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Research review paper

Immobilized invertase

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ABSTRACT

Invertase is a commercially important enzyme used for the hydrolysis of sucrose. The hydrolysis of sucrose yields an equimolar mixture of glucose and fructose, known as invert syrup, is widely used in food and beverage industries. This enzyme is also used for the manufacture of artificial honey, plasticizing agents used in cosmetics, pharmaceutical and paper industries as well as enzyme electrodes for the detection of sucrose. Immobilization of invertase and its biotechnological applications are reviewed.

Contents

1. Introduction

Use of enzymes as catalysts for large-scale industrial processes is limited by their high cost of production and stabilization on storage. During use, their stability decreases due to changes in pH, temperature, conformational changes as a result of friction, osmotic pressure imposed by the environs of their use and a cumulative effect of all these factors as a function of duration of their use. Secondly, since they are soluble, their recovery from a mixture of substrate and product for reuse is not economically practical rendering the costly enzymatic process even more costly. However, the advent of immobilized enzyme technology has led to increasing efforts to replace conventional enzymatic process with immobilized preparations as immobilization [a] allows the enzymes to process large amounts of substrate since it can be separated easily from the mixture of substrate and product(s) thus enabling the enzyme to be reused [b] in general, imparts greater stability to the enzyme, so that it can be used for the development of continuous process [c] affords greater control of the catalytic process and [d] permits the economical utilization of an otherwise cost-prohibitive enzyme.

Invertase (β-D-fructofuranoside fructohydrolase, β-fructofuranosidase, sucrase, invertin, saccharase; EC 3.2.1.26) which catalyses the hydrolysis of sucrose and related glycosides is one of the simplest commercial carbohydrases. Though they are widespread in distribution (Aleksanyan and Markosyan, 1986), the enzymes of commercial interest originate from strains of *Saccharomyces* sp. Yeast produces a β-fructosidase type of invertase whereas, the fungal invertase is a α-glucosidase. Both invertase and α-glucosidases hydrolyse sucrose but they differ in their mode of action viz. the former cleaves from the fructose end while the latter from the glucose end (Kulp, 1975). The hydrolysis of sucrose which yields an equimolar mixture of glucose and fructose (invert syrup) is sweeter than sucrose due to high degree

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of sweetness of fructose. Consequently the sugar content can be increased considerably without crystallization of the material. Hence, one of the important applications of invertase lies in the production of non-crystallizable sugar syrup from sucrose. Due to its hygroscopic nature, invert syrup is used as a humectant in the manufacture of soft-centered candies and fondants. Invertase is also used whenever sucrose containing substrates are subjected to fermentation viz. production of alcoholic beverages, lactic acid, glycerol etc. Due to the associated inulinase activity, it is also used for the hydrolysis of inulin (poly-fructose) to fructose. Other uses of the enzyme include, manufacture of artificial honey, plasticizing agents used in cosmetics, drug and paper industries and as enzyme electrodes for the detection of sucrose. Enzymatic hydrolysis of sucrose is preferable to acid hydrolysis as it does not result in the formation of undesirable flavoring agents as well as coloured impurities. Additionally, the use of immobilized invertase for the continuous hydrolysis of sucrose can be advantageous because the shifts in the pH brought about as a result of immobilization can be exploited to prevent the formation of oligosaccharides by the transferase activity associated with the soluble enzyme (Wiseman, 1978). In view of the high commercial potential of the enzyme, several attempts have been made to obtain a highly active and stable immobilized preparation suitable for commercial application. Invertase has also been immobilized to study different methods of immobilization and supports, protein structure and mechanism of enzyme action.

2. Matrices

In immobilization studies, the matrix characteristics and the mode of attachment of the enzyme to the matrix play an important role in determining the properties of the bound enzyme. In general, hydrophilic matrices viz. cellulose, Sephadex, Sepharose and their derivatives are extensively used since hydrophilic matrices tend to stabilize the bound enzyme. Invertase has been immobilized on several hydrophilic as well as inorganic supports and polystyrene resins. Compared to hydrophilic matrices, inorganic and polystyrene based matrices offer advantages like, resistance to mechanical compression and microbial attack. The latter characteristics are of significance because of the industrial importance of immobilized invertase.

3. Methods

3.1. Adsorption

Invertase was the first enzyme to be immobilized. In fact, using invertase bound to charcoal or aluminium hydroxide, Nelson and Griffin (1916) demonstrated that enzyme bound to an insoluble support retains its activity. Suzuki et al. (1966) bound yeast invertase to DEAE–cellulose and used the immobilized system to study the continuous hydrolysis of sucrose. Immobilization resulted in a shift in the optimum pH towards acid side and a decrease in the thermal stability. On continuous operation, at pH 3.6, the bound enzyme lost 50% of its initial activity after 10 cycles. Invertase bound to DEAE–cellulose retained approximately 25–30% of its initial activity. While immobilization brought about a decrease in the optimum temperature, no change in the activation energy was observed. The apparent K_m was dependent on the flow rate of the substrate (Usami et al., 1971). Maeda et al. (1973a) immobilized invertase on diethylaminoacetyl cellulose (DEAA–cellulose) but the bound enzyme lost approximately 55–70% of its initial activity. On continuous operation, the DEAA–cellulose–invertase complex exhibited higher stability at 30 °C compared to 40 °C. *Candida utilis* invertase bound to porous cellulose beads by ionic-guanidino bond showed a broad pH optimum between pH 4.0 and 5.4 compared to the soluble enzyme (pH 4.1). Both soluble and immobilized preparations showed inhibition at high substrate concentrations. The K_i for cellulose bound invertase was higher than that of the soluble enzyme suggesting diffusional barriers (Dickensheets et al., 1977). Woodward and Wiseman (1978) immobilized baker's yeast invertase onto microcrystalline cellulose, DEAE- and CM-Sephadex and Con A-agarose and the immobilized preparations exhibited 46%, 41%, 70% and 73% activity respectively, of the soluble enzyme. Moreover, the Con-A and microcrystalline invertase complexes showed greater stability than ionically bound enzyme. Chemical modification of amino and carboxyl groups of the pure enzyme brought about a decrease in its thermal stability compared to the untreated enzyme, indicating that salt linkages may have an important role in maintaining the conformational stability of the enzyme. Hradil and Švec (1981a,b) studied the inversion of sucrose using invertase bound to DEAHP–cellulose (i.e. cellulose containing the weak base *N*,*N*-diethylamino-2-hydroxypropyl groups). The binding was strong and no leaching was observed at an ionic strength of 0.1 M in the pH range of 3–6. Moreover, the amount of enzyme bound was proportional to the porosity and exchange capacity of the matrix. Continuous inversion of sucrose using DEAHP–cellulose–invertase conjugate revealed that substrate concentration, flow rate and reaction temperature affected hydrolysis. The bound enzyme exhibited good operational stability with a half-life of 215 days. Extracellular invertase from *Aspergillus athecius* bound to DEAE–cellulose showed approximately 45% increase in the activity, apart from greater temperature and storage stabilities, compared to the soluble enzyme. Immobilization also brought about a decrease in the apparent K_m (Madyastha et al., 1987). Recombinant invertase (re-INVB) from *Zymomonas mobilis* adsorbed onto totally cinnamoylated derivative of D-sorbitol revealed that parameters like enzyme concentration, coupling and irradiation time influenced the retention of activity. Evaluation of kinetic parameters showed a decrease in the apparent K_m but an increase in the apparent V_{max} and these changes were correlated to a better affinity of the bound enzyme to sucrose as well as a better hydrolytic rate. The good thermal and operational stability of the bound enzyme prompted the authors to suggest that cinnamic carbohydrate esters of D-sorbitol can be a useful support for immobilizing re-INVB and its application for the production of invert syrup (Vallejo-Becerra et al., 2008).

In an attempt to develop a novel method for immobilizing glycozymes, Gómez et al. (2006a) pre-coupled invertase to ionic polysaccharides via its carbohydrate moiety (neoglycoenzymes) and then adsorbed it onto various supports coated with opposite charged polyelectrolyte. Chitosan–invertase neoglycoconjugate adsorbed onto carboxymethyl cellulose(CMC)-modified chitin via electrostatic interaction, retained 68% activity of chitosan–invertase complex. Immobilization brought about 5 °C and 9 °C increase in the optimum temperature and temperature stability, respectively. Additionally, the bound enzyme exhibited high storage and operational stabilities. Subsequently, Gómez et al. (2006b) using the same method bound chitosan–invertase complex to pectin coated chitin support and the bound enzyme showed 97% activity of chitosan–invertase complex. The pectin–invertase adduct exhibited a 10 °C increase in the optimum temperature and temperature stability. In addition, the immobilized preparation showed increased resistance against high ionic strength and temperature apart from good stability to repeated and continuous use. The above authors (Gómez et al., 2006c) adsorbed chitosan modified invertase onto alginate-coated chitin and the bound enzyme retained 97% activity of chitosan–invertase complex. The optimum pH of chitosan modified and chitin bound enzyme preparations shifted towards acid side and this was attributed to the attachment of cationic polyelectrolyte to the enzyme. The bound enzyme also exhibited increased stability against temperature and high salt concentration apart from continuous operation. Furthermore, chitosan modified invertase bound to hyaluronic-acid-coated chitin via polyelectrolyte complex formation showed an efficiency of 80%. Immobilization brought about an increase of 5 °C and 10 °C in the optimum temperature and thermostability, respectively. In addition, bound enzyme exhibited good operational and storage stabilities (Gómez et al., 2008).

Mohmoud (2007) examined the applicability of wood waste as a support for the immobilization of invertase. Among different forms of wood waste viz. wood chips, shavings and sawdust, maximum adsorption

was observed with sawdust. The high capacity of sawdust was explained on the basis of its increased surface area. Additionally, autoclaved wood sawdust exhibited higher capacity than non-autoclaved one and this in turn was correlated to the partial removal of lignin resulting in an increase in the cellulosic surface area. The bound enzyme was stable and retained significant amount of its initial activity (97%) for a week even after washing with 6 M NaCl. Immobilization did not affect the optimum pH and temperature of the enzyme but it enhanced the pH and temperature stability. The bound enzyme was less sensitive to impurities present in molasses. Based on the observations the author concluded that sawdust–invertase adduct has the potential for the production of invert syrup. Yamasaki et al. (1984) developed a simple and safe method for immobilizing invertase on cotton flannel coated with polyethylenimine (PEI). The enzyme, after adsorption onto PEI coated cloth was fixed with glutaraldehyde. The retention of enzyme activity increased with increase in the incubation time and maximum specific activity was observed after 16 h of incubation. Column packed with invertase bound cotton segments exhibited good flow rates and efficient sucrose hydrolysis for 3 months. Yeast invertase adsorbed onto PEI coated cotton threads and then cross-linked with glutaraldehyde could hydrolyse high concentrations of sucrose. The immobilized preparation, when stored in presence of 80% (w/v) sucrose at 50 °C, retained its initial activity for 90 days. Packed bed reactor studies, at pH 4.6 and 50 °C, showed that the bound enzyme could effectively hydrolyse 80% (w/v) sucrose for 3 months without any significant loss in the conversion efficiency (Godbole et al., 1990). Subsequently, D'Souza and Godbole (2002) used invertase bound to PEI coated rice husk to evaluate the applicability of lignocellulosic materials for immobilization of enzymes. Under optimal conditions, the immobilized adduct showed good retention (56%) of activity. Immobilization resulted in a marginal shift in the optimum pH of the enzyme towards acid side (4.5) from that of the free enzyme (5.0) and this was correlated to the basic nature of the matrix. Although immobilization did not affect the optimum temperature of the enzyme (50 °C) it increased the temperature stability. The bound enzyme could be used 12 times without any significant loss in its initial activity. Based on the results the authors opined that this approach can be a good method for the immobilization of enzymes on lignocellulosic matrices.

Yeast invertase adsorbed onto PEI coated Sepabeads showed high efficiency (>95%). The binding was strong and no leaching was observed in presence of high concentrations of NaCl. Despite this, the inactive enzyme could be desorbed and the matrix recharged with fresh enzyme without significant effect on the capacity of the support. It was also noted that desorption of the enzyme from the flexible polymeric matrix led to freezing of some of the oligomeric structure of the enzyme. Interestingly, conditions which favoured oligomerisation of the enzyme viz. certain pH and high enzyme concentration, yielded immobilized adducts with better stability than those which did not favour oligomerisation. The bound enzyme showed good stability to storage and at pH 4.5 and 50 °C retained its initial activity for 15 days (Torres et al., 2002). When native and periodate oxidized invertase were adsorbed onto sepolite (a clay material made up of complex magnesium silicate), the periodate oxidized invertase–sepolite complex exhibited higher activity. Moreover, compared to native invertase–sepolite adduct, the periodate oxidized invertase–sepolite complex showed higher stability and retained 90% of its activity when washed with 1 M NaCl. The firm binding of the latter was attributed to oligomerisation of the enzyme on the surface of the matrix. Furthermore, this explanation was supported by the higher stability of the periodate oxidized enzyme than native invertase. In packed bed reactors, at pH 4.7 and 40 °C, the immobilized preparation could hydrolyse high concentration of sucrose (500 g/dm^3) at a conversion efficiency of 90% and 50%, at a flow rate of 0.1 and 1.0 bed volume per hour, respectively (Prodanović et al., 2003). Mansour and Dawoud (2003) adsorbed invertase on celite and polyacrylamide and the bound enzymes showed 92% and 81% efficiency, respectively. Amongst them, the celite bound enzyme exhibited better stability. Both celite and polyacrylamide bound enzyme preparations showed good pH, temperature, storage and operational stabilities suggesting their industrial potential. In an effort to increase the storage stability and repeated usability, Tümtürk and Tufan (2004) coupled invertase to 1,2-diamine ethane and 1,3-diamine propane activated dimmer acid-*co*-alkyl polyamine and examined its properties. Immobilization did not affect the optimum pH but it increased the optimum temperature to 50 °C from 45 °C for the soluble enzyme. A marginal increase in the apparent K_m and V_{max} of both 1,2-diamine ethane and 1,3-diamine propane–invertase adducts was explained on the basis of either immobilization induced structural changes in the enzyme or to reduced accessibility of the substrate. The bound enzyme exhibited good stability to storage and repeated use and retained 60–70% and 76–80% of its initial activity for 1 month at 4 °C and 43 cycles, respectively.

Iqbal and Saleemuddin (1983) investigated the activity and stability of various glucose oxidase and invertase preparations immobilized on Sepharose containing varying amounts of concanavalin A and noted a progressive decline in the efficiency with increase in the enzyme load. A similar decrease in the efficiency was also observed when the enzyme was bound to Con A-Sepharose containing higher amount of lectin. Compared to the soluble enzyme, all the immobilized preparations (bound either to low or high lectin matrices) exhibited marked stability against inactivation either by temperature or urea. Subsequently, the above authors (Iqbal and Saleemuddin, 1985) bound invertase to Con A-Sepharose and the immobilized preparation retained approximately 70% activity of the soluble enzyme. When the enzyme was treated with 0.2% glutaraldehyde, it lost about 10% activity. Compared to the uncross-linked immobilized preparation, the cross-linked Con A–Sepharose–invertase conjugate exhibited higher stability at 60 °C. Though Con A–Sepharose bound invertase showed higher stability in presence of urea, cross-linking imparted only a marginal increase in the stability. The immobilized preparation showed good operational stability and could hydrolyse relatively high concentrations of sucrose almost quantitatively for more than 60 days. Jafri et al. (1993) noted that, antisera of baker's yeast invertase could enhance the activity of the enzyme in vitro. Invertase immobilized either as insoluble antibody complex or bound to gamma globulin–Sepharose exhibited high efficiency in addition to high thermal stability and this in turn could be improved by glutaraldehyde cross-linking. Husain and Jafri (1995) bound invertase and horseradish peroxidase (HRP) to Con A–Serelose via their carbohydrate moiety and then cross-linked the lectin both intra- and inter-molecularly by treating with glutaraldehyde. Attempts were also made to introduce covalent linkages with intermolecular cross-linking. Both invertase and HRP immobilized preparations showed high coupling yield and efficiency apart from increased thermal stability. Ahmed et al. (2001) described a novel and inexpensive method for the immobilization of glycoenzymes with high mannose and glucose content viz. invertase, glucoamylase, glucose oxidase and cellulase on *Cajanus cajan* lectin (CCL)–Seralose 4B. All the immobilized preparations, with the exception of cellulase, exhibited high retention of activity. When the immobilized invertase was stabilized by cross-linking with glutaraldehyde, both cross-linked and uncross-linked preparations were resistant to inactivation against pH, temperature, denaturants and proteases. The bound enzyme showed good storage stability and retained a significant amount of its initial activity (>80%) after 60 days at 4 °C. Melo and D'Souza (2000) developed a simple procedure for the simultaneous isolation and immobilization of yeast invertase where, the crude yeast extract with invertase activity was mixed with Jack bean extract and the precipitate containing the adsorbed enzyme was cross-linked with glutaraldehyde to yield the immobilized preparation. The bound enzyme retained more than 60% activity of the soluble enzyme. Immobilization did not affect optimum pH (4.5) of the enzyme but it increased the optimum temperature to 65 °C from 55 °C for the soluble enzyme. Invertase from a thermophilic fungus viz. *Thermomyces lanuginosus* adsorbed onto phenyl–Sepharose, showed a marginal decrease in the optimum pH (6.0) but a significant decrease in the optimum temperature (40 °C) from that of the soluble enzyme (pH 6.5, 50 °C). However, the bound enzyme exhibited high thermal stability

and at 50 °C, retained more than 80% of its initial activity for 12 h. Additionally, the immobilized preparation showed increased resistance against metal ions. Compared to sucrose, both soluble and phenyl–Sepharose bound enzymes showed higher affinity towards raffinose. Based on this observation the authors suggested that the firm binding of the enzyme to phenyl–Sepharose is due to the presence of nonsulphated galactose on the matrix. This explanation was based on weak binding of the enzyme to phenyl–agarose which contains sulphated galactose. The above observations led the authors to conclude that the higher affinity of the enzyme for phenyl–Sepharose is probably due to hydrophobic interaction induced affinity (induced fit) towards galactose residues. Furthermore, the inability of yeast invertase to bind to phenyl–Sepharose coupled with the firm binding of *T. lanuginosus* enzyme to the matrix was correlated to high hydrophobic nature of *T. lanuginosus* invertase (Basha and Palanivelu, 2000). Yavuz et al. (2004) used Con A bound to poly(2-hydroxyethyl methacrylate) (PHEMA) beads to immobilize yeast invertase via its carbohydrate moiety. The PHEMA–Con A matrix showed high adsorption capacity and could bind 107 mg of the enzyme per gram of the matrix at pH 5.0. Immobilization resulted in an increase in the K_m pointing towards diffusional barriers. The bound enzyme exhibited good stability to pH, temperature and repeated use.

Boudrant and Cheftel (1975) immobilized invertase on several polystyrene cation and anion exchange resins and amongst them macroreticular resins yielded higher activity (32–53%) compared to gel type resins (13–28%). Efficiency of binding on different macroreticular type resins viz. IRA 93, IRA 900 and IRA 200 showed that while 83% and 87% of the enzyme leached out from IRA 200 and IRA 900 respectively, on washing with 10 mM acetate buffer pH 4.0, only 42% desorption was observed with IRA 93. Moreover, salt concentration had a greater influence on desorption than pH. Continuous hydrolysis of industrial sucrose solution (50% w/v), at pH 3.0 or 4.0 and 30 °C, revealed the bound enzyme to be stable for a month. Kobayashi and Moo-Young (1973) coupled invertase to polyamine type of ion exchange resin and investigated the kinetic behaviour of the enzyme bound to small pellets, in a packed bed reactor. At low flow rates, an effect of intra-particle diffusion on apparent K_m was observed. A new polystyrene based ion-exchange fiber (IONEX) having a large surface area per unit weight was tested for its ability to adsorb and immobilize biologically active proteins. Strong cation IONEX could effectively adsorb haemoglobin and albumin while invertase and glucose isomerase were readily adsorbed onto a strong anion IONEX. Moreover, the adsorption capacity of proteins enhanced significantly with an increase in the water-holding capacity of the fiber. Invertase bound to strong anion IONEX retained 40–50% activity of the soluble enzyme (Yoshioka and Shimamura, 1986). Invertase bound to weakly basic acrylic anion exchangers through ionic as well as covalent (i.e. secondary cross-linking of the ionically bound enzyme) linkages retained approximately 92% and 85% activity respectively, of the soluble enzyme. Simulated studies revealed the bound enzymes have good operational stability (Maxim et al., 1988). Tomotani and Vitolo (2004, 2006, 2007) studied the utility of Dowex® anion exchangers of varying mesh size (50–400) and crosslinking (1–8%) for the immobilization of invertase and noted that the maximum retention of activity (100%) is exhibited by Dowex® 1X4–200-invertase complex (DW1X4–200I). Characterization of DW1X4–200I showed a significant decrease (10 °C) in the optimum temperature as a result of immobilization and this was correlated to the requirement of less energy for the reaction. This observation prompted the authors to suggest that the less energy requirement coupled with the reusability of the immobilized system can lead to an overall reduction in the cost of the process. Immobilization brought about a marginal shift in the optimum pH towards acid side (4.5) from that of the soluble enzyme (5.0). Evaluation of the kinetic parameters of soluble and DW1X4–200I showed a marginal increase in the apparent K_m and a significant (47%) decrease in the apparent V_{max}. The comparable K_m values of both soluble and bound enzymes were correlated to the lack of substantial changes in enzyme–substrate interactions. On continuous operation (pH 5.5 and 30 °C at a dilution rate of 1.6 h^{-1}), on a membrane reactor fitted with either an ultrafiltration membrane (100 kDa cut off) or a microfiltration membrane with a pore diameter of 5 μm, DW1X4–200I hydrolysed 2.5 mM sucrose with a yield of 84% and 95% respectively and the bound enzyme was stable. The invert syrup, when chromatographed on Dowex-50, yielded 70% high fructose syrup suggesting the potential of the immobilized preparation for the production of high fructose syrup.

Invertase bound to poly(ethylene-vinyl alcohol) membrane surface modified with two aminoacetals of different chain length viz. 2-dimethyl-aminoacetoaldehyde dimethylacetal (AAA) and 3-(*N*,*N*-dimethylamino-*n*-propanediamine) propionaldehyde dimethylacetal (APA) exhibited higher thermal stability. Evaluation of Michaelis–Menten constant (K_m) revealed that the apparent K_m of the enzyme bound to APA with longer chain lengths was less than that of the AAA bound enzyme (Imai et al., 1986). Soluble complexes of yeast invertase bound to bovine serum albumin via glutaraldehyde retained approximately 50% activity of the free enzyme. When the co-reticulated enzyme preparation was adsorbed on the active surface of an ultrafiltration membrane, the K_m of the immobilized adduct was comparable to that of the soluble enzyme. In the opinion of the authors, this co-reticulated immobilized invertase can serve as a model to study the kinetic behaviour of a cofactor dependent enzyme or an enzyme in presence of inhibitors (Cantarella et al., 1977).

Parascandola et al. (1982) immobilized yeast cells with invertase activity on tuff granules with gelatin and compared it with the corresponding unsupported gelatin immobilized whole cells. Except for differences in the activity yields, no change in the kinetic properties was observed. Yeast cells immobilized on gelatin coated tuff granules adhered strongly as a monolayer on the surface of the support. From a commercial point of view, this system offered advantages like good operational stability and in addition, the proliferation of the cells on the matrix could be controlled (Parascandola et al., 1987). Yeast cells, when adsorbed onto glass surface using PEI for coating cells or glass or both, the PEI coated cells adhered strongly as a monolayer and were viable. The immobilized preparation was stable and the cells did not leach out under extreme conditions of pH, ionic strength and repeated use (D'Souza et al., 1986) Subsequently, Melo et al. (1992) adsorbed yeast cells onto PEI coated waste cotton threads at pH 4.5 and 45 °C and the immobilized system showed high stability and no leaching was observed with changes in temperature, ionic strength or in presence of substrate. In packed bed reactors (pH 4.5 and 45 °C), the immobilized cells could hydrolyse high concentration of sucrose (60% w/v) at a conversion rate of 75% for more than a month. Comparison of the conversion efficiency of the cotton bound yeast cells with that of Ca^{2+}-alginate entrapped and free cells revealed that while cotton adsorbed cells and free cells showed 75% hydrolysis, the Ca^{2+}-alginate entrapped cells could hydrolyse only 37% of the sucrose. The simple immobilization technique coupled with high stability and conversion efficiency of the immobilized system along with the non-toxic nature of the support and PEI led the authors to conclude that PEI-cotton thread-yeast cell system has the potential for the large scale production of invert syrup. A thermotolerant yeast namely, *Kluyveromyces marxianus* with invertase activity, when bound to glass wool reinforced silica aerogel, showed good stability to repeated use. Moreover, scanning electron microscopic studies on the immobilized system revealed the presence of growing and budding cells on the matrix. The ability of the cells to grow was attributed to the porosity of the matrix. Based on the observations, the authors suggested that the inert nature of the matrix offers advantages like resistance to degradation due to friction and microbial attack (Karandikar et al., 2006).

3.2. Covalent binding

Mason and Weetal (1972) coupled invertase to porous glass beads and examined the properties of the immobilized preparation. No change in

the apparent K_m was observed probably suggesting the absence of diffusional barriers. On continuous operation, the immobilized invertase showed a half-life of over 40 days. Invertase bound to glutaraldehyde activated aminoalkylsilylated magnetite (Fe_3O_4) showed very low activity due to inactivation of the enzyme. However, approximately 79% of the initial activity could be recovered when the enzyme was immobilized in presence of 1% sucrose. Compared to the soluble enzyme, the bound enzyme showed less activity towards oligosaccharides of raffinose family. The immobilized preparation could be recovered magnetically from the reaction mixture (Van Leemputten and Horisberger, 1974). Thornton et al. (1975) examined the suitability of hornblende (ferromagnesiumsilicate) as a support for immobilizing invertase with respect to physical stability of the support apart from thermal and operational stability of the bound enzyme. Hornblende bound invertase exhibited higher stability during long-term operation compared to the enzyme bound to porous glass. Yeast cells with invertase activity bound to alkylamine and aldehyde derivatives of titanium (Ti) activated porous silica exhibited inferior operational stability compared to the preparations obtained by simple metal link method. However, higher activity was observed when the cells were bound to Ti activated pumice stone and it was comparable to Ca^{2+}-alginate entrapped cells (Dias et al., 1982). Hu et al. (1985) studied the diffusion and adsorption phenomena in an immobilized enzyme reactor using invertase bound covalently to porous alumina containing an adsorbed polymer. Evaluation of the long term stability of the immobilized preparation showed a 14% decrease in the activity after 22 days. Adsorption of glucose, fructose or sucrose on porous alumina was greatly influenced by the presence of enzyme on the polymer matrix and also by the polymer alone (i.e. without enzyme). Sugar cane invertase adsorbed onto bentonite clay (BI) retained 55.5% activity of the soluble enzyme. However, when the enzyme was covalently linked to bentonite using cyanuric chloride (BCCI) and thionyl chloride (BTCI), a further 17% and 22% increase in the activity was observed. When BI, BCCI, BTCI were used for the hydrolysis of sucrose in batch process, they showed 53.1%, 57.4% and 59.6% conversions respectively in 12 h compared to 42.3% conversion in 24 h with the soluble enzyme (Tomar and Prabhu, 1985). Findlay et al. (1986) assessed the utility of poultry bone residue for the immobilization of enzymes since it is cheap, non-toxic, readily available, porous, mechanically strong and can be derivatised. Invertase covalently coupled to bone by acyl-azide method showed higher retention of activity (38 U/g) compared to the enzyme bound via glutaraldehyde (4.4 U/g) and carbodiimide (1.0 U/g). Rosa et al. (2000) compared the properties of invertase bound to aminopropyl silica (APTS-SiO_2) activated with humic substances (a high molecular weight complex mixture of heterogeneous substances having a number of oxygen containing functional groups; APTS-SiO_2-HS) with glutaraldehyde activated aminopropyl silica (APTS-SiO_2-GA) and noted that APTS-SiO_2-HS showed higher enzyme load. However, the retention of activity in both cases was fairly similar. Based on the results the authors concluded that activation of aminopropyl silica with humic substances offers a viable method for the immobilization of enzymes. Invertase bound to controlled pore glass via silane glutaraldehyde method showed low retention of activity (24%). The immobilized preparation was not affected by diffusion limitations, as evidenced by close activation energies of the free and bound enzymes. It was also observed that the experimentally obtained activation energy values are a complex function of pH (Bergamasco et al., 2000). Subsequently, Bassetti et al. (2000) examined the thermostability and deactivation energy of free and controlled pore silica-bound invertase at varying temperatures (i.e. 35 °C–65 °C) during the hydrolysis of 5% (w/v) sucrose solution and noted that while the soluble enzyme was stable up to 4 h at pH 5.0 and 50 °C, the bound enzyme was stable for the same period at pH 4.5 and 55 °C. Moreover, the immobilized preparation was more stable than the free enzyme with deactivation energies of 83.1 kcal./mol and 72.0 kcal/mol, respectively. However, the immobilization-induced stabilization of the enzyme was more at lower temperatures. Sabularse et al. (2005) immobilized invertase on white and black lahar (volcanic mudflow) by silane-glutarldehyde method and examined the properties of the enzyme bound to white lahar. The maximum activity of the immobilized preparation was observed at pH 3.5 and 40 °C, when assayed in presence of 300 mM sucrose. Immobilization brought about an increase in the K_m but a decrease in the V_{max} and these changes were correlated to immobilization induced conformational changes in the enzyme.

Sanjay and Sugunan (2005, 2006a,b) bound invertase to acid activated montmorillonite K10 by adsorption and covalent linkage and the immobilized adducts retained 29% and 36% activity respectively, of the soluble enzyme. The immobilized preparations exhibited good stability to repeated use and retained 85% of its initial activity after 25 cycles. Subsequently, the above authors (Sanjay and Sugunan, 2006a,b) characterized the immobilized adducts by various physical techniques. The XRD pattern, apart from showing the involvement of amino acid side chains in intercalation, revealed that the entire polypeptide backbone is located on the periphery of the matrix. NMR, on the other hand, indicated that while tetrahedral Al species are involved in adsorption, octahedral species are involved in covalent binding. Moreover, N_2 adsorption studies revealed that covalent binding of the enzyme leads to blocking of pores on the matrix as the protein is located on the surface of the matrix. In packed bed reactors, both adsorbed and covalently bound invertase showed 59% and 77% efficiency. Immobilization brought about an increase in the K_m and V_{max} and this was correlated to diffusional barriers. Among adsorbed and covalently bound enzyme preparations, latter exhibited superior storage and operational stabilities. David et al. (2006) bound yeast invertase on amino-chemically surface modified silica gel (N-CSMG), obtained by the co-condensation of silica sol with 3-aminopropyl(triethoxy)silane via glutaraldehyde (GA-N-CSMG) and used it as a model to study the characteristics of GA-N-CSMG. Interestingly, the modified matrix exhibited high binding capacity (~99%) with no loss of catalytic efficiency. Immobilization brought about a decrease in the optimum pH (4.0) from that of the soluble enzyme (5.0) and this was attributed to the basic nature of the functionalized silica surface. On the contrary, immobilization did not affect the optimum temperature of the enzyme. Immobilization brought about a decrease in the K_m indicating higher substrate affinity. The bound invertase showed good storage stability and could effectively hydrolyse sucrose. Based on the results the authors opined that, ease of fabrication of the silica gel with desirable morphology, pore-structure, micro-channels permitting substrate-ligand interaction coupled with the mechanical stability and chemically inert nature of the matrix makes N-CSMG a promising support for industrial applications.

Filippusson and Hornby (1970) bound invertase onto the surface of polystyrene beads and tubes by diazo method and observed a 2-fold increase in the apparent K_m in both cases. The pH activity curve of the bound enzyme was narrow without any change in the optimum pH and this was correlated to the hydrophobic nature of the support. Onyezili and Onitiri (1981) bound invertase onto *O*-alkylated nylon tubes modified with various amines via glutaraldehyde and noted that the immobilized adducts obtained with 1,2-diaminoethane or diaminohydroxypropane treated matrix showed maximum efficiency. It was also noted that the efficiency of the bound enzyme was dependent on amination of the matrix prior to coupling since a significant non-specific binding of the enzyme occurred in the absence of glutaraldehyde. Evaluation of the activation conditions revealed that, though increase in glutaraldehyde concentration brought about an increase in the enzyme load it also resulted in a decrease in the specific activity of the immobilized preparation. In addition, prolonged activation time gave comparable results. The decrease in the specific activity of the bound enzyme, under the above conditions, was correlated to over crowding of the enzyme on the matrix. Ooshima et al. (1980a,b) immobilized invertase onto porous glass and ion exchange resins and noted that while the enzyme bound ionically to IRA-94 (anion exchanger) and covalently to porous glass showed high activity, the enzyme bound covalently to IRC-50 (cation exchanger) was inactive. Kinetic analysis of the thermal stability of native, porous

glass and IRA-94 bound enzymes revealed that the thermal inactivation of all invertases obeyed first-order kinetics and is independent of substrate concentration. Marek et al. (1984) coupled invertase to three types of modified supports namely, glycidylmethacrylate, styrene–divinylbenzene copolymers and cellulose beads via aldehyde groups generated after selective periodate oxidation of the carbohydrate moiety, with amino groups on the support using modified Ugi reaction. Influence of various parameters viz. buffer, oxidation conditions and spacer length revealed that optimum results are obtained when appropriately oxidized enzyme is reacted with styrene–divinylbenzene copolymer having free amino groups. Invertase immobilized on macroporous polystyrene anion exchanger via benzoquinone and glutaraldehyde showed approximately 37–64% activity of the soluble enzyme. Determination of the kinetic parameters showed 1.4–5.7 fold increase in the apparent K_m. Since particle size and enzyme load on the matrix influenced the magnitude of K_m values, increase in the apparent K_m was attributed to internal diffusion. Packed bed reactor studies, using low and high concentrations sucrose, suggested that high specific and relative activities of the polystyrene–invertase adduct as well as the good hydrodynamic and mechanical properties of the polystyrene matrix is suitable for commercial exploitation (Mansfeld and Schellenberger, 1987). Mutlu et al. (1996) coated the external surface of polystyrene beads with a copolymer of phenylalanine and lysine [poly (phe-lys)] and bound invertase via glutaraldehyde. Optimization of coupling conditions revealed that maximum yield (24%) was obtained when 0.01% (w/v) poly(phe-lys) coated beads activated with 2% (v/v) glutaraldehyde is reacted with the enzyme at pH 4.5 and 25 °C. Immobilization induced marginal changes in the K_m and V_{max} and this was explained on the basis of efficient mass transfer of the substrate to the surface of the matrix and to minor conformational changes in the enzyme. Based on the results the authors concluded that the use of poly (phe-lys) coated polystyrene beads offer advantages like, simple coupling technique, higher operational stability and the use of a natural material as surface modifying agent. Crude extracellular invertase from *Sclerotium rolfsii* coupled to glutaraldehyde activated Indion 48-R (a cross-linked macroporous polystyrene anion exchanger) retained 70–80% activity of the soluble enzyme. Immobilization brought about marginal shift in the optimum pH towards acid side (pH 4.0) and a decrease in the optimum temperature (65 °C) compared to that of the soluble enzyme (pH 4.5, 75 °C). The immobilized invertase exhibited superior pH and temperature stabilities. Evaluation of kinetic parameters showed approximately 2- and 7-fold increase in the apparent K_m and V_{max} respectively, suggesting the absence of significant diffusional barriers. Both soluble and immobilized enzymes showed inhibition at high substrate concentrations (Kotwal and Shankar, 1997). Bahar and Tuncel (2002) examined the binding conditions as well as batch reactor performance of invertase bound to polyethylenimine coated poly(*p*-chloromethylstyrene) (PCMS) beads via glutaraldehyde. Maximum retention of activity was obtained when 2 mg of the protein was reacted with 1 g of 2.5% (v/v) glutaraldehyde activated PCMS at pH 4.7 and 4 °C. Although immobilization did not affect the optimum pH of the enzyme, it brought about a wider pH activity curve and this in turn was attributed to its better pH stability. On the contrary, immobilization resulted in an increase in the optimum temperature to 65 °C from 55 °C for the soluble enzyme and this was correlated to improved thermal stability of the enzyme. Evaluation of the kinetic parameters showed an increase in the apparent K_m accompanied by a decrease in the apparent V_{max} and these changes were explained on the basis of either immobilization induced conformational changes in the enzyme or to its deactivation. The PCMS-invertase adduct exhibited good stability to repeated use and retained approximately 80% of its initial activity after 25 cycles. Altinok et al. (2008) bound invertase to poly(styrene-2-hydroxyethyl methacrylate) [P(S-HEMA)] microbeads activated with epichlorohydrin. Immobilization brought about an increase in the pH and temperature optima to 5.5 and 55 °C from that of the soluble enzyme (pH 4.5 and 45 °C). The bound enzyme also exhibited better stability to storage and repeated use.

In an attempt to increase ethanol production by *Z. mobilis* using sucrose, Lee and Huang (1995) added polyacrylamide bound invertase to the fermentation broth and noted that a rapid accumulation of glucose in the medium inhibited levan formation and enhanced the growth rate during the initial stages of fermentation. The use of immobilized invertase also reduced sorbitol formation due to lowering of glucose and fructose levels. In the opinion of the authors, the combination of microb-immobilized invertase is advantageous because it eliminates the costly process of sucrose hydrolysis. Chiang et al. (1997) bound purified β-fructofuranosidases from *Aspergillus niger* ATCC 20611 and *Aspergillus japonicus* TIT-KJ1 on oxriane containing methacrylamide-based polymeric beads. Immobilization did not alter the optimum temperature of the soluble enzyme (60 °C) for the transglycosylation reaction but it increased the apparent K_m. The immobilized preparation could synthesize fructooligosaccharides from high concentration of sucrose (50% w/w) at a conversion efficiency of 60%, suggesting its commercial potential. Chen et al. (2000) covalently coupled invertase to emeraldine (EM) base form of polyaniline (PAN) films and powders with surface-grafted acrylic acid(AAc) polymer. X-ray photoelectron spectroscopic studies on the surface structure and composition of the grafted polymer with and without the bound enzyme showed that the enzyme load increased linearly with the concentration of the surfaced-grafted AAc polymer chains. However, the enzyme could be bound more efficiently to surface-grafted EM powders. The low efficiency (20–40%) of the EM powder bound invertase was attributed to reduced accessibility of the substrate and to immobilization induced conformational changes in the enzyme. Immobilization did not affect the optimum pH of the enzyme but it increased the optimum temperature. The bound enzyme exhibited good temperature and storage stability. The increase in the thermal stability was correlated to the reduction in its denaturation as a result of covalent binding. Invertase immobilized via its carbohydrate moiety to macroporus glycidyl methacrylate showed 45% efficiency. Immobilization did not significantly alter the optimum pH of the enzyme but it slightly increased the optimum temperature. The marginal increase in the K_m (~1.6 fold), on immobilization, was attributed to diffusion restrictions. In packed bed reactors, at 40 °C, the bound enzyme could hydrolyse 2.5 M sucrose with a specific productivity of 3 kg l^{-1} h^{-1} (Prodanović et al., 2001). Mateo et al. (2003) bound β-galatosidases, invertase, glucoamylase, lipase and glutaryl acylase to a new commercially available heterofunctional support viz. amino-epoxy-Sepabeads and compared it with these enzymes bound to conventional epoxy supports and noted that the enzymes bound to heterofunctional matrix showed high retention of activity and stability. Interestingly, *Aspergillus oryzae* β-galatosidase and *Candida rugosa* lipase lost significant amount of their initial activity when bound to conventional supports and this was correlated to the orientation of the bound enzyme or to the nature of the support. Moreover, the enzymes bound to amino-epoxy-Sepabeads exhibited better stability than those bound to conventional epoxy supports. Extracellular invertase from *Saccharomyces cerevisiae* bound to glutaraldehyde activated nylon-6 beads showed high retention activity (93%) when the coupling was carried out at pH 5.0. Immobilization did not alter the optimum pH of the enzyme but it increased the optimum temperature as well as temperature stability. Kinetic parameters of the free and nylon-6 bound enzyme showed marginal increase in the apparent K_m, V_{max} and K_i values. This observation coupled with high efficiency of the immobilized system indicated the absence of significant diffusional barriers. In a tubular fixed bed reactor, the immobilized system demonstrated very good productivity up to 2.0 M sucrose with conversion factors of 0.95 and 0.97 and this in turn was dependent on the concentration of sucrose in the feed. Based on these observations the authors concluded that this method can be adopted for the production of fructose rich syrup (Amaya-Delgado et al., 2006).

Uedaira et al. (1984) studied the effect of immobilization in photocrosslinked poly(vinylalcohol) (PVA) polymer on the thermal stability of invertase and observed an increase in the denaturation temperature of the enzyme. The stabilizing effect was correlated to high

concentration of the enzyme in the PVA films. de Queiroz et al. (1996) prepared a graft copolymer of poly(ethylene-g-acrylic acid) (LDPE-g-AA) by radiation induced graft copolymerization of acrylic acid onto low density polyethylene (LDPE) pallets. Characterization of the graft polymer by infrared photoacoustic spectroscopy and scanning electron microscopy indicated the presence of grafted poly(acrylic acid) (PAA). Invertase bound covalently to this grafted polymer retained 50% activity of the soluble enzyme. The comparatively low efficiency of the bound enzyme was correlated to the interaction of the active-site residues with the functional groups on the support. Immobilization did not affect the optimum pH of the enzyme but it increased its temperature stability. Immobilization also brought about an increase in the K_m and this was attributed to steric hindrance. Invertase bound to poly(2-hydroxyethyl methacrylate-glycidyl methacrylate) (pHEMA-GMA) membrane via epoxy groups of glycidyl methacrylate and amino groups of the enzyme retained 60% activity of the soluble enzyme. Immobilization resulted in an increase in the K_m but a decrease in the V_{max} and these changes were attributed to diffusion restrictions. Additionally, the bound enzyme exhibited superior pH, temperature and storage stabilities (Danisman et al., 2004). Mazi et al. (2006)synthesized poly(maleic anhydride-*alt*-hexen-1)poly(MA-*alt*-H-1)by radical polymerization and characterized it by different physical methods. The data obtained suggested that it is an alternating co-polymer and its composition is independent of the monomer composition. Invertase coupled to glutaraldehyde activated poly(MA-*alt*-H-1) membrane exhibited higher pH and temperature stability. Immobilization brought about an increase in the K_m but a decrease in the V_{max}. The increase in the apparent K_m was attributed to limited accessibility of the substrate due to spatial distribution of the enzyme on the polymer as well as immobilization induced conformational changes of the enzyme while the decrease in the V_{max} was correlated to diffusional barriers. The immobilized preparation exhibited good stability to repeated use and retained 66% of its initial activity after 15 cycles.

Kennedy et al. (1984) developed a simple procedure for the immobilization of enzymes on glutaraldehyde cross-linked gelatin beads where, glutaraldehyde was used as a cross-linking agent as well as an enzyme coupling agent. By this method, duel conjugates of amyloglucosidase and invertase were prepared by entrapment of one enzyme and surface binding of the other. This method offers a suitable procedure for the co-immobilization of enzymes acting on high and low molecular weight substrates. By immobilizing the enzyme acting on high molecular weight substrates on the surface of the beads, it is possible to avoid diffusional barriers. Invertase bound on the surface of glutaraldehyde cross-linked gelatin matrix retained 23.8% activity of the soluble enzyme. Monsan and Combes (1984) compared the efficiency of corn grits bound invertase for the inversion of concentrated sucrose solutions in a stirred-tank as well as plug-flow reactors and noted the latter to be more efficient. Continuous inversion of sucrose could be carried out efficiently in 0.1 and 1.0-L tubular reactors and then scaled up to 17.6-L pilot reactor for the production of invert syrup from highly concentrated sucrose solutions. Subsequently, Monsan et al. (1984) standardized the coupling conditions viz. oxidation, amination, reduction and activation for the covalent coupling of invertase to corn grits. Invertase bound to the chemically modified support exhibited 25 times higher activity than the enzyme bound to porous silica. Corn grits bound invertase showed high operational stability with a half-life of 365 days at 40 °C in presence of 2 M sucrose. The immobilization procedure could be scaled up for obtaining large amounts of the immobilized preparation. Invertase coupled to acylchitosan gel through glutaraldehyde, at pH 5.2, showed 37.7% activity of the soluble enzyme. Immobilization resulted in a shift in the optimum pH towards acid side in addition to an increase in the optimum temperature. On continuous operation, the immobilized preparation did not show any significant loss of activity after 20 days (Kusaoke et al., 1987). Simionescu et al. (1984, 1987) bound invertase onto CM-cellulose acid chloride and diazotized 4-aminobenzoylcellulose and noted that the binding is influenced by enzyme to substrate ratio and enzyme concentration respectively, apart from reaction time and pH of the reaction medium. In both cases, immobilization brought about an increase in the K_m suggesting diffusion restrictions. Štefuca et al. (1988) coupled invertase to porous cellulose by four different methods viz. diazo, hydrazide, chlorotriazine and adsorption and selected chlorotriazinylcellulose–invertase conjugate for reactor studies. Stirred tank reactor studies showed that due to localization of the enzyme on the external surface of the matrix, external diffusion did not exert any influence on the reaction kinetics. Husain et al. (1996) modified the amino groups and carbohydrate moiety of yeast invertase and noted that periodate followed by ethylenediamine treatment led to a significant loss of the carbohydrate moiety (~40%) with a marginal loss of enzyme activity. On the contrary, 2,4,6-trinitrobenzene sulfonic acid (TNBS) treatment resulted in a significant loss of activity (~60%) suggesting the involvement of lysine in the catalytic activity of the enzyme. The modified and unmodified enzymes were bound to CNBr-actvated Sepharose and evaluated for their resistance to temperature and urea induced denaturation, with a view to understand the effect of the nature of immobilization procedures on these processes. Amongst the immobilized preparations viz. native enzyme bound to Sepharose exclusively via side-chain amino groups (Sp-INV), periodate and ethanolamine treated enzyme bound to Sepharose via amino acid side-chain amino groups (Sp-PEA-INV), periodate oxidized and ethylenediamine treated enzyme coupled to Sepharose through both side-chain amino groups as well as amino groups incorporated in the glycosyl residues (Sp-PEDA-INV) and pretreatment of the enzyme with TNBS (to block the amino groups) followed by periodate and ethylenediamine treatment bound to Sepharose exclusively via amino groups incorporated onto the glycosyl residues (Sp-PEDA-TNBS-INV), Sp-PEDA-INV adduct showed maximum efficiency (96%) apart from high thermal and storage stabilities. On the contrary, Sp-INV exhibited high stability against urea. Based on the observations, the authors opined that additional stability against various denaturants is due to structural stability of the enzyme as a result of multipoint attachment to the support. Hsieh et al. (2000) coupled invertase via its carbohydrate moiety to chitosan and the bound enzyme, apart from showing high efficiency (91%), exhibited enhanced temperature stability at 60 °C, with a half-life of 7.2 h. On the contrary, invertase bound either to Sepharose or chitosan via its protein moiety showed less thermostability. Additionally, both free and the bound enzymes showed inhibition at high substrate concentrations. Based on these observations the authors concluded that the retention of activity, thermal stability and catalytic properties of the bound enzyme depends on the coupling method as well as on the nature of attachment to the support. *S. cerevisiae* invertase, after periodate oxidation and ethylenediamine or sodium borohydride treatment, when bound to carboxymethylcellulose using carbodiimide, retained approximately 56% activity of the soluble enzyme. Immobilization brought about an increase in the pH and temperature stability apart from increased resistance to 6 M urea (Ramírez et al., 2002). Cheng et al. (2005) bound partly purified β-fructofuranosidase from *Aspergillius japonicus* to chitosan beads either via glutaraldehyde or tris(hydroxymethyl) phosphine (THP) and noted that the latter showed higher stability against temperature and repeated use. However, the THP-invertase adduct produced less fructooligosaccharides (48%) compared to that of the soluble enzyme (58%) from 50% (w/v) sucrose at 50 °C and this was correlated to diffusional barriers.

3.3. Entrapment

Usami and Kuratu (1973) entrapped invertase in polyacrylamide gel and the bound enzyme retained 8–10% activity of the soluble enzyme. Immobilization did not bring about any changes in the pH and temperature optima but it increased the thermal stability of the enzyme. Nakagawa et al. (1975) compared the properties of tomato invertase

entrapped in polyacrylamide with the enzyme adsorbed onto CM-cellulose and noted an increase in the apparent K_m in both the cases. Additionally, the entrapped enzyme was less sensitive to inhibition by *p*-chloromercuribenzoate and Ag^{2+} ions compared to CM-cellulose bound enzyme. Invertase entrapped in acrylamide (mixed in various proportions) by aerobic radiopolymerisation retained 51–76% activity of the soluble enzyme. A shift in the optimum pH towards acid side and an increase in the optimum temperature and temperature stability were observed (Kawashima and Umeda, 1976). Yeast cells containing invertase activity when entrapped in polyacrylamide gels by γ-irradiation retained 85% of its initial activity. No change in the kinetic values were observed as a result of immobilization. In a packed bed reactor, the entrapped cells could hydrolyse sucrose for a month without significant loss of its activity (D'Souza and Nadkarni, 1980). Ghosh (1990) entrapped yeast cells in polyacrylamide and compared the physical parameters viz. bead and irregular shaped polymer matrices and noted that the bead shaped matrix had a higher intrusion volume, surface and pore area than that of irregular shaped support. Scanning electron microscopic pictures of the immobilized cell preparations revealed a uniform distribution of cells within the matrix. Entrapment broadened the optimum pH to 3.5–5.5 and also increase in the optimum temperature to 60 °C from that of the free cells (pH 4.5 and 55 °C). The immobilized cells showed higher temperature stability and retained 93% of their initial activity at 70 °C whereas, the free cells showed only 61% of their initial activity. However, high pressure brought about a reduction of inversion in both free and immobilized cells. Emregül et al. (1996) examined the utility of carboxymethyl cellulose–gelatin (CMC–G) composite matrix cross-linked with different chromium salts namely, chromium(III) acetate (CA), chromium(III) sulfate (CS) and potassium chromium(III) sulfate (PCS) for the immobilization of invertase. Effect of various parameters viz. CMC–gelatin ratio and cross-linker concentration showed that maximum retention of activity (78%) and stability is obtained when immobilized preparation with a CMC–G ratio of 0.111% w/w is cross-linked with 0.004 mol dm^{-3} of CS and 0.001 mol dm^{-1} PCS. Moreover, the immobilized adducts with cross-linker concentrations below the aforementioned values were not mechanically stable. Furthermore, decrease in the relative activities with increasing concentration of cross-linkers was correlated to either tightening of the matrix or to metal ion mediated inactivation of the enzyme. However, when the enzyme was bound to CS and PCS cross-linked gelatin, the immobilized preparations exhibited less efficiency. The immobilized adducts could be used 20 times with no detectable loss in its initial activity. Subsequently, the above authors (Emregül et al., 2006) immobilized invertase in polyacrylamide–gelatin (PAA–G) composite matrix by cross-linking with chromium(III) acetate (CA), chromium(III) sulfate (CS) and potassium chromium (III) sulfate (PCS) and the immobilized preparations showed an efficiency of 79%, 72% and 79%, respectively. In the present case, polyacrylamide was chosen as the filling matrix since it helps in increasing the surface area and secondly, it does not harden in presence of cross-linkers. As observed with CMC–gelatin system, the PAA–gelatin ratio and cross-linker concentration influenced the activity of the immobilized preparations. Optimum retention of activity was obtained when a PAA–gelatin ratio of 0.177 w/w is cross-linked with 0.01 mol dm^{-3} of CA, 0.004 mol dm^{-3} of CS and 0.001 mol dm^{-3} of PCS. Properties of invertase bound to PAA–G matrix cross-linked with CA showed a shift in the optimum pH towards alkaline side (7.2) from that of the soluble enzyme (6.0) and this shift was correlated to the secondary interactions between the enzyme and the matrix. However, immobilization did not affect the optimum temperature of the enzyme (i.e.55 °C). A slight increase in the apparent K_m was correlated to spatial distribution of the enzyme molecules on the polymer layer and immobilization induced conformation changes of the enzyme. However, the marginal decrease in the apparent V_{max} was attributed to diffusional barriers. All the immobilized preparations exhibited good operational stability.

Maeda et al. (1973b) entrapped invertase in poly(vinyl alcohol) gel matrix by means of electron beam irradiation and the bound enzyme retained approximately 44% activity of the soluble enzyme. No leaching of the entrapped enzyme was observed. Invertase immobilized in poly (vinyl alcohol) membrane gelled with UV irradiation showed no changes in the optimum pH and temperature compared to the soluble enzyme. On continuous reaction in a batch system, at pH 4.6 and 30 °C, the bound enzyme showed only marginal loss of activity after 10 cycles (Imai et al., 1983). Fukui et al. (1976) immobilized invertase in photosensitive polyethylene glycol dimethacrylate resin and the bound enzyme retained more than 40% activity of the soluble enzyme. Immobilization brought about a 5-fold increase in the K_m pointing towards diffusional barriers. Marconi et al. (1974) entrapped invertase in cellulose triacetate fibers and the bound enzyme showed 15–65% efficiency. The retention of activity was dependent on the amount of enzyme bound and the porosity of the fibers. The immobilized preparation showed high operational stability, with a calculated half-life of 5300 days. Tanaka et al. (1977) developed a simple procedure for the entrapment of enzymes or microbial cells using photocross-linkable resin oligomers viz. polyethylene glycol dimethacrylate. Invertase could be entrapped in the resin film, formed by exposing briefly to a lamp (λ_{max} 360 nm). The entrapped enzyme exhibited 40–50% efficiency. Interestingly, except exposure to light other conditions viz. proportion of water, enzyme and oligomers in the immobilization system, ratio of oligomers having one or two functional groups and chain length of the oligomers affected the retention of activity. The bound enzyme showed good stability to repeated use. Fukushima et al. (1978) developed a simple procedure for the entrapment of enzymes, whole cells and organells, in hydrophilic urethane prepolymers. Invertase entrapped in this system showed higher optimum pH compared to the soluble enzyme. On repeated use, a slight increase in the activity was noted which was correlated to the relaxation of the polymer. Invertase entrapped inside the reversed micelles, formed by either sodium tauroglycocholate or sodium lauryl sulfate in various organic solvents viz. hexane, *n*-decanol, benzene and carbon tetrachloride, exhibited more than 4-fold increase in the activity compared to the soluble enzyme. The bound enzyme also showed higher velocity, low K_m and longer active life. The observed changes in the properties were attributed to the stabilizing effect of the active enzyme conformation by the surfactants (Madamwar et al., 1988). Phadtare et al. (2004) entrapped invertase in octadecylamine film and the process was highly pH dependent suggesting the role of attractive electrostatic interactions between the enzyme and the lipid in the formation of biocomposite film. The entrapped enzyme showed high retention of activity and stability to repeated use.

Selampinar et al. (1997) prepared enzyme electrodes by entrapping invertase in conducting matrices during electropolymerisation of pyrole. Immobilization brought about a decrease in the optimum pH and temperature but the bound enzyme showed good stability to repeated use and storage. Evaluation of the kinetic parameters revealed an increase in the apparent K_m and V_{max} and they were correlated to diffusional barriers as well as increased concentration of the product within the pores of the matrix. Erginer et al. (2000) prepared enzyme electrodes by entrapping invertase in pyrole-capped polyazotetrahydrofuran and polytetrahydrofuran-block-polystyrene copolymer matrices during electrochemical polymerization of pyrole using sodium dodecyl sulphate as supporting electrolyte. Scanning electron microscopic studies on the surface morphologies of enzyme entrapped films revealed that while on the solution side of the films showed considerable damage to the cauliflower-like structure of the polymer matrix after enzyme entrapment, enzyme clusters were not visible on the electrode side. Evaluation of the kinetic parameters showed an increase in the apparent K_m but a decrease in the apparent V_{max} and these changes were attributed to diffusional restrictions. Among the immobilized preparations viz. polyazotetrahydrofuran/polypyrole/invertase and polytetrahydrofuran-block-polystyrene/invertase, the latter exhibited higher stability to repeated use. Yeast cells with invertase activity entrapped in different polymer matrices namely, polypyrole(PPy), poly(methyl methacrylate)/polypyrole (PMMA/PPy) and thiophene-capped poly(methyl methacrylate)/

polypyrole (TPMMA/PPy) showed maximum activity in co-polymer bound cells than pure PPy bound cells. Moreover, the highest activity was observed with PMMA/PPy graft polymer matrix. Furthermore, immobilization did not lead to cell damage or lysis. Entrapment brought about a marginal increase in the optimum temperature (60 °C) from that of the free cells (55 °C). The enzyme electrodes exhibited good operational and storage stabilities (Balci et al., 2002). Yildiz et al. (2005) prepared enzyme electrodes using invertase entrapped in three types of polymethyl methacrylate-*co*-polymethyl thienyl methacrylate random copolymer matrices during electrochemical polymerisation of pyrole (PMMA-*CO*-PMTM/PPy) viz. MT1, MT2 and MT3, having the same segments but with different copolymer compositions with respect to mole percents. Entrapment brought about an increase in the optimum pH of MT2/PPy and MT3/PPy to 5.0 and 6.0 for MT1/PPy, from that of the soluble enzyme (pH 4.6). This shift in turn was attributed to partitioning effects. On the contrary, immobilization decreased the optimum temperature of the free enzyme (50 °C) to 40 °C for MT1/PPy–invertase and MT2/PPy–invertase adducts whereas, no change was observed for MT3/PPy bound enzyme. Evaluation of kinetic parameters of soluble and entrapped enzymes revealed that while the apparent K_m and V_{max} of MT2/PPy and MT3/PPy–invertase complexes were comparable, that of MT1/PPy–invertase adduct was close to that of PPy–inveratase conjugate. These changes were correlated to very low thienyl group content in MT1 and more polypyrole chains in MT1/PPy copolymer matrix. Studies on the operational stability showed that at 25 °C, the entrapped enzyme could be used 40 times without any significant loss of its initial activity. Based on the results the authors concluded that conducting polymers can be used for immobilizing invertase. Arslan et al. (2006) entrapped invertase in polyacrylic acid (PAA) and poly(1-vinyl imidazole) (PVI) complex system for its potential use as a biosensor. Entrapment brought about an increase in the K_m but a decrease in the V_{max} and these changes were correlated to diffusional barriers. Though the entrapped enzyme exhibited good operational stability, the storage stability was poor. Moreover, surface morphologies of the polymer electrolyte matrices before and after enzyme entrapment showed damage in the smooth structure of the PAA/PVI matrix as a result of entrapment and this was attributed to the large structure of the enzyme.

Toda and Shoda (1975) entrapped yeast cells with invertase activity in spherical agar pallets and studied the continuous hydrolysis of sucrose in a fluidized bed reactor and noted that the magnitude of apparent K_m and V_{max} values for cell bound invertase activity depends on both pallet size and intrapellet concentration of the enzyme. Nakane et al. (2001) entrapped invertase in a composite gel fiber of cellulose acetate and zirconium tetra-*n*-butoxide and noted that the activity of the bound enzyme increased with increase in the fiber diameter. This led the authors to suggest that the hydrolysis of sucrose might be taking place near the fiber surface. The entrapped enzyme also exhibited higher thermal stability. Mansfeld et al. (1991) entrapped free and polystyrene bound invertase in semi permeable membranes (symplex membranes) made up of cellulose sulfate and poly(dimethyldiallyl-ammonium chloride) and compared the performance of the immobilized preparation with that of the soluble enzyme. Although, the optimum pH of the free and encapsulated enzymes showed no change (pH 5.0), the optimum pH of the polystyrene bound enzyme shifted slightly towards the acid side (4.0). This shift in turn was correlated to the positive charge on the matrix. Encapsulation resulted in the broadening of the optimum temperature (60–65 °C) from that of the soluble enzyme (60 °C). It also brought about an increase in the apparent K_m and this was dependent on the capsule diameter because reduction in capsule diameter led to a decrease in the apparent K_m and this in turn was attributed to diffusional barriers. The encapsulated enzyme exhibited good storage stability but the operational stability was dependent on the concentration of sucrose in the feed solution i.e. lower the sucrose concentration, higher was the stability.

Husain et al. (1985) entrapped glucose oxidase, invertase and amyloglucosidase in calcium alginate gels as Con-A complexes, in order to prevent leaching of the enzyme from the matrix. Both soluble and gel entrapped Con A–gycozyme conjugates exhibited relatively high efficiency, indicating good accessibility of the substrate. It was also noted that considerable amounts of invertase, as Con A complexes, could be entrapped without any significant decrease in the efficiency. A study on the effect of gelation method, cell loading, gel surface area, gel concentration and alginate composition on invertase in an immobilized whole cell system revealed that cell loading and specific surface area play an important role in determining the apparent activity. However, gelation method, piece geometry and alginate composition did not have any effect on the activity of the enzyme if the immobilized preparations had the same specific surface area and alginate to cell ratios (Johansen and Flink, 1986). In an effort to integrate cell growth and immobilization, Chang et al. (1996) entrapped recombinant *S. cerevisiae* cells with invertase activity in liquid–core alginate capsules and cultured to high density. In batch culture, the immobilized preparations with cloned invertase exhibited marginally higher activity than the free cells. In addition, the entrapped cells showed good storage and operational stabilities. Protoplasts of a high invertase producing strain of yeast viz. *Saccharomyces cerevisiae* IFO0309, entrapped in strontium alginate gels could secrete invertase. However, the enzyme levels dropped after 24 h due to regeneration of cell wall suggesting that prevention of cell wall regeneration is important to prolong the operational life span of the immobilized system. This problem could be alleviated, to some extent, by the addition of aculeacin (an inhibitor of β-1,3 glucan synthesis) in the medium. In addition, *S. cerevisiae* T7 (a hyper invertase producer) protoplasts when assessed for invertase production in a bubble column reactor yielded a steady level of invertase (45 U/ml) over a period of 72 h (Tanaka et al., 2000). Tanriseven and Doğan (2001) entrapped yeast invertase in calcium alginate capsules and the bound enzyme retained 87% activity of the soluble enzyme. Although immobilization did not affect the optimum pH and temperature of the enzyme (i.e. 4.3 and 60 °C), it increased its pH and temperature stability. High retention of activity (87%) on immobilization coupled with marginal changes in the kinetic parameters suggests the absence of significant diffusional barriers. The bound enzyme was stable and retained its initial activity after 36 days. These results led the authors to suggest that this procedure can be used for the production of invert syrup. Rossi-Alva and Rocha-Leão (2003) studied free and calcium alginate entrapped cells of *S. cerevisiae* mutants with respect to their growth pattern and invertase activity using enzyme repression by glucose as well as glucose consumption ability as the criteria for the selection of mutants. Amongst them, entrapped cells previously grown inside the matrix yielded higher activity. Interestingly, Ca^{2+}-alginate entrapped mutant strain of Q6R2 cells exhibited high invertase activity with glucose and sucrose as the carbon source. The immobilized preparation showed good operational and storage stabilities. Based on the results, the authors opined that entrapped yeast cells with decreased ability to consume sugar can be employed for the production of invert syrup. Invertase from *Cucumis melo*.L. entrapped in agarose–guar gum biopolymer matrix showed high efficiency (91%) apart from enhanced stability against temperature, storage and reuse. Fluorescence spectroscopic characterization of the membrane bound enzyme revealed that the matrix provides conformational stability to the enzyme without any modification. The excellent mechanical strength of the biopolymeric membrane coupled with its non-toxic nature led the authors to suggest this matrix can be useful for the fabrication of biosensors for application in food industry (Bagal and Karve, 2006).

Gianfreda et al. (1980) observed a decrease in invertase activity per mg dry cell for increasing concentrations of whole yeast cells immobilized in gelatin particles and attributed it to probable reduction in the cell surface area available for enzyme reaction with increase in cell number. Parascandola and Scardi (1981) entrapped yeast invertase in gelatin and examined various parameters like, pH profile, K_m, saturation velocity and activation energy of the immobilized system. The results indicated that though the entrapment

procedure did not influence the affinity of the enzyme for sucrose, it resulted in some loss of activity probably due to either enzyme inactivation or cell impairment, in addition to diffusion limitations. Knauze and Krauze (1981) immobilized invertase in gelatin and then combined it with various inorganic carriers by covalent binding. Reactor studies showed that the activity of the bound enzyme is influenced by the substrate concentration and flow rate. Comparison of the activities of alginate, κ-carrageenen and gelatin entrapped yeast cells with invertase activity revealed that maximum retention of activity (77%) is observed with gelatin entrapped cells (Dhulster et al., 1983). de Alteriis et al. (1988) entrapped invertase in gelatin and subsequently subjected the beads to hardening with formaldehyde. The observed loss of activity was correlated to steric hinderance, conformational changes and blocking of the active site. Subsequently, de Alteriis et al. (1999) examined the expression of *SUC2* gene encoding invertase using free and gelatin entrapped yeast cells to assess the high activity observed in case of immobilized cells when grown in nutrient medium. The results indicated that, while expression of *SUC2* gene is maintained throughout the growth in immobilized cells, its expression is only transient in free cells. Additionally, the entrapped cells were less susceptible to endogenous proteolytic attack than the free cells. The resistance of invertase from entrapped cells to proteolysis in turn was correlated to the maintenance of its high glycosylated state. Venkatasubramanian and Veith (1973) studied the effect of pressure on the hydrolysis sucrose using invertase immobilized in collagen membranes and observed that pressure enhances the activity of the enzyme.

Invertase and α-galactosidase entrapped in hollow fiber cartridges could effectively hydrolyse sucrose and raffinose respectively. When the hollow fibers were preconditioned with bovine serum albumin, the stability of the bound enzyme approached that of the soluble enzyme (Korus and Olson, 1977). Shiomi et al. (1988) bound invertase ionically in the inner surface of aminoacetalized poly(ethylene-co-vinyl alcohol) hollow fiber membrane and noted that enzyme concentration and coupling time affected the binding efficiency. Optimum retention of activity (89%) was obtained when 5 μg/ml of invertase solution is reacted with the matrix for 4 h. The K_m of the immobilized system was dependent on the flow rate of the substrate. Nakajima et al. (1988) developed a new enzyme reactor system for low molecular weight substrates, utilizing forced flow through an enzyme immobilized membrane. The enzyme was physically immobilized within a porous ceramic membrane by filtering the enzyme solution and forcing the sucrose solution through the membrane using a cross flow filtration method. The reaction rate of the forced flow system was ten times more than that of diffusion controlled system. Ghosh (1988) entrapped radiation killed yeast, with invertase activity, in plaster of Paris and compared it with cells entrapped in polyacrylamide. The plaster of Paris entrapped cells were approximately 20% more active than the cells immobilized in polyacrylamide. However, the operational stabilities of plaster of Paris and polyacrylamide entrapped systems remained unchanged even after 1 month of continuous use. de Almeida et al. (2005) used intracellular invertase of *Cladosporium cladosporioides* as a low cost auto-immobilized system to study the kinetic parameters as well as operational conditions for the hydrolysis of sucrose employing factorial planning method. In the present case, factorial planning was used since the production of invert syrup is a complex process involving a number of variables. Kinetic parameters showed an increase in the apparent K_m and V_{max} and this was attributed to diffusional barriers. Evaluation of the operational parameters revealed that optimum hydrolysis of sucrose is obtained when the reaction was carried out at pH 7.0 and 70 °C, under mild agitation. Watermelon cell suspension with invertase activity, after permeabilization with Tween 80, was immobilized by glutaraldehyde treatment and optimum activity was observed at pH 4.6 and 50 °C. The bound cells exhibited high activity and good storage stability. Since the kinetic parameters and physico-mechanical properties of glutaraldehyde cross-linked cells were comparable to that of the matrix bound enzyme, the authors concluded that the former offers an ecofriendly alternative for biotransformation process (Stano et al., 2006).

4. Conclusion

Extensive investigations have been carried out on the enzymatic isomerisation of glucose to fructose mainly due to the increasing prices of sucrose in the world market. In view of this, high fructose syrup (HFS) has gained considerable commercial importance as an alternate sweetener (Veith and Venkatasubramanian, 1976). HFS, made up of approximately 50% glucose and 42% fructose, is sweeter than sucrose due to the presence of fructose. However, HFS production requires hydrolysed starch as the raw material. In this respect, in countries where sucrose is readily available, utilization of sucrose can be advantageous. In addition, molasses, a by product of sugar industry, can serve as a cheap source of sucrose due to its high sucrose (~50%) content. Since immobilization offers several advantages, use of immobilized invertase presents a suitable alternative for the production of isomerized syrup. Moreover, using suitable enrichment techniques, the fructose content in the invert syrup can be increased, thus enhancing its commercial potential.

Although numerous attempts have been made on the immobilization of invertase as a laboratory model for the production of invert syrup, it is yet to find industrial application primarily due to the high cost of the enzyme as well as availability of sucrose. Moreover, advances in immobilized glucose isomerase technology and the resultant low cost and availability of HFS has limited the development of immobilized invertase. Hence efforts towards reducing the cost of the enzyme is required if immobilized invertase were to find successful industrial application.

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