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A N I N T E R N A T I O N A L J O U R N A L

Role of Glutaraldehyde in Imparting Stability to Immobilized β-Galactosidase Systems

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ABSTRACT

This review article highlights the role of glutaraldehyde as a matrix activator/stabilizer in imparting higher operational and thermal stability to β-galactosidase (βG) for biotechnological applications. Glutaraldehyde has been used extensively as a crosslinking agent as well as for functionalization of matrices to immobilize β-galactosidase. Immobilized β-galactosidase systems (IβGS) obtained as a result of glutaraldehyde treatment has been employed to hydrolyze whey and milk lactose in batch reactors, continuous packed-bed and fluidized bed reactors under various operational conditions. Moreover, these IβGS have also been utilized for the production of galactooligosaccharides in food, dairy and fermentation industries. It was observed that glutaraldehyde provided remarkable stability to immobilize βG against various physical/chemical denaturants, thus enhancing thermal/operational stability and rendering it more suitable for repeated utilization in industrial scale operations.

Key words: β-galactosidase; Crosslinking; Glutaraldehyde; Immobilization; Stability

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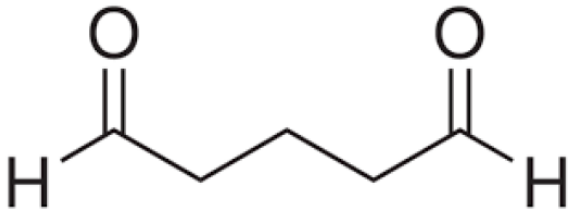
INTRODUCTION

Glutaraldehyde: A versatile organic compound

Glutaraldehyde [CH2(CH2CHO)2] is a 5-carbon linear dialdehyde (Fig 1) pungent oily liquid exhibiting excellent solubility in all proportions in water, alcohol and organic solvents. It is commercially available in varying concentration (2-70% w/v) as acidic aqueous solutions [1].

It reacts rapidly with N-terminal amino groups of peptides, amino acids via α-amino groups and sulfhydryl group of cysteine at neutral pH and form pyrans, hydrates and other polymers which show excellent thermal and chemical stability. The major reaction site in amino acids (lysine, tyrosine, histidine and sulfhydryl residues) occurs through the ε-amino group which may lead to the formation of precipitates. In glutaraldehyde-mediated aqueous reaction systems, a reversible equilibrium has been observed between the polymeric, cyclic and open chain forms [2].

Glutaraldehyde promoted crosslinking in 2 ways – intermolecularly and intramolecularly. When crosslinking occurs among groups placed on different protein molecules, it is known as intermolecular crosslinking. However, if crosslinking takes place between groups located in the same molecule, it is termed as intramolecular crosslinking. Glutaraldehyde was initially used to preserve and fix tissues in combination with formaldehyde by the formation of intermolecular crosslinking, the features arise as a result of their capacity to react with itself or with protein groups already modified with a glutaraldehyde molecule [3]. Presently, glutaraldehyde serves as one of the most potent crosslinker reagents in plethora of clinical, biomedical and biotechnological applications. Several researchers have excellently and critically reviewed the importance and properties of glutaraldehyde in these applications [4,5]. Recent studies have demonstrated the utilization of glutaraldehyde pre-activated supports for imparting stability to various enzymes in food industry owing to their Generally Recognized as Safe status [6].



Molecular Formula: $C_5H_8O_2$

CAS Number: 111-30-8

Molecular Weight: 100.12

Synonyms: 1,5-Pentanedial; Glutaral; Glutaric dialdehyde; 1,3-Diformylpropane

Fig 1: Chemical properties of glutaraldehyde

Glutaraldehyde was earlier utilized to stabilize glutaryl acylase, D-aminoacid oxidase and glucose oxidase (GOX) and it was observed that GOX gained 400-fold stabilization as a result of glutaraldehyde treatment. Glutaraldehyde activation significantly reduced the ability of these enzymes to desorb from the supports due to their high ionic strengths, thereby suggesting that glutaraldehyde promoted strong support-protein reaction. This approach provided a simple strategy for obtaining excellent enzyme immobilization rate and desired stability for various biotechnological applications [7,8]. Moreover, glutaraldehyde was also exploited for

preparing crosslinked enzyme aggregates (CLEAs) mainly in case of enzymes that have low density of surface reactive groups and therefore may pose problem to obtain a final solid catalyst [9,10]. The process of CLEAs formation involves the treatment of enzymes immobilized on supports that are non-reactive to glutaraldehyde in order to prevent enzyme leakage followed by the crosslinking of enzymes adsorbed on aminated supports. This method was used to prepare CLEAs of trehalose synthase via co-aggregation with polyethyleneimine [11] and pectin CLEAs of glucoamylase [12] . Thus, glutaraldehyde remains the most widely used compound for designing biocatalysts to be utilized in a large number of biomedical and biotechnological applications.

β-galactosidase – sources and application

β-galactosidase (βG) is an enzyme that belongs to glycoside hydrolase 2 families of carbohydrate active enzymes. It is involved in the process of hydrolysis of lactose to glucose and galactose and has been extensively used in food, dairy and fermentation industries [13]. In clinics, βG is being used as a drug to treat a condition called "lactose intolerance" caused by a genetic deficiency that results in the absence of intestinal lactase [14,15]. It has been recommended as an essential ingredient to hydrolyze lactose in several milk-based food products for consumption by lactose intolerant individuals. They are used extensively as food ingredients in the production of ice-cream and prebiotics (substances that stimulate growth of beneficial gut microorganisms for assisting digestive process) such as galactooligosaccharides (GOS) in Japan and Europe [16].

Thus, βG makes milk and milk products more accessible and suitable for consumption by people that are intolerant to lactose. In addition, lactose present in whey can be hydrolyzed to produce sweet syrup which in turn can be utilized in confectionery, baking and soft drinks industries [13,17].

βG occurs widely in nature including plants, animals, birds, insects and microorganisms (yeast, bacteria and fungi). This enzyme has been isolated from various species, purified and extensively characterized in terms of stability, specificity and kinetics [18-20]. Though Bg obtained from various sources are non identical, but they display unique chemical, physical and immunological properties. It has been observed that microorganisms produce βG in high yields as compared to animals and plants source. Therefore, their major commercial source arises from microorganisms such as yeasts (*Kluyveromyces fragilis*, *Kluyveromyces lactis* and *Candida pseudotropicalis*), fungi (*Aspergillus niger* and *Aspergillus oryzae)* and bacteria (*Bacillus* species) [21-24]. However, commonly used microbes include *Kluyveromyces sp.* and *Aspergillus sp.* Moreover, numerous plants such as almonds, peach, apricots, apples and peas have also been employed for the isolation of βG. While *E. coli* βG (encoded by the *lacZ* gene) has been extensively studied in order to understand its mechanism of action, but its application in food industry has not been permitted due to possible toxicity associated with coliforms [25]. Therefore, this enzyme is usually isolated from microorganisms that are generally considered as safe for large scale use in industrial production of milk and dairy production. Amino acid sequences of βG obtained from several bacterial species has been determined and it has been observed that it displayed a high level of homology and highly conserved regions across the bacterial species [25,26]. Similarly, enzyme isolated from *Kluyveromyces lactis* exhibited remarkable structural identity with *E. coli* enzyme, thereby suggesting their close structural, functional and phylogenetic relationships.

Immobilization of βG on polymeric/mesoporous matrices via glutaraldehyde

Glutaraldehyde is used as an activator/stabilizer for several immobilization matrices to impart stability to βG (Fig 2). In order to immobilize βG, various techniques have

been employed which include (a) physical adsorption, (b) covalent attachment, (c) chemical aggregation, (d) entrapment, (e) microencapsulation, and (f) bioaffinity layering.

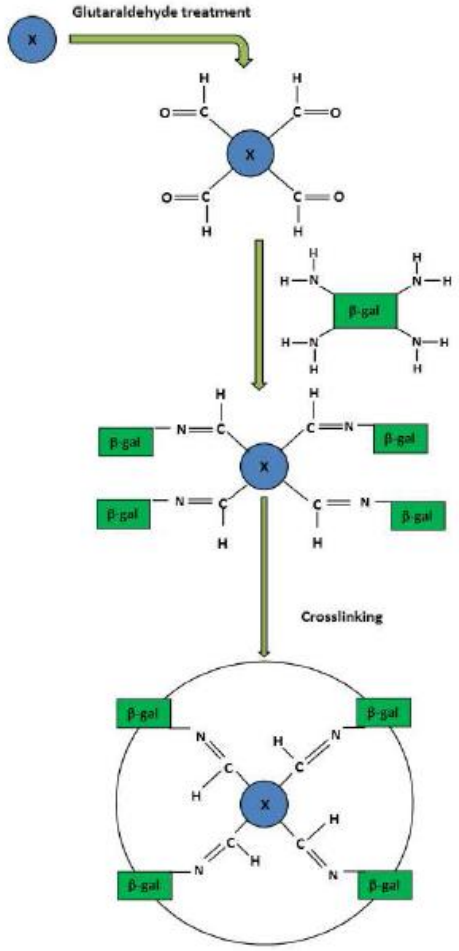


Fig 2: Schematic representation of βG binding on X (immobilization matrix) via glutaraldehyde

Matrices used to immobilize βG for various applications have been 143 listed in Table 1. Chitosan is the most primitive and ideal support that has been used for the adsorption of βG for obtaining lactose free dairy products due to its better biodegradability, excellent biocompatibility, strong hydrophilicity and good antibacterial properties. Klein et al. [27] immobilized *Kluyveromyces lactis* βG on chitosan which was activated by glutaraldehyde for hydrolyzing lactose and GOS production. The activated biocatalyst exhibited enhanced operational stability at optima pH and temperature, and pronounced thermal stability in the presence of substrate and products. This IβGS was able to hydrolyze lactose completely in continuous reactor operated at 2.6 ml/min flow rate at 37°C. However, maximal GOS concentration of 26 g/l was obtained at a flow rate of 3.1 ml/min with a productivity of 186 g/l/h.

Chen and co-workers have studied the effect of glutaraldehyde concentration, crosslinking time, crosslinking pH and crosslinking temperature on the activity of βG immobilized on chitosan beads. Enzyme activity was measured in terms of the absorbance at 420 nm. Maximum immobilized enzyme activity was observed in 0.3% glutaraldehyde solution while the optimal cross-linking time, optimal crosslinking pH, optimal crosslinking temperature were found to be 3h, pH 6.5 and 25°C, respectively [24]. Similarly, *Aspergillus oryzae* βG was crosslinked by

glutaraldehyde via entrapment in sodium alginate gel. The resulting IβGS exhibited orthogonal axial value of 1.3 in analyzing optimal concentrations of immobilization procedure by orthogonal central composite design. It was also noted that the process of crosslinking resulted in only 25% decrease in the enzymatic activity even after 25 cycles of repeated use thereby indicating the higher level of stability achieved as a result of glutaraldehyde involvement [28]. In another study, βG was immobilized on novel _-carrageenan gel beads activated by glutaraldehyde and full-factorial central composite design was studied to optimize the conditions for maximum enzyme loading. It was observed that 11.443 U of enzyme/g was 166 retained by gel beads after 8h incubation. This method of enzyme immobilization process increased the temperature-optima from 50oC to 55o168 C and pH-optima from 4.5 to 5.5 as compared to free enzyme. Km value for immobilized enzyme increased from 22.9 mM (for free enzyme) to 61.6mM while _max value was decreased from 177.1 _mol/min (for free enzyme) to 131.2mol/min. The full conversion experiment showed that the developed IβGS was active as compared to the free enzyme, and achieved 100% lactose hydrolysis after 4h of cycle operation [29].

The chitosan-hydroxyapatite beads were used as a matrix for immobilizing *Kluyveromyces lactis* βG by using glutaraldehyde as a crosslinking agent. This IβGS retained 81% enzyme activity and exhibited pH-optima at pH 7.5, and demonstrated enhanced thermostability and excellent repeated use efficiency [30]. Glutaraldehyde was also used to crosslink βG from *Aspergillus oryzae* on concanavalin A (Con A)-cellulose. The results were compared between βG immobilized on Con A-cellulose adsorbed and crosslinked βG. The crosslinked βG exhibited 84% enzyme activity even after its sixth repeated use as compared to 75% activity for simply adsorbed matrix. Moreover, crosslinked βG showed higher lactose hydrolysis from solution in batch process at 60°C and in continuous packed bed reactors as compared to uncrosslinked enzyme [31]. Similar studies were carried out successfully for the hydrolysis of lactose in milk by Mateo et al. [32]. Giacomini and coworkers exploited controlled pore carrier-silica (CPC-silica) which was activated with glutaraldehyde and observed that higher amounts of enzyme were retained on glutaraldehyde activated CPC-silica [33].

Importance of glutaraldehyde to immobilize βG was also studied on Con A-celite. The activity yield of crosslinked enzyme was 71%. Michaelis constant, Km was 5.58 mM for crosslinked adsorbed βG while Vmax was 0.38 mM/min. Stability studies 189 suggested that theobtained IβGS showed 71% activity after its seventh repeated use and lost only 10% enzyme activity after 1 month under refrigerated conditions. Immobilized enzyme was less affected by product inhibitors (glucose and galactose) and promoted the hydrolysis of lactose from milk and whey in batch/continuous packed-bed reactors in an efficient and controlled manner [34]. Ansari and coworkers utilized glutaraldehyde to crosslink βG form various plant sources including *Amygdalus communis* and *Prunus armeniaca kaisa* on Con A layered calcium alginate-cellulose beads. Immobilized βG retained 72% of the initial activity after crosslinking by glutaraldehyde and showed remarkable broadening in pH and temperature-activity profiles as compared to the native enzyme. Glutaraldehyde crosslinked βG showed improved hydrolysis of lactose from milk and whey in batch processes at higher temperature as well as in continuous reactors operated at different flow rate for one month [35,36]. Glutaraldehyde crosslinked layered beads were also exploited for immobilizing *Aspergillus oryzae* βG. It exhibited improved pH and temperature202 activity profiles and higher stability for longer durations at 4°C, and lost only 35% enzyme activity in the presence of 5% galactose as a completive inhibitor. Lactose hydrolysis in continuous spiral bed reactor by this IβGS exhibited nearly 90% conversion rates at various flow rates even after one month of operation [37]. In another study, cellulose acetate polymethylmethacrylate

membranes were activated by 2% glutaraldehyde solution for 2h to provide a highly efficient matrix for immobilization of βG [38]. The temperature optimum for crosslinked adsorbed βG was increased by 10°C while the preserved activity of free and immobilized βG was found to be 45% and 83%, respectively, after five weeks of storage at 4°C.

Reusability of immobilized βG was observed to be 86% even after fifth repeated use, thereby signifying its application in lactose hydrolysis in various dairy products including milk and whey.

In another approach of crosslinking, βG was entrapped in silica-based nanospheres via glutaraldehyde in order to obtain high enzyme loading capacities and enhanced mechanical stability. It resulted in 3.5-fold increase in enzyme loading capacity as a result of 3D network of silica and glutaraldehyde. IβGS prepared was found to be highly stable and lost only 20% activity at 25°C after 10 days [39]. Chen and coworkers utilized recombinant βG for hydrolyzing milk lactose in a packed-bed reactor by exploiting the crosslinking property of glutaraldehyde on chitosan. IβGS generated showed greater stability for prolonged durations even in the presence of various ions present in milk. This model could hydrolyze 80% lactose from milk after 2h of operation [24]. Similarly, 95% lactose was hydrolyzed in a pilot scale reactor after 2h by *Kluyveromyces lactis* βG immobilized on cotton fabric using glutaraldehyde as the crosslinking agent [40].

Glutaraldehyde was used to entrap Con A-βG complex for analyzing their stability in the presence of product inhibitors, glucose and galactose [23]. Entrapped glutaraldehyde crosslinked concanavalin A-βG complex showed greater retention of enzyme activity in the presence of 4.0M urea at room temperature as compared to soluble βG. Moreover, this IβGS exhibited 62% enzyme activity at 5% galactose concentration, and showed excellent stability and reusability properties. Furthermore, alginate and gelatin fibres were also utilized for βG immobilization and crosslinking was achieved by glutaraldehyde [41,42]. Graphite was also used as an immobilization matrix to conjugate *Kluyveromyces lactis* βG by using glutaraldehyde as crosslinking agent. It exhibited improvement in immobilization yield apart from imparting excellent stability and reusability attributes. *K*m of IβG showed five folds increase as compared to its soluble counterpart. Seventy percent lactose (5% w/v) was hydrolyzed at 37°C in 3h by this IβGS [43].

Szczodrak reported 90% immobilization yield for *Kluyveromyces fragilis* βG on glutaraldehyde modified silanized porous glass. Significant change in pH and temperature optima was observed for this IβGS. Moreover, it showed remarkable high lactose hydrolysis of 86-90% in whey permeate in a recycling packed bed bioreactor [44].

Table 1: Mesoporous matrices utilized for immobilizing βG via glutaraldehyde mediated crosslinking

Matrix type	**Application**	**Reference**
Gelatin	Lactose hydrolysis	21
Calcium alginate	Lactose hydrolysis	23
Chitosan	Lactose hydrolysis, GOS production	24, 27, 45, 46
Sodium alginate and gelatin	Lactose hydrolysis	28
κ-Carrageenan gel beads	Lactose hydrolysis in milk	29
Chitosan-hydroxyapatite microspheres	-	30

Con A-cellulose	Lactose hydrolysis	31
Agarose	Lactose hydrolysis	32
Silica/Agarose	Lactose hydrolysis	33
Con A-celite	Lactose hydrolysis	34
Con A layered cellulose-alginate hybrid gel	Lactose hydrolysis	35-37
Cellulose acetate-polymethylmethacrylate membrane	Hydrolysis of lactose whey from milk and	38
Silicon surface	-	39
Cotton fabric	Lactose hydrolysis	40
Alginate and gelatin fibres	Lactose hydrolysis	42
Graphite	Lactose hydrolysis	43
Silanized porous glass	Whey hydrolysis	44
Chitosan beads	Lactose hydrolysis in milk	47
Silica	Whey hydrolysis	48
Cellulose beads	Lactose hydrolysis	49
Cotton	GOS production	50

Immobilization of βG on nanoparticles via 264 **glutaraldehyde**

In biotechnology industry, cost of the raw materials such as enzymes remains one of the major economic considerations. Therefore, numerous technologies and novel carrier molecules have been adopted to improve βG immobilization and enhance enzyme loading, activity and stability in order to reduce overall cost of biocatalytic activity at industrial scale [51]. These approaches encompass formation of crosslinked enzyme aggregates wherein active enzyme aggregates are immobilized using a multifunctional crosslinker that provides extra stability and enhanced their recyclability during their use as biocatalysts [52]. Other approaches include microwave-assisted covalent immobilization of enzymes, click chemistry technology, mesoporous supports and most recently nanoparticle-based immobilization of enzymes [53]. Nanoparticle-based technology of enzyme immobilization has been widely used to make them suitable against harsh micro-environmental conditions involved in biotechnological processes. Enzymes immobilized on nanoparticles retained their catalytic activity at a broader pH and temperature range, and demonstrated significantly higher thermal stability as compared to that of native enzyme. Nanoparticle based immobilization methodology has several advantages over conventional methods of enzyme immobilization such as high quality nano-enzyme particles can be obtained without using surfactants and toxic reagents, they are homogenous with well-defined characteristics (nature of core-shell nanoparticles enzyme shell features) and particle size can be conveniently modulated according to the requirement of specific applications [54]. In addition, it is well recognized that co-immobilization of multi-enzymes is possible using advanced nanoparticle based technologies [55].

Various nanoparticles that have been utilized earlier to immobilize βG 286 via glutaraldehyde mediated crosslinking have been shown in Table 2. Zinc oxide

nanoparticles were used for the immobilization of βG from *Lactobacillus plantarum* HF571129 by using glutaraldehyde as crosslinking agent. Immobilized enzyme exhibited broad spectrum of pH-optima from pH 5 to 7.5 and temperature optima from 50°C to 60°C. Km and Vmax values were also increased for immobilized βG as compared to soluble counterpart. Moreover, glutaraldehyde increased the long term storage activity for crosslinked adsorbed enzyme. Activation energy and half life of the prepared IβGS was 24 kcal/mol and 2h at 35°C while the rate of lactose hydrolysis for batch and packed-bed reactor was observed as 0.023 and 0.04 min-1, respectively [56]. In another study, glutaraldehyde was utilized to immobilize βG on chitosan296 coated magnetic Fe3O4 nanoparticles for GOS production. It was observed that immobilized enzyme retained higher activities at a wider range of temperatures and pH, and obtained GOS production with a maximum yield of 50.5% from 36% lactose solution [57]. Similarly, *Kluyveromyces lactis* βG was immobilized on glutaraldehyde functionalized multiwalled carbon nanotubes (MWCNTs) for binding greater amount of enzyme. Thermogravimetric analysis revealed the stability of glutaraldehyde modified MWCNTs as an ideal matrix for βG immobilization. The optimal pH for soluble and immobilized βG was observed at pH 7.0 while the optimal operating temperatures were observed at 40oC and 50oC, respectively [58].

Similarly, magnetic polysiloxane polyvinyl alcohol composite was used to immobilize *Kluyveromyces lactis* βG. This support was functionalized by glutaraldehyde to promote covalent immobilization of enzyme. This IβGS exhibited greater operational and thermal stability and was more effective in hydrolyzing lactose from milk in batch and continuous reactors [59].

Aspergillus oryzae βG was immobilized on Fe3O4-chitosan nanoparticles by using glutaraldehyde as activating agent to impart excellent storage, pH and thermal stability to the enzyme. The developed IβGS yielded 15.5% (w/v) GOS after 50% lactose was hydrolyzed [60]. More recently, novel carbon based nanoparticles in the form of nanodiamonds (NDs) were utilized by Ansari and co-workers for hydrolyzing lactose in batch reactors by functionalizing it with glutaraldehyde. βG activity retained was 7420 U/gm on glutaraldehyde modified NDs. The optimal pH and temperature for soluble and immobilized βG was observed at pH 4.5 and at 50°C, respectively. However, significant stability was observed at both higher and lower limits of pH and temperature for the enzyme immobilized on glutaraldehyde modified NDs. Moreover, it was observed that enzyme immobilized on glutaraldehyde modified NDs retained greater biocatalytic activity even after 2 months of storage and at higher galactose concentration, and upon repeated uses as compared to native enzyme in solution. Modified NDs bound βG showed improved lactose hydrolysis from solution in batch processes at various temperatures even after 10h for hydrolyzing lactose in dairy products [61]. In another approach, glutaraldehyde was used as a surface functionalizing agent to modify the surface of silver nanoparticles. *Aspergillus oryzae* βG bound to this glutaraldehyde modified nanomatrix exhibited pronounced increase in pH stability toward acidic and alkaline sides, and increased temperature resistance. Immobilized βG was more stable against galactose mediated product inhibition and showed improved operational and thermal stability, and greater lactose hydrolyzing capacity [62].

Table 2: Nanoparticles utilized for immobilizing βG via glutaraldehyde

Nanoparticles	**Application**	**References**
Zinc oxide	Lactose hydrolysis	56
Fe_3O_4-chitosan	GOS production	57
Multiwalled carbon nanotubes	Lactose hydrolysis	58
Polysiloxane polyvinyl alcohol magnetic composite	Lactose hydrolysis	59
Magnetic	GOS production	60
Nanodiamonds	Lactose hydrolysis	61
Silver	Lactose hydrolysis	62

CONCLUSION

Glutaraldehyde has been extensively used as an effective enzyme crosslinking agent because of its unique characteristics. Commercially, glutaraldehyde is readily available at relatively low cost and is highly reactive with amine groups at/around neutral pH. It has been found to be more efficient and effective as compared to other aldehydes in the formation of stable crosslinks. This compound has been frequently utilized for immobilizing βG by various mechanisms such as aldol condensation and Michael-type addition for biotechnological applications. Significant efforts have been made to immobilize βG from various sources on a large number of immobilization matrices by either using glutaraldehyde as a crosslinker or surface modifying agent for imparting higher stability in order to extend their application in food, fermentation and dairy industries. Thus, glutaraldehyde is an effective crosslinking agent that may be exploited for regular industrial application.

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