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Preparation and Properties of *Sclerotium rolfsii* Invertase Immobilized on Indion 48-R

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ABSTRACT

Crude extracellular invertase from *Sclerotium rolfsii*, when coupled to glutaraldehyde activated Indion 48-R, retained 70–80% activity of the soluble enzyme. Immobilization resulted in a decrease in the pH and temperature optima but it increased the temperature stability. K_m and V_{max} also increased as a result of immobilization. Both soluble and immobilized invertase showed inhibition at high substrate concentrations. The bound enzyme showed excellent stability to repeated use and retained approx 90% of its initial activity after 8 cycles of use.

Index Entries: Invertase from *Sclerotium rolfsii*; binding via glutaraldehyde; immobilization on polystyrene resin; immobilized invertase.

INTRODUCTION

Invertase (EC 3.2.1.26), which catalyses the hydrolysis of sucrose and related glycosides, is one of the simplest commercial carbohydrases and is used for the production of invert syrup. Extensive investigations have been carried out on the enzymatic isomerization of glucose to fructose, mainly due to the increasing sugar prices in the world market. Hence, as an alternative to sucrose, high fructose syrup (HFS) has gained considerable commercial significance *(1)*. However, HFS production requires hydrolyzed starch as the raw material. In this respect, in countries like India where sucrose is readily available, utilization of sucrose would be advantageous. In addition, molasses (a byproduct of sugar industry) can serve as a cheap source of sucrose due to its high sucrose (approx 50%) content. On this background, use of immobilized invertase for the production of invert syrup offers a suitable alternative to the production of isomerized syrup. Enzymatic hydrolysis of sucrose to invert sugar is preferable to acid hydrolysis since it does not

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result in the production of furfural and other undesirable flavoring agents as well as oligosaccharides. Moreover, immobilization-induced shifts in the optimum pH can be beneficial as it will prevent the formation of reversal products by the transferase activity associated with the soluble enzyme *(2)*. *Sclerotium rolfsii* (a sclerotial state of *Corticium rolfsii*) produces an acidophilic and thermophilic extracellular invertase *(3)*. This enzyme was selected for immobilization studies because the above properties of the enzyme will permit the reaction to be carried out at a high temperature and low pH, a condition, which will discourage microbial contamination. If an immobilized enzyme is to be used for the production of food and pharmaceuticals, the matrix and the reagents used for immobilization should be nontoxic. Therefore, Indion 48-R (a cross-linked macroporous anion exchange resin) was selected because polystyrene resins show good chemical and mechanical stability, are resistant to microbial contamination and can be easily derivatized. Moreover, binding the enzyme via glutaraldehyde was preferred as this method is simple, nontoxic and can be carried out over a wide range of pH. Hence attempts were made to immobilize invertase to glutaraldehyde-activated Indion 48-R so as to obtain a highly active and stable immobilized preparation suitable for commercial exploitation.

MATERIALS AND METHODS

Sucrose (Loba-Chemie Indoaustranal, Bombay, India); glutaraldehyde (25% v/v, Fluka AG, Buchs, Switzerland); dinitrosalicylic acid (Riedel-Dehan, Seelze, Germany); agar, peptone, malt extract and yeast extract (Difco, Detroit, MI) and Tween 80 (Sisco Research Laboratories, Bombay, India) were used. Indion 48-R (a crosslinked macroporous polystyrene anion exchange resin) was from Ion-Exchange India, Bombay, India. All other chemicals used were of analytical grade. *Sclerotium rolfsii* (NCIM 1084) was routinely maintained on potato dextrose agar (2% w/v PDA) slants.

Enzyme Production

This was carried out in 500-mL flasks containing 100 mL of the medium (g/L): 2g, KH_2PO_4; 7g, $(NH_4)_2HPO_4$; 3g, urea; 0.3g, $MgSO_4 \cdot 7H_2O$; 0.3g, $CaCl_2 \cdot 2H_2O$; 0.25g, proteose peptone; 0.1g, yeast extract; 0.33g, Tween 80; 10g, sucrose and 1 mL of trace metal solution, adjusted to pH 6.5. Trace metal solution composed of 5mg $FeSO_4 \cdot 7H_2O$, 1.5 mg $MnSO_4 \cdot 7H_2O$, 3.34 mg $ZnSO_4 \cdot 7H_2O$ and 2 mg $CoCl_2 \cdot 6H_2O$, in 1 L of distilled water.

The medium was inoculated with a 7-d-old mycelium and incubated on a rotary shaker (150 rpm) at 30°C for 7 d. The broth obtained after filtration was lyophilized and stored frozen. Before use, the lyophilized broth was extensively dialyzed against 20m*M* sodium acetate buffer, pH 4.5, to remove the low molecular weight impurities and used as the source of enzyme. The specific activity of the crude enzyme which ranged from 5.5–6.0, was used for immobilization studies.

Enzyme Assays

For soluble enzyme activity, the standard reaction mixture contained 1 mL of 500m*M* sucrose and 1 mL of 200m*M* sodium acetate buffer, pH 4.5, preincubated at 50°C and 1 mL of appropriately diluted enzyme. The reaction was initiated by the addition of the enzyme followed by incubation at 50°C for 15 min. After the incubation period, the reaction was terminated by the addition of 1 mL of dinitrosalicylic acid reagent and the reducing sugars were estimated spectrophotometrically, at 540 nm, according to Miller *(4)*.

The immobilized enzyme was assayed in a similar manner by incubating appropriate amounts of the matrix (40–50 mg wet weight) with 3 mL of the standard reaction mixture, at 50°C and pH 4.0, in a thermostated shaker water bath (100 rpm) for 15 min followed by measuring the reducing sugars formed.

One unit of the enzyme is defined as the amount of enzyme required to liberate 1 μmol of reducing sugars/min under the assay conditions.

Determination of Protein

Protein was estimated according to Lowry et al. *(5)*, using bovine serum albumin as standard.

Immobilization Technique

Indion 48-R was regenerated, by alternately washing with 0.5*N* HCl and 0.1*N* NaOH. The regenerated resin (2 g wet weight) was activated by incubation with 10 mL of 2% (v/v) glutaraldehyde, in 25 m*M* sodium bicarbonate buffer, pH 9.5, at 10°C for 3–4 h, under mild agitation. The resin was then washed free of excess glutaraldehyde and used immediately for coupling the enzyme.

In a typical experiment, 30–35 U of crude invertase, in 5 mL of 25m*M* sodium acetate buffer, pH 5.0, was incubated with 1 g (wet weight) of the activated resin, at 10°C for 16 h, under mild agitation. The unbound enzyme and protein was removed by decantation and the matrix was washed successively with the coupling buffer, 1*M* NaCl in coupling buffer and finally with dilute assay buffer (25 m*M* sodium acetate buffer, pH 4.5) till the washings showed no invertase activity. The amount of enzyme and protein bound to the matrix was determined by estimating the difference in the enzyme activity and protein before loading on the matrix and after coupling.

Determination of Efficiency

The efficiency of the immobilized enzyme was determined by assaying appropriate amounts of the immobilized preparation (40–50 mg wet weight), under standard assay conditions, followed by calculating the ratio of measured activity to bound activity. The specific activity of the immobilized preparation is defined as measured activity/mg protein/g matrix.

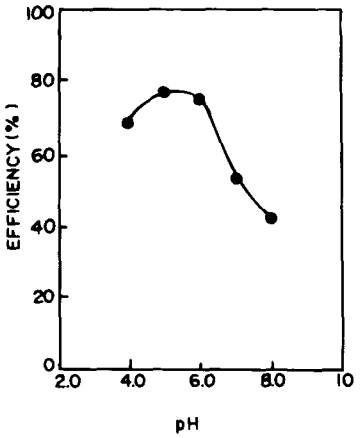

Fig. 1. Effect of coupling pH on the activity of Indion 48-R bound invertase. One gram (wet weight) of 2% glutaraldehyde activated matrix was incubated with approximately 30 U of crude invertase at different pH (4.0–8.0) and 10°C for 16 h and efficiency of the immobilized enzyme was determined as described under Materials and Methods.

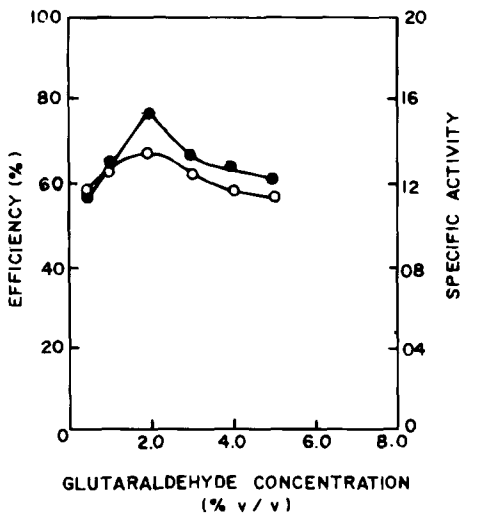

Fig. 2. Influence of glutaraldehyde concentration on the activity of Indion 48-R bound invertase. Efficiency (●) and Specific activity (○). One gram (wet weight) of the matrix was activated with varying concentrations of glutaraldehyde (0.5–5.0% v/v) at pH 9.5 for 3–4 h. Coupling of invertase to the activated matrix was carried out by incubating approximately 21 U of the enzyme with 0.5 g (wet wt.) of the activated matrix at pH 5.0, followed by determination of the efficiency of the bound enzyme.

RESULTS AND DISCUSSION

In the present studies, attempts were made to bind invertase covalently to Indion 48-R, as it is possible to obtain a stable immobilized preparation. Optimization of binding conditions viz. coupling pH, glu-

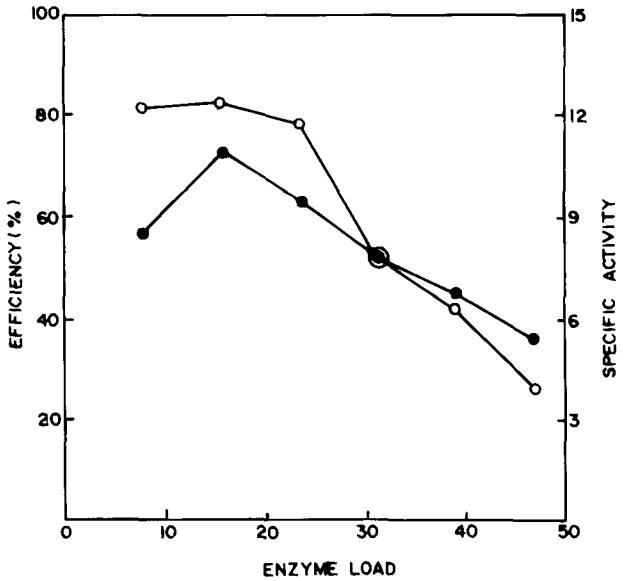

Fig. 3. Effect of enzyme load on the activity of Indion 48-R bound invertase. Efficiency (●) and Specific activity (○). 0.5 gram (wet weight) of 2% (v/v) glutaraldehyde activated matrix was incubated with varying amounts of the enzyme (7.8–39.0 U) at pH 5.0 and 10°C for 16 h and efficiency of the immobilized system was determined.

taraldehyde concentration and matrix to enzyme ratio, showed that the most active preparations are obtained when 1g (wet weight) of 2% (v/v) glutaraldehyde activated resin is incubated with approximately 30U of the enzyme, at pH 5.0 and 10°C, for 16 h (Figs. 1–3). Influence of activation conditions of the matrix revealed that optimum results are obtained when the coupling is carried out with 2% (v/v) glutaraldehyde activated matrix. The decrease in the efficiency of the matrix activated with higher concentrations of glutaraldehyde (>2%) can be due to the overcrowding of the enzyme on the matrix, since there was a decrease in the specific activity of the bound enzyme (Fig. 2). Similarly, studies on matrix to enzyme ratio on the activity of Indion 48-R invertase conjugate showed that after an increase, the efficiency steadily decreased with increase in the enzyme load. The decrease in the efficiency at higher enzyme load can be correlated to overcrowding of the enzyme on the matrix as there was a decrease in the specific activity of the bound enzyme (Fig. 3). When 33 U of crude invertase was reacted with 1 g (wet weight) of 2% (v/v) glutaraldehyde activated Indion 48-R, at pH 5.0 and 10°C for 16 h, 13.48 U were bound. The effectiveness factor (η) of the immobilized preparation was 0.72, indicating the efficiency of the immobilized preparation to be 72% (Table 1). Our efficiency values are higher compared to the values (37–64%) reported by Mansfeld and Schellenberger *(6)* for invertase bound to macroporous polystyrene resin by different methods.

Table 1
Immobilization of Invertase on Glutaraldehyde-Activated Indion 48-R

Enzyme loaded		Enzyme bound		Activity of the complex	Efficiency[a]
U	Protein (mg)	U	Protein (mg)	U expressed	%
33.0	6.0	13.48	1.88	9.72	72

[a]Efficiency = Activity of the complex/Bound activity × 100.

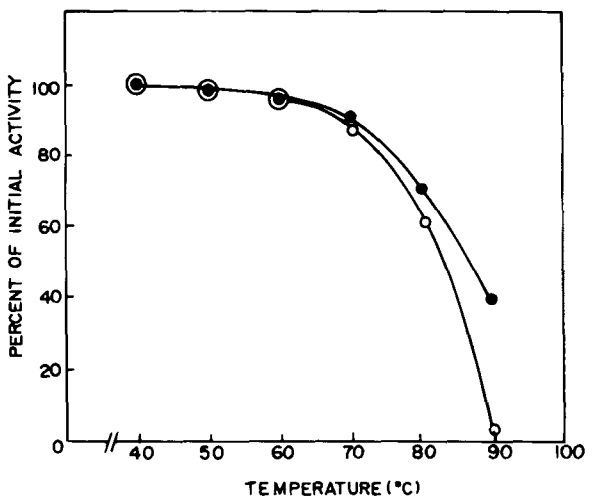

Fig. 4. Temperature stability of soluble (○) and immobilized (●) invertase. Both soluble and immobilized enzymes (0.4–0.6 U) were preincubated at different temperatures (30–90°C) for 1 h at pH 4.5 and their activities were determined under standard assay conditions.

The pH activity profiles of soluble and immobilized enzyme showed a slight shift in the optimum pH towards acid side (pH 4.0) compared to that of the soluble enzyme (pH 4.5). This shift can be correlated to the polycationic nature of the matrix. Immobilization also brought about a decrease in the optimum temperature to 65°C from 75°C for the soluble enzyme. However, immobilization resulted in an increase in the thermal stability of the enzyme, compared to its soluble counterpart, as indicated by its inactivation pattern. While the soluble enzyme lost more than 95% of its activity at 90°C, the bound enzyme retained approx 40% of its activity (Fig. 4). The superior temperature stability of the bound enzyme indicates the rigidity of the enzyme structure in the bound form. The bound enzyme showed high stability to repeated use and retained approximately 90% of its activity after 8 cycles of use (Fig. 5).

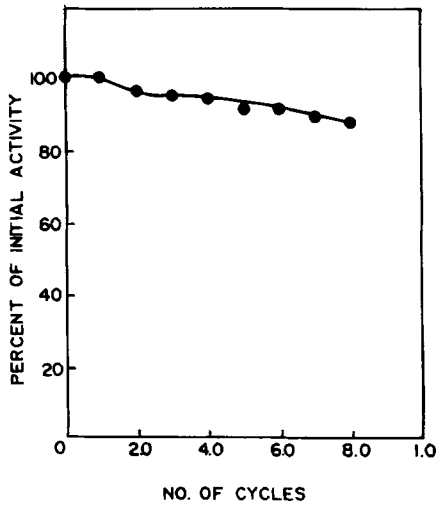

Fig. 5. Effect of number of assay cycles on the activity of immobilized invertase. 0.3 U (50 mg wet wt.) of the immobilized preparation was assayed under standard conditions. After every cycle the immobilized enzyme was washed free of substrate and product and used for the next assay.

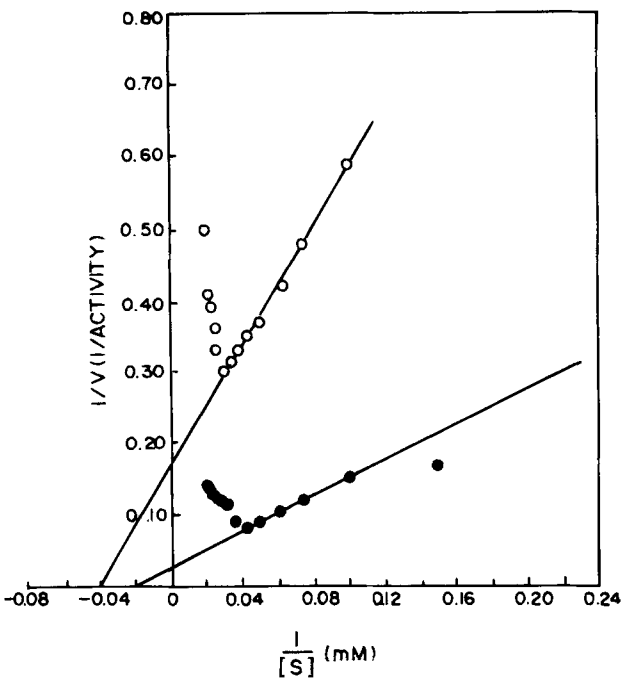

Fig. 6. Lineweaver-Burk plots for soluble (○) and immobilized (●) invertase. Soluble and immobilized enzymes (0.4–0.6 U) were assayed in a series of substrate concentrations (10–130 m*M*) at pH 4.5 and 50°C. K_i was calculated according to Dickensheets et al. (*7*).

Table 2
Kinetic Data of Soluble and Immobilized Invertase

Parameter	Soluble	Immobilized
K_m (m*M*)	25.00	50.00
V_{max} (U/mL; U/g IME[a] (wet weight)	5.88	40.00
K_i (m*M*)	1.70	56.17

[a]Immobilized enzyme

Evaluation of the kinetic parameters of Indion 48-R invertase conjugate showed a two and sevenfold increase in the K_m and V_{max}, respectively. Moreover both soluble and immobilized enzymes showed inhibition at high substrate concentrations (Fig. 6, Table 2). Diffusional restriction has generally been considered undesirable in pratical enzyme applications (as it reduces the efficiency of the immobilized system) but in the present case, it seems beneficial because it helps to reduce inhibition at high substrate concentrations. Increase in the K_m and K_i of the bound enzyme suggests diffusional barriers.

A simple and efficient method has been developed for obtaining a highly active and stable immobilized invertase. The polystyrene matrix used in the present studies is stable, can afford good flow rates and is nonbiodegradable. The high activity and stability of Indion 48-R bound invertase suggests that it has the potential for commercial exploitation.

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