Highly efficient production of inverted syrup in an analytical column with immobilized invertase

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Abstract This paper presents a procedure by which a simple and economical analytical column containing immobilized invertase was developed. This column has high efficiency of converting sucrose into inverted syrup rapidly. Gelatine beads were used for the immobilization of invertase. The enzyme was entrapped efficiently and was found to be stable and retained its activity over a period of 3 months. Immobilization parameters for maximum enzyme activity were estimated as temperature optima at 60 °C, pH optima 7.0 and 30 mg/mL enzyme concentration was found to give maximum immobilization (72 %). The reusability of the gelatine immobilized invertase was found to be seven times with a time interval of 24 h. The immobilized invertase presented a K_M of 51.28 mM and V_{max} of 0.334 mM/min. The time required to hydrolyse 50 % sucrose solution by a column of length 10 cm and diameter of 1.5 cm was found to be 15 min at room temperature. The column was found effective for inversion of biological samples like sugar cane juice.

Keywords Sucrose · Gelatine beads · Glutaraldehyde · Invertase · Immobilization

Introduction

In recent years, Invertase (E.C.3.2.1.26, β -Dfructofuranosidase) gained considerable attention in food industry, owing to its ability to hydrolyse sucrose into a mixture of glucose and fructose, named inverted syrup. These syrups are mainly used

in confectionaries, beverage industries, pharmaceuticals and bakeries. Owing to its widely uses, a lot of methods have been incorporated for the efficient production of the syrup. The conventional method of producing invert syrup is to hydrolyse sucrose in the presence of acid, however such acid hydrolysis is often contaminated with coloured oxidation compounds due to high temperature and low pH (de Queiroz et al. 1996). Beside this, low conversion efficiency and high energy consumption are some other factors which make the acid hydrolysis, an avoidable method.

Bioproduction of inverted syrup with the help of enzymes is a better alternative as compared to conventional methods and with further advancement in immobilization technology, numerous approaches have been explored for the preparation of immobilized enzymes because they have considerable advantages over enzymes in bulk solution (Kotwal and Shankar 2009). For the commercial production of invert sugar, immobilized yeast invertase has been the major enzyme (Mahmoud 2007) and infact, it is the first enzyme to be immobilized (Kotwal and Shankar 2009). Among different methods of immobilization, entrapment is widely used and has been carried out in silica particles (Rai et al. 2012), calcium alginate (Tanriseven and Doğan 2001; Vu and Le 2008; Arruda and Vitolo 1999), cellulose triacetate fibres (Marconi et al. 1974), wood waste (Mahmoud 2007), resins (Ribeiro and Vitolo 2009), nylon beads (Amaya-Delgado et al. 2006) and Germania nanospheres (Regan and Banerjee 2007). Although numerous attempts have been made on the immobilization of invertase as a laboratory model for the production of High fructose syrup (Monsan and Combes 2004; Mansfeld et al. 1992; Amaya-Delgado et al. 2006; D'Souza and Melo 2001; Tomotani and Vitolo 2007; Melo et al. 1992) but in most cases, the stability of enzymes has increased while in some cases there have been reports of loss of activity.

In the present work, we developed a simple and highly efficient analytical column containing immobilized invertase,

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for the production of inverted syrup. The cost of production is very low as there is no requirement of any sophisticated instrument and moreover, the developed column is efficient in terms of reusability and the shelf life of immobilized enzyme.

Materials and methods

Chemicals

Invertase (EC.3.2.1.26 *β -D-fructofuranosidase*) was purchased from Sigma- Aldrich while Gelatine, Glutaraldehyde, Dinitrosalicylic acid, Trichloro acetic acid (TCA) and Tris were purchased from HiMedia, India. Sucrose, Sodium hydroxide, Sodium Potassium Tartrate and Hydrochloric acid were of food grade, while other chemicals were of analytical grade from Merck, India. Double distilled water was used for the preparation of all solutions and throughout the process.

Enzyme assay

The soluble invertase was assayed in a 2 ml reaction volume, containing 0.2 ml enzyme, 0.8 ml sucrose (500 mM) and 1 ml Tris HCL buffer (0.01 M, pH7.0). After an incubation period of 30 min, 10 % TCA was added to stop further reaction and 1 ml is withdrawn for assay. The activity of invertase was then determined by employing DNS (dinitrosalicylic) method (Miller 1959). A reddish orange complex was formed as a result of reaction between DNS and reducing sugars which was spectrophotometrically measured at 540 nm. For each assay, three readings were taken and the average was used as the final reading. A standard curve of glucose allows the association of absorbance with the concentration of reaction products.

Immobilization in gelatine beads

Gelatine beads were prepared by dissolving 0.15 gm of invertase in 4.5 % gelatine solution and mixing it gently. Using a micropipette, the enzyme-gelatine solution was dropped in 25 ml of glutaraldehyde solution (0.35 %v/v) under chilled condition with constant stirring. The stirring was continued for 20 min and then excess of glutaraldehyde solution was discarded and the beads were thoroughly washed with Tris HCL buffer (0.01 M) and stored in the same buffer till further use.

Enzyme assay for immobilized invertase

The activity of immobilized invertase was determined by spectrophotometric assay. The desired numbers of beads were incubated at 30 °C for half an hour in standard assay

medium containing 1.0 ml of Tris HCL buffer (0.01 M, pH 7.0) and 1.0 ml sucrose (500 mM) solution. Following the incubation, an aliquot of 1.0 ml was withdrawn and assayed using DNS method. A reddish orange complex was formed as a result of the reaction between DNS and reducing sugars, which was spectrophotometrically measured at 540 nm. For each assay, three readings were taken and the average was used as the final reading. The beads were then recovered from the reaction mixture, rinsed thoroughly with the buffer and stored at 4 °C.

Optimization of gelatine concentration

Varying concentration of gelatine (2 to 8 %) was prepared in Tris HCL buffer with continuous stirring for 30 min at room temperature. For immobilization, invertase was mixed with gelatine solution and dropped with the help of micropipette into continuously stirred glutaraldehyde solution (0.35 % v/v) in an ice bath. The beads, thus obtained were checked for activity and leaching.

Optimization of glutaraldehyde concentration

Glutaraldehyde in the concentration range of 0.05–0.5 % was prepared in Tris HCL buffer under chilled conditions. Beads were prepared by dropping fixed concentration of invertase and gelatine with the help of micropipette in the above varying concentration of glutaraldehyde. The beads were then assayed for activity and leaching.

Kinetic study comparison of soluble and immobilized invertase

pH effect on invertase activity

The effect of pH on the activity of immobilized invertase was carried out by varying the pH of assay buffer from 3 to 8 at room temperature. In a standard assay medium, which contain 1 ml sucrose solution (500 mM) and 1 ml Tris HCL buffer (0.01 M) with varying pH, 5 beads of immobilized invertase were added and incubate for half an hour. Following incubation 1 ml was withdrawn from the medium and assayed. Control experiments were carried out using the free enzyme in a similar manner. The above experiment was carried out in four repeats ($n=4$) and the results were analysed through analysis of variance (ANOVA) for their significance.

Temperature effect on invertase activity

To determine the thermal stability of soluble and immobilized enzyme, the reaction was carried out at different

temperatures (0 °C to 100 °C). Following incubation of half an hour, 1 ml is removed from the medium and assayed. Control experiments were carried out using the free enzyme in a similar manner. The above experiment was carried out in four repeats ($n=4$) and ANOVA was used to analyse the results for their significance.

Effect of substrate concentration on invertase activity

The effect of substrate concentration on the soluble and immobilized invertase was studied by using different concentrations of sucrose. Assay buffers with varying concentration of sucrose, ranging from 25 mM to 250 mM was incubated with free and immobilized invertase for half an hour at 37 °C.

Following the incubation, the sample is removed and assayed. The results were expressed in Lineweaver Burk plot in order to determine the K_m and V_{max} values.

Shelf life and operational stability of immobilized invertase

The multiple use stability of immobilized invertase was studied by assaying the activity of same beads for continuous 8 days with a time interval of 24 h between each assay. The experiment was carried out in triplicates ($n=3$) and their statistical significance was calculated using ANOVA. The long term storage of the analytical column was investigated by evaluating the activity of immobilized invertase at every 3–4 days, for a period of three months, at two different

Fig. 1 Diagrammatic representation of the development of an analytical column for sucrose inversion

temperatures (4 °C and RT) using the same reaction conditions. The beads and the storing media were then assayed separately for the activity.

Lab scale inversion of sucrose by gelatine immobilized invertase in analytical column

Immobilized invertase-gelatine beads were filled in a syringe column supported with a thin layer of wool at the base. The dimension of the column was 10 cm in height and 0.75 cm radius (Fig. 1). This beads packed column was then washed with six bed volume of the buffer. Column was always filled with 1 cm buffer volume on the top of bead level and the precaution was taken that the column was never in dried condition. During laboratory inversion, the buffer was slowly eluted out and sucrose solution (50 % w/v) was poured from the top. After certain time intervals, small volumes were eluted from the bottom of column and checked for inversion. The set up was then stored at low temperature for further use.

Statistical analysis

For each parameters studied which were replicated either three times or four times, results were statistically analysed by analysis of variance (ANOVA). A mean and standard deviation was calculated and reported using Origin Pro (Version 8).

Results and discussions

Optimization of gelatine concentration

In order to search the appropriate concentration of gelatine, its concentration was varied from 2.0 to 8.0 %. With low concentration, the beads obtained were fragile in nature and were burst opening after a single assay. Whereas at higher concentration, the beads formed were not of uniform shape and size and hence lacks uniformity in the activity. The beads obtained with 4.5 % solution of gelatine were homogenous with respect to size and activity and exhibit 72 % immobilization. Therefore 4.5 % gelatine was used for further study.

Optimization of glutaraldehyde concentration

Optimum concentration of glutaraldehyde was explored in the range of 0.05–0.5 %. Low molarities of glutaraldehyde resulted into fragile beads with maximum leaching as 80 % enzyme was leached out within 12 h. However, high concentration of glutaraldehyde resulted into hardening of beads, thus a fall in the enzymatic activity was reported.

This activity loss can be explained by using high concentration of cross-linkers which blocks the enzyme active sites (Emregul et al. 2006). With 0.35 % concentration of glutaraldehyde, beads show maximum stability, activity and were good enough to handle.

Effect of pH on invertase activity

The pH dependence activity of immobilized enzyme at different pH was compared with the free enzyme. From the activity–pH profiles (Fig. 2) of the free and immobilized enzyme, an increment in the activity at higher pH has been reported on immobilization. The optimal pH region of free enzyme was found to be 5.5 ($p < 0.05$) while in the case of immobilized enzyme, it was 7.0 ($p < 0.05$). In the case of immobilized invertase, activity decreases sharply after pH7 while a slower decrement in activity was reported for soluble enzyme after pH5.5. Table 1 shows the analysis of

Fig. 2 pH and temperature kinetics of soluble and gelatine immobilized invertase. Each observation is an average of four repeats ($n=4$)

Table 1 Analysis of variance (ANOVA) for optimum kinetic variables of soluble and immobilized invertase

Parameters	Invertase form	SS ^a	DF ^b	MS ^c	F-Value	P-Value [*]	Remarks
pH	Soluble	0.4465	9	4.961×10^{-2}	71.64	< 0.05	Significant
	Immobilized	0.5937	9	6.5961×10^{-2}	127.5	< 0.05	Significant
Temperature	Soluble	0.5768	9	6.409×10^{-2}	140.6	< 0.05	Significant
	Immobilized	1.089	9	0.1210	228.2	< 0.05	Significant
Re-usability	Immobilized	5473	7	781.8	288.7	< 0.05	Significant

^a sum of square^b degree of freedom^c mean square^{*} p-value is significant at $\alpha=0.05$

variance for the experimental data and the results were found to be statistically significant at $\alpha=0.05$. A shift toward higher pH has also been reported in literature (Erginer et al. 2000; Danisman et al. 2004; Jen Tien and Huang Chiang 1999). This pH alteration may be due to the building-up of large amount of product in the reaction layer of immobilized enzyme. Moreover, these changes also depend upon the surface and residual charge on the matrix used for immobilization.

Effect of temperature on invertase activity

The effect of temperature on invertase activity was investigated in the range from 0 °C to 100 °C. As shown in Fig. 2, the temperature optima were found to be 40 °C and 60 °C for free and immobilized enzyme respectively ($p<0.05$) which clearly indicate that immobilization imparts thermal stability to the enzyme. Enzyme stabilization by immobilization may also be caused by the existence of a local environment which is much favourable than bulk solution conditions. The matrix also provides a masking effect to the immobilized enzyme. Both the enzymes decreased their activity above 70 °C due to protein denaturation, however,

Fig. 3 Double reciprocal plot for determination of K_m and V_{max} values of soluble and immobilized invertase

the immobilized enzyme was found to be more stable than free enzyme at this temperature. Similar results were also obtained, when the invertase was immobilized in wood saw dust waste (Mahmoud 2007), nylon-6 microbeads (Amaya-Delgado et al. 2006) and Germania matrix (Regan and Banerjee 2007).

Effect of substrate concentration on invertase activity

Effect of substrate concentration on enzymatic activity was carried out by varying the sucrose concentration from 25 mM to 250 mM and, the K_m and V_{max} values were calculated using Lineweaver-Burk method (Fig. 3). From the data, an apparent K_m value of 51.8 ± 0.5 mM was obtained for immobilized invertase which was similar to that obtained for soluble enzyme. It indicates that immobilization did not alter the affinity of invertase for its substrate. However, the V_{max} value tends to increase by 1.37 fold. The V_{max} value was found to be 0.334 mM/min and 0.243 mM/min for immobilized and

Fig. 4 Variation in the activity of invertase immobilized beads with reuse for continuous 8 days. Each observation is an average of three repeats ($n=3$)

soluble enzyme respectively. Immobilization of invertase within calcium alginate capsules also reported to have same K_m values (Tanriseven and Doğan 2001). However, some increasing K_m values have also been reported in literature (Akgöl et al. 2001; Tümtürk et al. 2000; Vu and Le 2008).

Shelf life and operational stability of immobilized invertase

The stability of gelatine immobilized invertase was found to be more at 4 °C than room temperature, suggesting that the immobilized preparations and column storage should be done preferably at low temperature. The reusability was found to be good as after 8 times of utilization of same beads, there was less than 40 % loss in enzyme activity (Fig. 4). An apparent increase in the shelf life of immobilized invertase to 90 days was found with practically no leaching over a period of 45 days. It indicates that the enzyme was immobilized efficiently in the gelatine beads which provide it a stable environment and prevent the activity loss.

Efficiency of analytical column

The developed analytical column showed high efficiency of converting sucrose to inverted syrup. Initially, when a 50 % sucrose solution was filled in the column containing gelatine beads, the beads raised up due to the high density of sucrose solution. As the reaction proceeded with the conversion of sucrose to glucose and fructose, the beads settled down due to the reduction in the density of the solution. After 15 min, the beads completely settled down and the solution was floating above the beads as the density of glucose and fructose was comparatively lower than that of sucrose. It took almost 15 min by the column to hydrolyse the sucrose solution (50 %) into inverted syrup at room temperature.

Conclusion

In this study, enzyme invertase was immobilized on gelatine beads which were further used to prepare an analytical column for the production of inverted syrup. From the data, it has been concluded that the column is more efficient in terms of conversion ability as compared to the free enzymes. Also the immobilization method involved, provides thermal and pH stability to the enzyme so that the column can be used at varying parameters. The shelf life of immobilized invertase was 90 days and it showed hardly 40 % loss in enzyme activity after 8 times reuse of the same beads with a time interval of 24 h. Without the use of any sophisticated instruments, this approach provides a cost effective and stabilized system that can be scale-up at industrial level.

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