clear, amber liquid with 3000 lactose units per ml (as per supplier's product sheet). Cellulose beads were purchased from Sigma Chemical Co., St. Louis, USA. Epichlorohydrin and glucose detection kit were products of Merck, Germany. o-Nitrophenyl-b-D-galactopyranoside (ONGP) was obtained from the Council for Scientific and Industrial Research, India. All other chemicals used were of analytical grade (Table 1).

Table 1
Binding of b-galactosidase from *K. fragilis* on an expanded bed of Streamline DEAE

	Activity (U)	Protein (mg)
Crude	27 450	266.9
Wash	1854	66.5
Leaching (0.2 M NaCl)	12 850	60.9
Leaching (0.5 M NaCl)	3600	16.7

Streamline DEAE (bed volume 10.2 ml) was equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. Thirty milliliter (of diluted) LactozymT^{M} was loaded on the column after the bed had expanded to a height equal to 1.2 times the settled bed height. The column was then washed with the equilibration buffer till the absorbance (at 280 nm) decreased and leveled off (at 0.02). The pump was switched off and the bed allowed to settle down. The column was then washed with 0.05 M potassium phosphate buffer, pH 7.5 containing 0.2 M NaCl, followed by 0.05 M potassium phosphate buffer, pH 7.5 containing 0.5 M NaCl. Fractions of 4.8 ml each were collected and analyzed for enzyme activity and amount of protein.

2.2. Methods

2.2.1. Estimation of enzyme activity

The activity of the enzyme using ONGP was determined as described by Craven et al. [10]. One enzyme unit liberates 1 mmol of p-nitrophenol per min at 25 8C and pH 6.5. The amount of product formed was monitored at 410 nm. Activity of b-galactosidase was also estimated using milk whey or milk as the substrate. In this case, the amount of glucose produced by the hydrolysis of lactose was measured using a glucose detection kit based on the Trinder reagent [11]. One unit is defined as the amount of enzyme required to produce 1 mmol of glucose under assay conditions.

2.2.2. Estimation of amount of protein

Protein was estimated using a dye-binding method [12], using bovine serum albumin as the standard protein.

2.2.3. Preparation of whey [13]

The milk was skimmed by centrifuging the cold milk at 8000 x *g* for 20 min. The fat layer was removed. Whey was prepared from the skimmed milk by acidifying with HCl until the pH reached 4.5. The casein was removed by centrifugation. The pH of the whey supernatant was adjusted to 6.6. It was stored at 4 8C until further use.

2.2.4. Activation of cellulose beads by epichlorohydrin

Cellulose beads were washed thoroughly with distilled water. The beads (15 ml) were suspended in 0.6 N NaOH containing sodium borohydride and stirred. This was followed by the addition of 11.5 ml of epichlorohydrin, added slowly over a period of 10 min, with continuous stirring. The stirring was continued overnight.

2.2.5. Immobilization of b-galactosidase on epicholorohydrin-activated cellulose beads

The epichlorohydrin-activated beads were washed thoroughly with distilled water until the oily layer disappeared. Varying volumes (0.1-1 ml) of Lactozym, 3000L were added to 1 ml of activated beads. The final volume was made up to 2.0 ml (matrix + enzyme + buffer) with 0.03 M sodium carbonate buffer, pH 9.5. After incubation for 48 h at 25 8C (120 r.p.m., orbital shaker), the unbound enzyme was estimated in the supernatant after allowing the beads to settle. The enzyme-bound beads were then washed with 0.03 M sodium carbonate buffer, pH 9.5 until no enzyme activity could be detected in the washings. The enzyme activity was measured in the supernatant and washings.

The 'effectiveness factor' for an immobilized preparation was calculated as follows:

$$\text{Effectiveness factor} = \frac{\text{Activity units observed for the immobilized preparation}}{\text{Amount of enzyme bound to the matrix}}$$

The amount of enzyme bound to the matrix was obtained by subtracting the unbound activity in the supernatant (and washings) from the initially added enzyme.

2.2.6. Determination of pH and temperature optima

Effect of pH on free and immobilized b-galactosidase was studied by assaying both preparations at different pH values. The effect of temperature was studied by incubating the enzyme preparations with ONGP at that particular temperature and measuring the activity.

2.2.7. Measurement of thermal stability

Thermal stability was studied by incubating the two enzymes at 37 and 55 8C using ONGP and milk whey as the substrates. Appropriate aliquots of free and immobilized b-galactosidase were withdrawn at different time intervals and the activities determined.

2.2.8. Continuous hydrolysis of ONGP in the batch mode

The operational stability of the immobilized enzyme was studied by incubating the enzyme preparations with ONGP under shaking conditions. After particular intervals, the shaking was stopped to allow the enzyme-bound beads to settle down. Aliquots were withdrawn to measure the amount of product formed till that time. In another experiment, the effect of product inhibition was studied by removing the supernatant, washing the enzyme-bound beads with 3 ml of assay buffer (the product was estimated by combining the supernatant and wash of each cycle) and adding fresh substrate to the settled beads and resuming the shaking till the completion of the next cycle.

2.2.9. Hydrolysis of milk and milk whey in the batch and fluidized bed modes

The immobilized enzyme was incubated with milk whey and shaken continuously. Aliquots were withdrawn at regular intervals to measure the amount of glucose formed. In the fluidized bed mode, milk and milk whey were passed through the column. The effluent was again pumped into the column, thus forming a continuous loop. Two hundred and fifty microliter aliquots from the effluents were withdrawn at regular intervals to determine the amount of glucose formed.

2.2.10. Determination of K_m and V_{max}

K_m and V_{max} values of native and immobilized b-galactosidase were determined by measurement of enzyme activity at various concentrations of ONGP. The Michaelis constant was calculated using the LEONORA software program [14]. This software uses the Lineweaver-Burk equation to calculate the values of K_m and V_{max}.

3. Results and discussion

Cheese whey, the major byproduct of the dairy industry, contains about 6.5% solids of which 70% is lactose. Lactose can be hydrolyzed by chemical or enzymic methods. As pointed out by Gekas and Lopez-Leiva, a straight comparison between the economics of the two approaches is difficult [1]. The use of enzymes does not affect the proteins and other components of whey. Similarly, milk treated by enzymic methods retains its original nutritional value, especially since glucose and galactose (arising out of lactose hydrolysis) are not removed. The enzyme from *K. fragilis* has been used at pilot-plant scale in Novo process for whey hydrolysis [1]. The Tetra-Pak process of UHT milk containing low lactose is an industrial scale process that also utilizes lactase from *K. fragilis* [1]. In both cases, lactase has been used in the free form. The use of immobilized enzyme, as in other cases, offers the advantage of reusability.

3.1. Immobilization of the enzyme

In order to immobilize Lactozym^{TM}, cellulose beads were activated with epichlorohydrin, a procedure which creates an in-built spacer between the carrier and the enzyme [15]. Various amounts of the enzyme were then coupled to the activated matrix to measure the 'effectiveness factor' of the immobilized preparations with the synthetic substrate, ONGP. An 'effectiveness factor' of 0.82 was obtained with an enzyme load of 85 U ml^{-1} of activated beads (Fig. 1). The variation of 'effectiveness factor' with the enzyme load followed the trend observed in numerous cases [16,17]. The decrease in

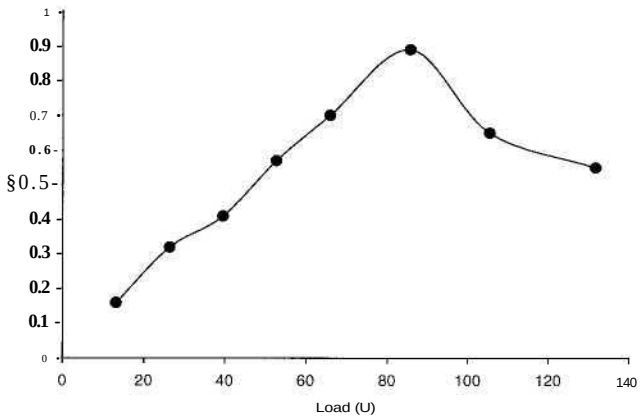

Fig. 1. Calculation of the effectiveness factor (BA^{-1}) of the immobilized preparation using ONGP as the substrate. Various aliquots of the free enzyme were coupled to the activated beads. 'A' represents the amount of enzyme theoretically bound to the matrix. This is calculated by subtracting the unbound activity in the supernatant from the initially added enzyme. 'B' represents the expressed activity of the particular immobilized preparation, measured after incubating the immobilized enzyme with the substrate.

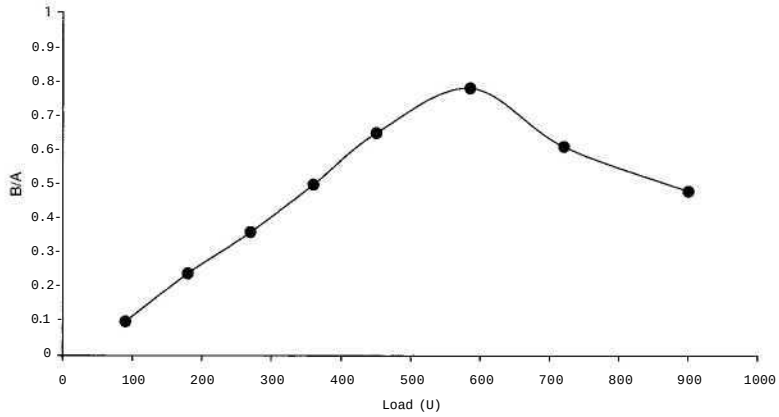

Fig. 2. Calculation of BA~' of the immobilized preparation using whey as the substrate. Measurements have been done as described in Fig. 1.

'effectiveness factor' beyond an optimum value has generally been ascribed to 'overcrowding' on the carrier. This 'overcrowding' is presumed to result in conformational distortion of the enzyme molecule [18]. That may be a contributory factor but 'mass transfer control' is likely to be an equally important factor [16,19]. The same trend in 'effectiveness factor' versus enzyme load was observed when whey lactose was used as the substrate. Here the optimum 'effectiveness factor' was 0.79 at 583 U ml^{-1} of activated beads (Fig. 2). There was a marginal decrease in the optimum 'effectiveness factor' with this different substrate. As both the synthetic substrate ONGP as well as the natural substrate lactose are low molecular weight substances, this marginal decrease is more likely to be due to whey being a more complex substrate than just lactose solution in aqueous medium [20].

It may be noted that this immobilized enzyme shows a much better 'effectiveness factor' than 0.5 reported by Ladero et al. [5] for the same enzyme immobilized on a commercial silica-alumina matrix. Szczodrak immobilized the *K. fragilis* enzyme on silanized porous glass modified by glutaraldehyde. A retention of more than 90% activity, as measured by lactose solution in aqueous buffer, was observed [6]. The author did not mention the effectiveness factor with whey lactose. Ladero et al. did not observe any change in pH optimum upon immobilization [5] whereas Szczodrak reported a shift in pH optimum from 7.0 to 6.0 as a result of immobilization [6]. In the present case, there was an increase in the pH optimum of the enzyme upon immobilization, it changed from 6.5 to 7.0 (Fig. 3). Such changes are normally analyzed in terms of charge on the matrix and a straightforward correlation of charge on the matrix and change in pH optimum is available [21]. Here, as the matrix (cellulose) was neutral, the effect of epichlorohydrin modifying amino groups on the enzyme becomes important. Such effects have often been overlooked while interpreting pH versus activity profile of immobi-

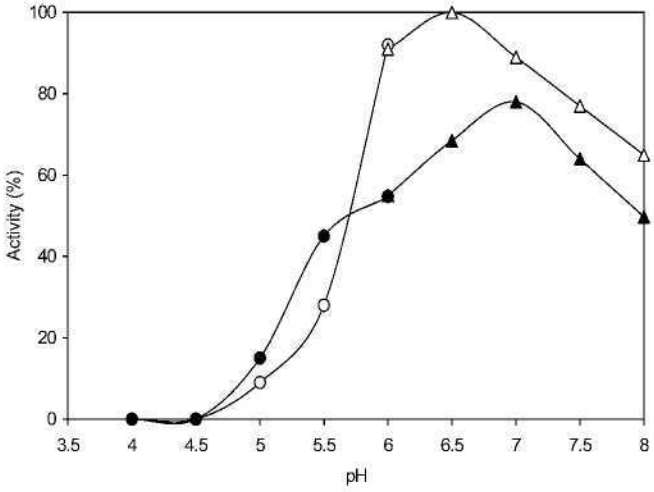

Fig. 3. pH optima of lactase, using ONGP as the substrate. Buffers used are 0.5 M acetate buffer (filled and empty circles, pH 4.0-6.0) and 0.15 M phosphate buffer (filled and empty triangles, pH 6.0-8.0); native enzymes (open symbol), immobilized enzyme (closed symbol).

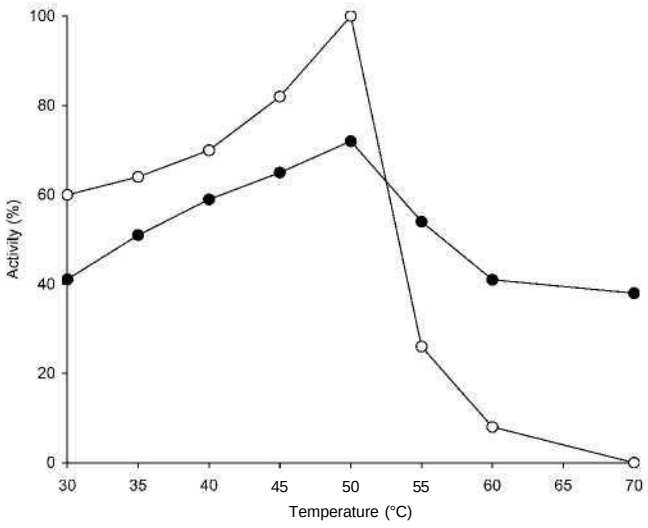

Fig. 4. Temperature optima of lactase, using ONGP as the substrate. Native enzyme (open symbol), immobilized enzyme (closed symbol).

lized enzymes. Linking the enzyme to cellulose beads via epichlorohydrin converts primary amino groups (on the enzyme surface) to secondary amines. Unfortunately, as pointed out by Price and Stevens [22], any change in the pH of the reaction affects both K_m and V_{max} and this makes it difficult to predict the change in pH as a result of such modifications. From the application point of view, the immobilized enzyme continues to be suitable for milk and sweet whey hydrolysis [1].

The temperature optimum of the enzyme remains unchanged at 50 8C upon immobilization. However, the activity of the immobilized enzyme decayed less rapidly with increase in temperature (Fig. 4).

The K_m of the free enzyme changed from 1.7 to 2.3 mM upon immobilization on the soluble polymer. V_{max} of the immobilized enzyme showed a marginal decrease to 63.7 mmol min^{-1} (from 77.5 mmol min^{-1} for the free enzyme). The values for the free enzyme agree with those reported in literature [23]. These authors however report a 9000-fold decrease in the value of V_{max} upon immobilization of the enzyme on graphite. The immobilized preparation reported here, on the other hand, shows only a slight decrease in V_{max}.

The thermal stabilities of the free and immobilized preparation were measured at 37 8C with ONGP and whey (lactose) as substrates (Figs. 5 and 7). In both cases, detectable thermostabilization could be observed. Both free and immobilized enzymes show similar kinetics of inactivation. There is rapid inactivation initially in the first eight hours or so; the decline in activity is much slower beyond this. It is well-established that thermal inactivation starts with the unfolding of the protein molecule which is followed by irreversible changes due to aggregation and formation of 'scrambled' structures [24,25]. As the solution remained clear in the case of free enzyme, aggregation can be ruled out. In the case of the immobilized preparation, aggregation does not take place as protein-protein

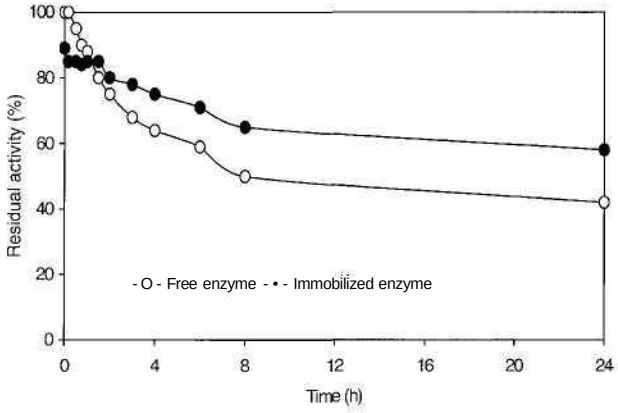

Fig. 5. Thermal stability of lactase at 37 8C using ONGP as a substrate. The enzyme is incubated at 37 8C for the desired time, cooled to 25 8C and assayed for residual activity. The starting activity at 25 8C has been taken as 100%.

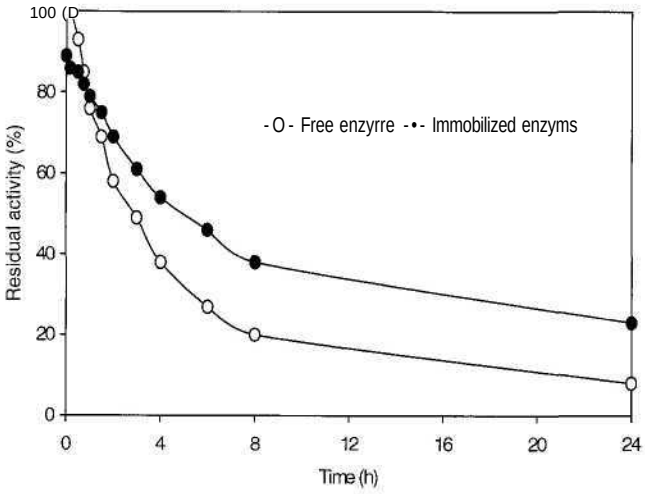

Fig. 6. Thermal stability of lactase at 37 8C using whey as a substrate. The experiment has been carried out as described in Fig. 5.

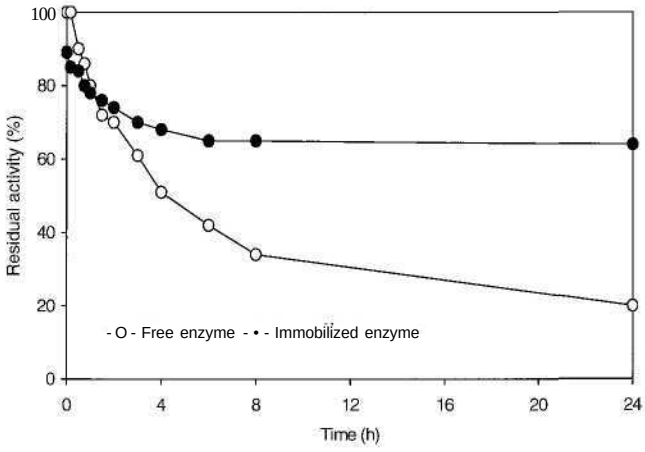

Fig. 7. Thermal stability of lactase at 55 8C using ONGP as a substrate. The enzyme is incubated at 55 8C for the desired time, cooled to 25 8C and assayed for residual activity. The starting activity at 25 8C has been taken as 100%.

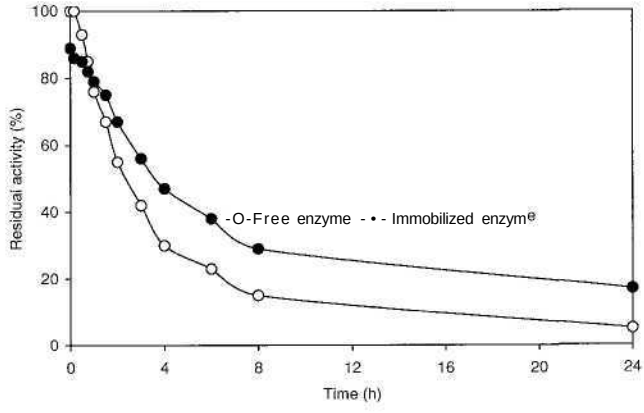

Fig. 8. Thermal stability of lactase at 55 8C using whey as a substrate. The experiment has been carried out as described in Fig. 7.

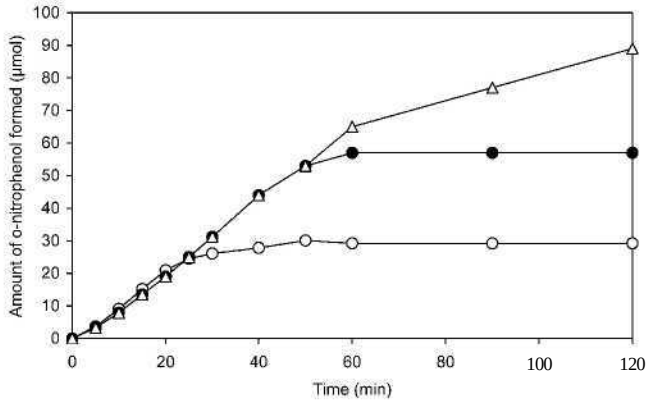

Fig. 9. Continuous hydrolysis of ONGP by b-galactosidase. Both the free (k) and immobilized (m) enzymes were shaken with the substrate in different vials. After a fixed time, the amount of product formed was evaluated in the vials. This was continued till a steady state in product formation is reached. In one case, the immobilized enzyme was checked for activity after removing the product and the undegraded substrate (A) by stopping the shaking and allowing the immobilized enzyme to settle down. Fresh substrate was then added to the immobilized enzyme and the shaking was continued.

contact is minimized. Greater inactivation is observed when the enzyme is assayed with whey lactose as the substrate. This has been reported before and is believed to be due to the components present in whey [26]. The corresponding thermal stabilities (with ONGP and whey as substrates) at 55 8C are shown in Figs. 6 and 8. At this temperature, the effect of immobilization is more significant. However, when the assay is carried out with whey as the substrate, immobilization does not seem to have helped. This confirms that short exposure to the components present in the whey inactivate the enzyme and immobilization can not prevent this inactivation.

Fig. 9 shows the continuous hydrolysis of ONGP by free as well as immobilized enzyme in the batch mode. The hydrolysis by the free enzyme reached a plateau at 26% conversion after about 30 min of hydrolysis whereas the immobilized preparation showed increased hydrolysis up to about 50 min and 59% hydrolysis was observed at the end of 50 min. Removal of the unhydrolyzed substrate and the product formed and charging the enzyme with fresh substrate showed that the enzyme had not lost its activity and the plateauing of the product formation was presumably due to product inhibition.

The hydrolysis of whey lactose by the immobilized enzyme was attempted in continuous batch mode at 30 8C (Fig. 10). About 90% conversion of the lactose present in whey could be achieved by 48 h. Most of the work with whey has been carried out after some pre-processing of the feed. Szczodrak, for example, used

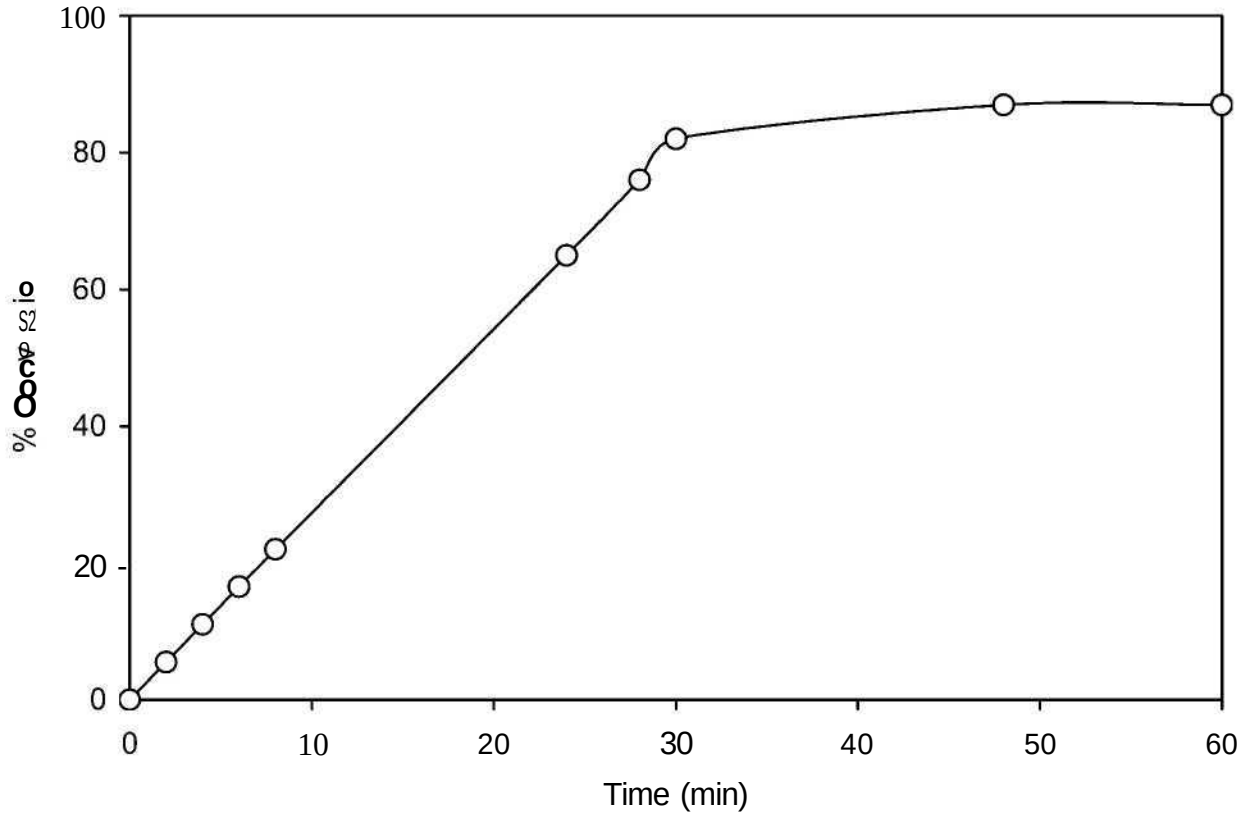

Fig. 10. Continuous hydrolysis of lactose present in milk whey (batch mode). Five milliliter of beads were incubated with 60 ml of milk whey on an orbital shaker at 30 8C. The amount of glucose produced was estimated at different time intervals by stopping the shaking and withdrawing small aliquots of the supernatant.

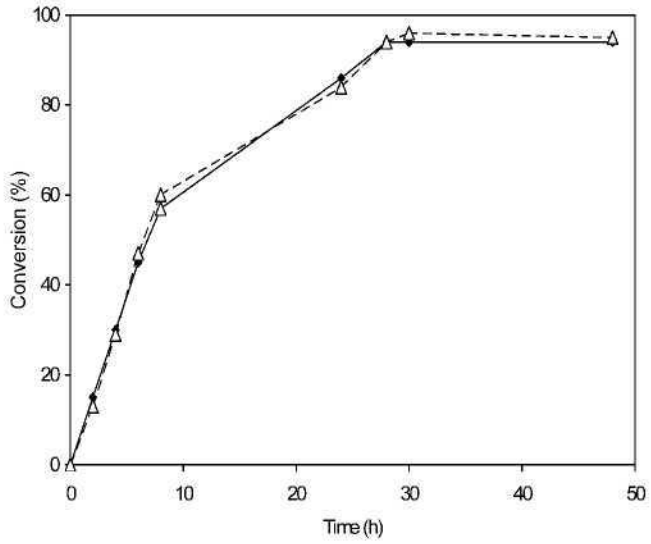

Fig. 11. Continuous hydrolysis of lactose present in milk whey (fluidized bed mode). Ninety milliliter of milk whey was loaded on a fluidized column of cellulose beads (bed volume 5 ml) at a flow rate of 2 ml min~'. The preparation used here had 'effectiveness factor' of 0.50. The effluent was recirculated through the column. The effluent was monitored at regular intervals for amount of glucose produced. Continuous line represents the first run, dashed line represents the second run.

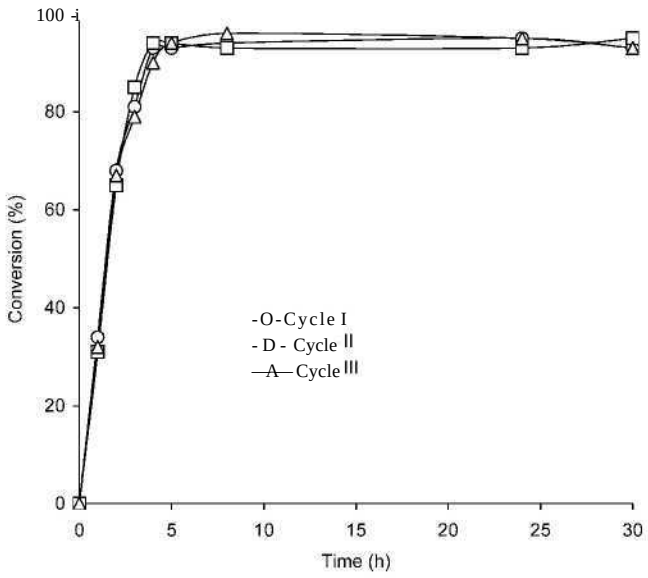

Fig. 12. Continuous hydrolysis of lactose present in milk whey (fluidized bed mode). Ninety milliliter of milk whey was loaded on a fluidized column of b-galactosidase immobilized on activated cellulose beads (bed volume 5 ml) at a flow rate of 2 ml min~[1]. The preparation used here had 'effectiveness factor' of 0.78. The effluent was recirculated through the column. The effluent was monitored at regular intervals for amount of glucose produced. The three runs were carried out without any sanitization step in between.

spray-dried whey permeate which was obtained from pasteurized raw sweet whey by ultrafiltration, demineralization and drying [6]. The use of fluidized beds, as opposed to packed bed format, allows the use of feed without preclarification. In a fluidized bed, the suspended matter does not clog the column as the space between the biocatalyst containing carrier particles allows this suspended matter to pass through the column. In fact, one of the first pilot-plants for whey hydrolysis utilized *A. niger* lactase (adsorbed on a porous alumina and crosslinked with glutaraldehyde) in a fluidized bed reactor [9]. As the cellulose beads used here as immobilization matrix form a fluidized bed, the hydrolysis of whey lactose was attempted in the fluidized bed mode. The fluidized bed reactor showed ~94% conversion by about 30 h even though a less efficient biocatalyst, with an 'effectiveness factor' of 0.5, was used in this case (Fig. 11).

With the optimized immobilized biocatalyst, with an 'effectiveness factor' of 0.78, the time for >90% conversion was drastically cut down to about 5 h (Fig. 12). Thus, the fluidized bed was found to be a considerably more efficient format for whey lactose hydrolysis. The immobilized biocatalyst could be used three times without any change in the performance of the fluidized bed reactor (Fig. 12).

Fig. 13 shows the continuous hydrolysis of lactose present in the whole milk. Using the fluidized bed mode, it was possible to use whole milk as substrate. About 60% conversion could be obtained within 5 h but

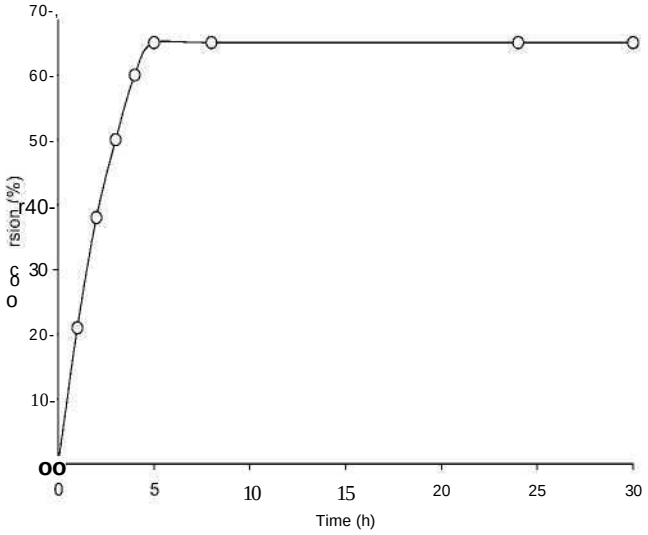

Fig. 13. Continuos hydrolysis of lactose present in whole milk (fluidized bed mode). Sixty milliliter of milk was loaded on a fluidized column of b-galactosidase immobilized on activated cellulose beads (bed volume 5 ml) at a flow rate of 2 ml min⁻'. The preparation used here had 'effectiveness factor' of 0.78. The effluent was recirculated through the column. The effluent was monitored at regular intervals for amount of glucose produced.

conversion did not proceed beyond this point. The presence of fat has been reported to impair the performance of fluidized bed in bioseparation [7,27,28]. One solution to this may be to use milk after

removal of fat for lactose hydrolysis and put back the fat, if so desired.

Lately, considerable experience with fluidized beds has been gained in the context of bioseparation [29,30]. It may be useful to have a 'second look' in the use of fluidized beds for bioconversion. The present work shows that LactozymT^{M} which is generally used (and recommended by manufacturers) as a free enzyme can be gainfully employed in immobilized form. The immobilized enzyme works better in fluidized bed format for hydrolysis of lactose in whey.

Acknowledgements

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