Current trends of ß-galactosidase research and application

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Beta-galactosidase (ß-D-galactoside galactohydrolase, EC 3.2.1.23), has tremendous potential in research and application in various fields like food, bioremediation, biosensor, diagnosis and treatment of disorders. The sources of the beta-galactosidase are microorganisms, plants and animals. Microbial beta-galactosidase is of much interest because of their thermostability, thermoacidophilic and thermoresistant properties. Whey, a by-product of cheese industry possess a challenge in terms of down-stream processing and has been utilised for ethanol, exopolysaccharide and single cell protein production by employing microbial beta-galactosidases. Transglycosylation and transgalactosylation properties of microbial beta-galactosidases have been utilized for production of glucose, galactose, heteropolysaccharide, galacto-oligosaccharides. Beta-galactosidase has wide range of medical and industrial applications. Immobilization of beta-galactosidase improves its stability and reusage. Single molecule analysis of beta-galactosidase of *Escherichia coli* give in-sights into its kinetic properties. Thus, research and development processes in beta-galactosidase have significant applications.

Keywords beta-galactosidase; cold-adaptation; galactooligosaccharide; immobilization; lactose; single molecule; thermos-table; transgalactosylation; whey

1. Introduction

Beta-galactosidases (EC 3.2.1.23) are present in a wide variety of organisms including plants, animals and microorganisms, and are known to catalyze both hydrolytic and transglycosylation reactions. The thermostable beta-galactosidase from *Aspergillus niger*, *Bacillus stearothermophilus*, *Pyrococcus woesei*, *Thermus sp* are relatively stable from 35–80 °C. Cold-active and cold-adapted beta-galactosidase from psychrophilic microorganisms like *Arthrobacter psychrolactophilus*, *Pseudoalteromonas haloplanktis* are in general quite efficient in compensating the reduction of reaction rates by induced low temperatures through improvement of the turnover number (k_{cat}) or of the physiological efficiency (k_{cat}/K_m) and are relatively stable from 0–25 °C. Whey has been utilised for the production of ethanol, exopolysaccharide and single cell protein by employing beta-galactosidase from microorganisms like *Aspergillus oryzae*, *Kluveromyces lactis*, *Kluveromyces marxianus*, *Lactobacillus delbrueckii subsp. bulgaricus, Saccharomyces cerevisiae*. Transglycosylation and transgalactosylation properties of beta-galactosidase from *A. niger*, *Bacillus megaterium*, *Beijerinckia indica, Bifidobacterium infantis*, *Bifidobacterium longum*, *Enterobacter cloacae*, *Geobacillus stearothermophilus*, *K. marxianus*, *Lactobacillus sp*, *Lactobacillus reuteri*, *Penicillium expansum* have been utilized for production of glucose, galactose, heteropolysaccharide, galacto-oligosaccharides. Beta-galactosidase based medical and industrial applications include cleavage of blood group A and B glycotopes, biosensor for specific lactose determination in milk and disease diagnosis, treatment of lactose malsorption, production of lactose hydrolysed milk. Immobilization of beta-galactosidase on anion exchange resin, cellulose-gelatin carrier system, DEAE agarose, glyoxyl / epoxy / BrCN groups, glutaraldehyde, polyelectrolyte surfaces, silicon surface, sepabeads-epoxy supports partially modified with boronate, iminodiacetic, metal chelates, and ethylenediamine improves its stability and reusage. Co-production of beta-galactosidase with other enzymes like amylase, beta-glucosidase has been demonstrated in *G. stearothermophilus*.

Thus, the various beneficial properties of beta-galactosidase and its application across fields are discussed in this chapter.

2. Cold-active and thermostable beta-galactosidases

The production of cold-stable β-D-galactosidases and microorganisms that resourcefully ferment lactose is of high biotechnological interest, particularly for removal of lactose in milk and dairy products at low temperatures, cheese whey bioremediation and bio-ethanol production (Table:1). Recently, a gene encoding β-D-galactosidase was isolated from the genomic library of Antarctic bacterium *Arthrobacter* sp. 32c. Although, the highest activity of this purified enzyme was found at 50 °C, 60 % of the highest activity of this enzyme was determined at 25 °C and 15 % of the highest activity was detected at 0 °C. The cold-stable properties of *Arthrobacter* sp. 32c β-D-galactosidase could be useful for commercial, industrial conversion of lactose into galactose and glucose in milk products and could be an exciting substitute for the production of bioethanol from lactose-based feedstock [1].

Nakagawa et al. (2007) have overexpressed a cold-stable beta-galactosidase from *A. psychrolactophilus* strain F2 in *E. coli* using the cold expression system. The purified recombinant enzyme, rBglAp exhibited similar enzymatic properties to the native enzyme, it had high activity at 0 ºC, its most favourable temperature was 10 ºC, and it was

achievable to swiftly inactivate the rBglAp at 50 ºC in 5 min. rBglAp was capable to hydrolyze both ONPG and lactose with K_m values of 2.7 and 42.1 mM, respectively, at 10 ºC. rBglAp is a cold-active and extremely heat labile enzyme and has major possible application to the food industry [2]. Hoyoux et al. (2001) have purified beta-galactosidase from the Antarctic Gram-negative bacterium *P. haloplanktis* TAE 79. The purified enzyme is characterized for optimum activity at low temperature. Heat-induced unfolding examined by intrinsic fluorescence spectroscopy shown lower melting point values for both *P. haloplanktis* wild-type and recombinant beta-galactosidase compared to the mesophilic enzymes. Assays of hydrolysis of lactose in milk showed that *P. haloplanktis* beta-galactosidase is comparatively better than current commercial beta-galactosidase, suggesting that the cold-active beta-galactosidase could be used in lactose hydrolysis in dairy products processed in refrigerated plants [3].

Chen et al. (2008) have cloned and expressed a thermostable beta-galactosidase gene *bga*B from *B. stearothermophilus* in *B. subtilis* WB600. The optimum temperature for this beta-galactosidase activity was 70 °C. Kinetics of thermal inactivation and half-life times for this thermostable enzyme at 65 and 70 °C were 50 and 9 h, respectively. This enzyme exhibited a high level of transgalactosylation activity in hydrolysis of milk lactose. The results suggest that this recombinant thermostable enzyme may be suitable for the processes such as hydrolysis of lactose and production of galacto-oligosaccharides [4]. Lauro et al. (2008) have cloned, expressed, purified and characterized a beta-galactosidase (Aaβ-gal) from thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*. The recombinant Aaβ-gal is optimally active and stable at 65 °C [5].

Table 1 Cold-active, thermostable beta-galactosidases and their properties

Beta-galactosidase producers	Thermostability (ºC)	Reference
Arthrobacter sp. 32c	0-60	[1]
A. psychrolactophilus F2	0-50	[2]
P. haloplanktis TAE 79	0-40	[3]
B. stearothermophilus	45-80	[4]
A. acidocaldarius	40-90	[5]
T. ethanolicus	40-80	[6]

Volkov et al. (2005) have determined the nucleotide sequence of a 4936-bp genomic DNA fragment from the thermophilic bacterium *Thermoanaerobacter ethanolicus*. The fragment contained three open reading frames (ORFs). One of the ORF corresponded to the *Lac* A gene for a thermostable ß-galactosidase. Native recombinant LacA showed the highest activity at 75–80 °C. Immobilized on aldehyde silochrome, LacA was even more thermostable and retained its high activity [6]. Thus, the cold-active and thermostable beta-galactosidases play a vital role in hydrolysis of lactose, bioethanol and galacto-oligosaccharide production because of their thermostable property.

3. Immobilization of beta-galactosidase

Immobilization has shown to improve beta-galactosidase's stability and reusage (Table: 2).The immobilization of the ß-galactosidase of *Thermus* sp. T2 was performed using ionic adsorption onto two different supports: a new anionic exchanger resin, based on the coating of Sepabeads internal surfaces with polyethylenimine (PEI) polymers, and conventional DEAE-agarose. Immobilization proceeded unusually rapid in both cases, but the adsorption strength was much greater in the case of PEI-Sepabeads than in DEAE-supports at both pH 5 and 7. Interestingly, the PEI-derivatives remained wholly active at pH 5 and 7 after several weeks of incubation at 50 ºC, conditions that permit the lactose hydrolysis in milk [7].

Pessela et al. (2004) have used a battery of new heterofunctional epoxy supports to immobilize beta-galactosidase. The capability of a standard Sepabeads-epoxy support to immobilize beta-galactosidase from *Thermus* sp. strain T2 can be equal with other Sepabeads-epoxy supports partially modified with boronate, iminodiacetic, metal chelates, and ethylenediamine. Immobilization yields depended on the support, ranging from 95 % using Sepabeadsepoxy-chelate, Sepabeads-epoxy-amino, or Sepabeads-epoxy-boronic to 5 % using Sepabeads-epoxy-IDA. In count, rate of immobilization differed when using different supports. Amazingly, the immobilized beta-galactosidase derivatives showed outstandingly improved but different stabilities after favoring multipoint covalent attachment by long-term alkaline incubation. The enzyme immobilized on Sepabeadsepoxy-boronic was found to be the most steady. The crosslinking with aldehyde-dextran allowed the stabilization of the quaternary structure of the enzyme. The optimal derivative was extremely active in lactose hydrolysis even at 70 °C (over 1000 IU/g), maintaining its activity after extended incubation times under these conditions and with no risk of product contamination with enzyme subunits [8]. An immobilized preparation of the beta-galactosidase of *E. coli* using diverse supports and immobilization strategies (bearing glyoxyl, epoxy, BrCN groups or by glutaraldehyde crosslinking on matrices containing primary amino groups) have been obtained. In each and every one cases, the immobilization yield was 100 % with activity recoveries between 50 % and 100 % (using *o*-NPG as substrate). The enzyme immobilized on Eupergit 250 L exhibited an increase in the

enzyme activity by a factor of 2. Synthetic activity / hydrolytic activity ratio (*Vs*/*Vh*) was lower than 0.1 with the enzyme immobilized on BrCN at 4 ºC and pH 7, while the soluble enzyme gave a ratio of 0.46 and the immobilized enzyme on Eupergit 250 L gave a ratio of 0.8. Eupergit C immobilized enzyme and soluble enzyme showed enhanced *Vs*/*Vh* ratio when temperature was decreased [9].
Immobilization of ß-galactosidase-producing permeabilized dead cells of *K. lactis* ATCC 8583 into gelatin using glutaraldehyde as cross-linker has been performed. Thirty percent activity was obtained by immobilized cells relative to free disrupted cells [10]. Hamlin et al. (2007) have constructed an electrostatic self-assembly (ESA) for the immobilization of beta-galactosidase onto polyelectrolyte multilayer assemblies of the polyanion poly [1-[4-(3-carboxy-4 hydroxyphenylazo) benzenesulfonamido]-1, 2-ethanediyl, sodium salt] (PAZO) and the polycation poly (ethylenimine) (PEI) [11].

Table 2 Immobilization agents of beta-galactosidases

Beta-galactosidase producers	Immobilization agents	Reference
Thermus sp. T2	Sepabeads-epoxy supports, Sepabeads-epoxy-chelate, Sepabeads-epoxy-amino, Sepabeads-epoxy-boronic, Sepabeads-epoxy-IDA, PEI-sepabeads	[7, 8]
E. coli	Glyoxyl, epoxy, BrCN groups	[9]
K. lactis ATCC 8583	Gelatine	[10]
A. oryzae	Silica	[12]
Streptococcus thermophilus	Calcium alginate (CA), K-carrageenan, gellan-xanthan (GX)	[13]
K. lactis , *A. oryzae*	Poly (vinylalcohol) hydrogel	[14]

Mariotti et al. (2008) have considered the hydrolysis of whey lactose by immobilized beta-galactosidase of *A. oryzae* on silica. The most excellent immobilization results were attained by using glutaraldehyde as support activator and enzyme stabilizer. The optimized enzyme concentration for immobilization was 15-20 mg g^{-1} of support [12]. The usage of calcium alginate (CA), K-carrageenan and gellan-xanthan (GX) gel beads for entrapment of cells of *Streptococcus thermophilus* containing ß-galactosidase enhanced the stability of enzyme at higher temperatures (>55 ºC) [13]. Grosová et al. (2009) have immobilized ß-galactosidases of *K. lactis* and *A. oryzae* and yeasts in poly vinylalcohol hydrogel lens-shaped capsules. In the process of SSF with co-immobilized enzyme of *K. lactis* and *S. cerevisiae*, the galactose output increased from 3 g l^{-1} h^{-1} to 4.1 g l^{-1} h^{-1}, thus condensed the time of preparation of D-galactose [14]. Thus, immobilization has shown to improve the stability of beta-galactosidase and reduces the processing time in food and other industries.

4. Notable industrial applications of beta-galactosidase

Microbial beta-galactosidases have a prominent position in terms of their role in production of various industrially-relevant products like biosensor, lactose hydrolyzed milk, ethanol. Marrakchi et al. (2008) have developed a biosensor associating two distinct enzymatic activities, that of the beta-galactosidase and that of the glucose oxidase, in order to apply it for the quantitative detection of lactose in commercial milk samples. To eliminate interferences with glucose, a differential mode of measurement was used in this biosensor [15]. A putative beta-galactosidase from *Streptococcus mitis* with a choline-binding domain was recently identified. This peculiar property makes it useful for biotechnological applications [16]. Panesar et al. (2007) have carried out trialling to overcome the problem of enzyme extraction and poor permeability of cell membrane to lactose. Permeabilized *K. marxianus* NCIM 3465 cells were used for the production of lactose-hydrolyzed milk. The ethanol-permeabilized yeast cells gave 89 % hydrolysis of milk lactose under optimized conditions [17].

Domingues et al. (2005) have investigated the constant production of extracellular heterologous beta-galactosidase and ethanol by a recombinant flocculating *S. cerevisiae*. Jointly with extracellular beta-galactosidase production, an ethanol productivity of 9 g/1 h was obtained for the bioreactor fed with 50 g/l initial lactose concentration at 0.45 h^{-1} dilution rate. In adding together to beta-galactosidase and ethanol production, this system allowed for complete lactose metabolism [18]. In 2005, a kinetic analysis of alcoholic fermentation of lactose using strain NCYC869-A3/T1, a recombinant *S. cerevisiae* flocculent strain expressing both the LAC4 (coding for ß-galactosidase) and LAC12 (lactose permease) genes of *K. Lactis* was carried out. The lactose was wholly utilized in all the fermentations. The increase in ethanol production improved linearly when the initial lactose concentration was increased between 5 and 200 g L^{-1}.

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Ethanol productivity improved with increasing initial lactose concentration up to 150 g L^{-1} (1.23 g L^{-1} h^{-1}) [19]. Rodríguez et al. (2006) have constructed and analyzed two hybrid proteins from the beta-galactosidase of *K. lactis*, intracellular, and its *A. niger* homologue that is extracellular. One of the hybrid proteins obtained has interesting properties for its biotechnological utilization that increases the yield of the protein released to the growth medium. Changes introduced in the construction, besides to improve secretion, conferred to the protein biochemical characteristics of biotechnological interest [20]. Thus, beta-galactosidase plays a key role in food and other allied industries.

4.1 Galacto-oligosaccharides production by beta-galactosidase

In recent years, much investigation has been carried out in the field of pro- and prebiotics as functional foods. Galacto-oligosaccharides (GOS) are used as nondigestible, carbohydrate-based food ingredients in human and animal nutrition. Much of the research is focussed upon microorganisms that produce beta-galactosidases with improved quality for production of galacto-oligosaccharides (Table: 3). The synthesis of GOS with a high yield of 55 % from 275 g/L lactose at 50 °C for 12 h was performed using transglycosylating beta-galactosidase producing *E. cloacae*. The enzyme showed a extensive range of acceptor specificity for transglycosylation and catalyzed glycosyl transfer from ONPGal to various chemicals resulting in novel saccharide yields from 0.8% to 23.5 % [21]. Wu et al. (2006) have screened a mutant strain of *B. indica* L3 for the production of heteropolysaccharide-7 (PS-7). The highest amount of PS-7 formed by the mutant was 2.88 g/L with a viscosity of 4530 cP in lactose-based MSM medium. The PS-7 manufacture was enhanced by the addition of 4 g/L glucose into lactose-based MSM medium, reaching 5.52 g/L with a viscosity of 39531 cP. PS-7 of 6.18 g/L with a viscosity of 45772 cP was produced from the mutant grown in whey medium. The PS-7 production from the mutant reached 7.04 g/L when 4 g/L glucose was added to the whey medium [22].

Li et al. (2009) have cloned a novel gene encoding transglycosylating beta-galactosidase (BGase) from *P. expansum* F3 and subsequently expressed on the cell surface of *S. cerevisiae* EBY-100 by galactose induction. The BGase-anchored yeast could directly utilize lactose to produce GOS, as well as the by-products glucose and a small quantity of galactose. The glucose was consumed by the yeast, and the galactose was used for enzyme expression, thus to a great extent facilitating GOS synthesis. The GOS yield reached 43.64 % when the recombinant yeast was cultivated in yeast nitrogen base-Casamino Acids medium containing 100 g/liter initial lactose at 25 °C for 5 days [23]. In 2007, the process by which GOS formed from lactose was optimized using beta-galactosidase from *Lactobacillus* sp. It proved to be beneficial to directly apply the crude cell-free enzyme extract for the conversion, since similar GOS yields and composition were obtained as when using the pure enzyme preparation, but expensive purification could be avoided [24]. Hsu et al. (2007) have studied the production of GOSs by transgalactosylation using ß-galactosidase of *B. longum* BCRC 15708. Two types of GOSs, tri- and tetrasaccharides, were formed after ß-galactosidase action on 40 % lactose. Trisaccharides were the major type of GOS formed. In general, an increase of the initial lactose concentration in the reaction mixture resulted in a higher GOS production. A highest yield of 32.5 % (w/w) GOSs could be achieved from 40 % lactose solution at 45 °C, pH 6.8, when the lactose conversion was 59.4 %. The corresponding productivity of GOSs was 13.0 g / L^{-1} h^{-1} [25].

A mutagenesis advance was applied to the beta-galactosidase (BgaB) of *G. stearothermophilus* KVE39 to improve its enzymatic transglycosylation of lactose into oligosaccharides. A straightforward screening strategy based on the reduction of the hydrolysis of a potential transglycosylation product (lactosucrose), provided mutant enzymes possessing enhanced synthetic properties for the autocondensation product from nitrophenyl-galactoside and GOS from lactose. Alter of one arginine residue to lysine (R109K) augmented the oligosaccharide yield compared to that of the wild-type BgaB. Consequently, Site-saturation mutagenesis at this position demonstrated that valine and tryptophan further enlarged the transglycosylation performance of BgaB. During the transglycosylation reaction with lactose of the evolved ß-galactosidases, a key trisaccharide [ß-D-galactopyranosyl-(1→3)-ß-D-galactopyranosyl-(1→4)-D-glucopyranoside (3'-galactosyl-lactose)] was formed [26]. Spletchna et al. (2007) have investigated GOS formation from lactose in discontinuous and constant modes of conversion using beta-galactosidase (ß-gal) from *L. reuteri*. In the continuous stirred tank reactor, ß-gal from *L. reuteri* showed up to 2-fold higher specificity toward the formation of ß-(1→6)-linked GOS, with ß-D-Galp-(1→6)-D-Glc and ß-D-Galp-(1→6)-D-Gal being the main GOS components formed under these conditions [27].

Cheng et al. (2006) have used *Bacillus sp* for the production of low-content GOS from lactose that resulted in the highest yield of trisaccharides and tetrasaccharides. GOS production was improved by mixing beta-galactosidase with glucose oxidase. The low content GOS syrups, produced by beta-galactosidase was subjected to the fermentation by *K. marxianus*, whereby glucose, galactose, lactose and other disaccharides were at a low level, resulting in up to 97 % and 98 % on a dry weight basis of high-content GOS with the yields of 31 % and 32 %, respectively [28].

In 2008, a procedure was proposed for producing non-monosaccharide and high-purity GOS from lactose by *P. expansum* F3 β-galactosidase immobilized in calcium alginate. A purity of 28.7 % (w/w) GOS was obtained from 380 g/L lactose solution at pH 5.4 and 50 ºC. The immobilized enzyme was used for repeated GOS synthesis and showed good working stability. Digestible sugars in the GOS were dwindling after fermentation with *S. cerevisiae* L_1 or *K. lactis* L_3 entrapped in the calcium alginate. Purity greater than 37 % with yields greater than 27 % of non-monosaccharide GOS were obtained by *S. cerevisiae* L_1 for 19 batches and purity greater than 97 % with a yield greater

than 20 % of high-purity GOS was produced using *K. lactis* L_3 for two batches [29]. Layer and Fischer (2006) have performed *in vitro* glycosylation of peptides and proteins by trans-galactosylation of protected serine and threonine by β-D-galactosidase. The trans-mono-galactosylation of serine with a surplus of lactose produced 28 % of *N-tert*-butoxycarbonyl-1-*O*-β-D-galactopyranosyl-L-serinemethylester. The same transformational conditions, when applied to threonine, produced *N-tert*-butoxycarbonyl-1-*O*-β-D-galactopyranosyl-L-threonine-methylester in lower quantities. Mono-galactosylated serine and threonine are further galactosylated in the examined experimental setup to yield bi-galactosylated products also, especially at 50 °C with completely dissolved lactose [30].

Table 3 Galactooligosaccharides production by beta-galactosidase

Beta-galactosidase producers	Galactooligosaccharides (GOS) and by-products	Reference
E. cloacae	GOS, glucose, galactose	[21]
B. indica L3	Heteropolysaccharide-7	[22]
P. expansum F3	GOS, glucose, galactose	[23,29]
Lactobacillus sp	ß-D-Galp-(1→6)-D-Glc, ß-D-Galp-(1→6)-D-Lac, ß-D-Galp-(1→6)-D-Gal, β-D-Galp-(1→3)-D-Lac, β -D-Galp-(1→3)-D-Gal	[24]
B. longum BCRC 15708	tri-, tetrasaccharides, lactose, galactose, glucose	[25]
G. stearothermophilus KVE39	Lactosucrose, ß-D galactopyranosyl-(1→3)-ß-D-galactopyranosyl-(1→4)-D-glucopyranoside (3'-galactosyl-lactose)]	[26]
L. reuteri	ß-D-Galp-(1→6)-D-Glc, ß-D-Galp-(1→6)-D-Gal, β -D-Galp-(1→3)-D-Gal β-D-Galp-(1→6)-D-Lac β-D-Galp-(1→3)-D-Lac	[27]
L. bulgaricus	Sialyllactose, 14 other oligosaccharides	[31]
L. delbrueckii subsp. bulgaricus	Galactose, lactic acid , acetic acid, ethanol	[35]
B. infantis	GOS, lactose, monosaccharides	[37]
Lactobacillus plantarum	ß-D-Galp-(1→6)-D-Lac, ß-D-Galp-(1→6)-D-Glc	[38]
Bacillus circulans	*N*-acetylactosamine, N-acetylglucosamine	[39]

In 2009, oligosaccharides in bovine cheese whey permeate was characterized by a combination of nanoelectrospray Fourier transform ion cyclotron resonance mass spectrometry and matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry. In adding together to sialyllactose (the most abundant oligosaccharide in bovine colostrum), 14 other oligosaccharides were identified, half of which have the same composition of human milk oligosaccharides. These oligosaccharides could potentially be used as additives in infant formula and products for the pharmaceutical industry [31]. Spletchna et al. (2006) have formed prebiotic GOS from lactose using the β-D-galactosidases (β -Gals) of *L. reuteri* L103 and L461. Greatest GOS yields were 38 % when using an initial lactose concentration of 205 g/L and at 80 % lactose conversion. Disaccharides other than lactose and trisaccharides made up the enormous majority of GOS formed. The main products were identified as β-D-Galp-(1→6)-D-Glc (allolactose), β-D-Galp-(1→6)-D-Gal, β-D-Galp-(1→3)-D-Gal, β-D-Galp-(1→6)-Lac, and β-D-Galp-(1→3)-Lac. There were no key products with β1→4 linkages. Both intermolecular and intramolecular transgalactosylation were observed. D-Galactose proved to be a very competent galactosyl acceptor; thus, a relatively large amount of galactobioses was formed [32].

Li et al. (2008) have analyzed a novel beta-galactosidase BgaBM from *B. megaterium* that displayed wide acceptor specificity for transglycosylation with a series of acceptors, including pentose, hexose, hydroxyl, and alkyl alcohol using o-nitrophenyl-β-D-galactoside (ONPG) as a donor [33]. Kwon et al. (2007) have established that β-D-galactosidase displayed on *Bacillus* spores by fusion to the spore coat proteins may be used as a whole-cell immobilized biocatalyst for transgalactosylation in water-solvent biphasic reaction systems resulting in the synthesis of octyl-β-D-galactopyranoside at concentrations up to 27.7 mM (8.1 g/liter) with a conversion yield of 27.7 % (wt/wt) after 24 h from 100 mM lactose and 100 mM octanol dissolved in phosphate buffer and ethyl ether, respectively [34]. In 2007, an exopolysaccharide producing strain of *L. delbrueckii subsp. bulgaricus* was isolated from yogurts grown at dilution

rates between 0.06 and 0.8 h^{-1}. A major fraction of the galactose moiety from lactose from deproteinized whey was metabolized; molar yield of galactose from lactose varied between 0.173 and 0.791 with increasing dilution rates. The process was heterofermentative with maximum concentrations of lactic acid (30.7 g L^{-1}), acetic acid (11.7 g L^{-1}) and ethanol (0.96 g L^{-1}) obtained at dilution rates of 0.12, 0.36 and 0.12 h^{-1}, respectively. Greatest EPS concentration (830 mg L^{-1}) and maximum specific EPS production rate [188 mg (g biomass h)$^{-1}$] were obtained at a dilution rates of 0.36 h^{-1} and 0.67 h^{-1} respectively [35]. Nguyen et al (2007) have established the synthesis of prebiotic GOS from lactose, with the maximum GOS yield of 38.5 % of total sugars at about 75 % lactose conversion using heterodimeric beta-galactosidase from the probiotic strain *Lactobacillus acidophilus* R22 [36].

In 2008, synthesis of GOS from 36 % lactose using a recombinant beta-galactosidase of *B. infantis* in *Pichia pastoris* was investigated. The trangalactosylation ratio reached up to 25.2 % with 83.1 % conversion of initial lactose and the highest yield of GOS was 40.6 %. The GOS syrup was possessed of a 13.43 % GOS, 5.06 % lactose, and 8.76 % monosaccharides. The prebiotic effect of GOS promoted the growth of *B. breve* ATCC 15700 and *L. acidophilus* ATCC 33323 [37]. Iqbal et al. (2010) have observed that a recombinant beta-galactosidase from *Lactobacillus plantarum* has a high transgalactosylation activity and was used for the synthesis of prebiotic GOS. The maximal GOS yield was 41 % (w/w) of total sugars at 85 % lactose conversion (600 mM initial lactose concentration). The main individual products formed were ß-D-Galp-(1→6)-D-Lac, accounting for 34 % of total GOS, and ß-D-Galp-(1→6)-D-Glc, totalling up 29 % of total GOS [38]. In 2002, the use of ionic liquids as substitute solvents for enzyme catalysis was investigated. Beta-galactosidase from *Bacillus circulans* catalysed the synthesis of *N*-acetylactosamine starting from lactose and *N*-acetylglucosamine in a transglycoslyation reaction. The adding up of 25 % v/v of 1, 3-di-methyl-imidazolmethyl sulfate as a water-miscible ionic liquid suppresses the secondary hydrolysis of the formed product, resulting in doubling-up the yield to almost 60 %. The enzyme can be reused a number of times after ultrafiltration of the reaction mixture without loss of activity [39]. Thus, beta-galactosidase plays a significant role in production of galacto-oligosaccharides that can be used as food and feed for human-beings and animals respectively.

4.2 Role of beta-galactosidase in whey utilization

Whey has enormous therapeutic applications due to its composition in terms of proteins, lactose, minerals and valuable milk nutrients. The disposal of whey remains a major problem for the dairy industry especially in developing countries where a relatively insignificant part of whey is used for production of protein concentrates or permeates and a significant part of it is disposed off into the water streams causing severe water pollution resulting in high BOD and 5–6 % dissolved solids. The major options for treatment or bioconversion of whey into commercially important products, ethanol and β- galactosidase (Table: 4) which finds an increasing use because of growing lactose intolerant population. Thus, microbial beta-galactosidases production is an important area in whey utilization.

Oberoi et al. (2008) have found that *K. marxianus* NCIM 3465 showed greatest beta-galactosidase activity of 1.62 IU mg^{-1} dry weight using whey and cauliflower waste. Although a minor increase in enzyme production was seen by incorporating 5 % to 10 % cauliflower waste in whey, nearly 15% increase in beta-galactosidase production was observed when cauliflower waste level was increased to 20%. Supplementing whey with 20 % cauliflower waste also lowered the production time. Lactose concentration in whey, mainly responsible for increasing the BOD of the effluent water, decreased from 4.2 % to nearly 0 % at 24 h. Thus, this study established that both these by-products ⁄ residues could be effectively used for beta-galactosidase production at commercial scale [40]. In 2004, a strain of *K. lactis* M2 obtained from whey samples has maximum enzyme activity (up to 8103 EU/ml). This yeast strain could be of valuable application in bioconversion of whey [41]. Oda and Nakamura (2009) have found that NBRC 1963 of *K. marxianus* converted lactose [in media containing 20 % (w/v) sugar cheese whey] most economically to ethanol [42].

Several mathematical models have been developed for ethanol production from whey using beta-galactosidase. In 2007, mathematical models for semi-continuous ethanolic fermentation in a whey medium employing coimmobilized *S. cerevisiae* strain and β-D-galactosidase was developed. Kinetic parameters of biomass growth, experimentally determined fluxes of ethanol and water, kinetic constants of ethanol separation, the degree of sugar utilization and ethanol productivity, the time of ethanol separation were predicted using this model [43]. Hatzinikolaou et al. (2005) have meticulously examined kinetics and stability of beta-galactosidase of **a** wild type strain of *A. niger* in the direction of its potential use for the hydrolysis of acid whey permeate lactose. An incorporated process, concerning the simultaneous hydrolysis–ultrafiltration of whey lactose that included the specific kinetic properties of the beta-galactosidase was developed and modelled [44]. Ozmihci and Kargi (2007) have used lactose utilizing yeast strain, *K. marxianus* DSMZ-7239 for ethanol production from cheese-whey powder (CWP) solution in batch experiments and developed a kinetic model describing the rate of sugar utilization and substrate inhibition as function of the initial substrate and the biomass concentrations [45]. In 2009, a mathematical model for ethanol fermentation with yeast *S. cerevisiae* and beta-galactosidase on whey was developed [46].

Rech and Ayub (2007) have reported the consequence of simple feeding strategies to obtain high-cell-density cultures of *K. marxianus* maximizing β–galactosidase productivity using cheese whey as basic medium. The best fed-batch strategy was established to be the feeding of three-fold lactose concentration in the cheese whey-medium during 25 h, resulted in beta-galactosidase productivity of 291 U/L h, signifying an increase of more than 50 % compared to batch cultivations [47]. Bansal et al. (2007) have carried out studies related to beta-galactosidase production using whey

containing 4.4 % (w/v) lactose inoculated with *K. marxianus* MTCC 1389 and alleviated water pollution problems caused due to its disposal into the water streams [48]. Saad (2004) has demonstrated that submerged culture of *Aspergillus japonicus* produced β-D-galactosidase, with 2.95 U mg^{-1} protein specific activity, when developed on cheese whey permeate fortified with 0.5 % yeast after 4 days incubation at 28 °C. Rates of lactose hydrolysis in whey was about 55 %, after 4 h incubation at 45 °C. This enzyme was found suitable for obtaining fermentable sugars from whey wastes [49].

Table 4 Beta-galactosidases used for ethanol production from whey

Beta-galactosidase producers	Reference
K. marxianus NCIM 3465	[40]
K. lactis M2	[41]
K. marxianus NBRC 1963	[42]
S. cerevisiae	[43]
A. niger	[44]
K. marxianus DSMZ-7239	[45]
S. cerevisiae	[46]
K. marxianus	[47]
K. marxianus MTCC 1389	[48]
Aspergillus japonicus	[49]
Streptococcus thermophilus 95/2, *Lactobacillus delbrueckii subsp. bulgaricus* 77	[51]

In 2009, appropriate conditions for the production of beta-galactosidase from whey permeate has been evaluated. This enzyme is to be used in the production of lactose-hydrolyzed milk [50]. Tari et al. (2009) have investigated beta-galactosidase production by *Streptococcus thermophilus* 95/2 (St 95/2) and *Lactobacillus delbrueckii* ssp. *bulgaricus* 77 (Lb 77) in a medium containing whey (5%), corn steep liquor (4%), potassium phosphate (2 %) and peptone (2 %) at 43 ºC for 8 h using RSM. The associative growth provided 6.4 % and 39 % more beta-galactosidase activity using pure St 95/2 and Lb 77 strains, respectively [51]. Ozmihci and Kargi (2008) have studied ethanol fermentation of cheese whey powder solution using the pure culture of *K. marxianus* (DSMZ 7239). Total Sugar of 50 gL^{-1} was fermented to ethanol in a continuously operated packed column bioreactor (PCBR) using olive pits as support particles for cell attachment [52]. Thus, whey utilization by beta-galactosidase reduces the burden of water pollution and provides beneficial products like ethanol and protein concentrates.

4.3 Medical and veterinary applications of beta-galactosidase

Beta-galactosidase with its transgalactosylation property finds prominent medical applications such as treatment of disorders and development of digestive supplements. Anderson et al. (2005) have isolated an endo beta-galactosidase from *Clostridium perfringens* ATCC 10543 capable of liberating both the A trisaccharide and B trisaccharide from glycoconjugates containing blood group A and B glycotopes, respectively. Recombinant EABase damaged the blood group A and B antigenicity of human type A and B erythrocytes and also released A-Tri and B-Tri from blood group A^+- and B^+-containing glycoconjugates. The exceptional specificity of this beta-galactosidase should make it useful for studying the structure and function of blood group A- and B-containing glycoconjugates [53]**.** In 2009, a recombinant endo-beta-galactosidase (ABase), which releases A/B antigen was developed. It removed 82 % of A antigen and 95 % of B antigen in human A/B red blood cells, and concealed anti-A/B antibody binding and complement activation effectively. It was also found to remain active at 4 °C. *in vivo* infusion into a blood type A demonstrated a marked reduction of A antigen expression in the glomeruli of kidney (85 % at 1 h, 9 % at 4 h and 13 % at 24 h) and the sinusoids of liver (47 % at 1 h, 1 % at 4 h and 3 % at 24 h) without grave adverse effects. This substitute approach might be useful for minimizing antibody removal and anti-B cell immunosuppression as an adjuvant therapy in ABO-incompatible kidney, liver and possibly heart transplantation [54]. Liu and Roffler (2006) have examined the expression of *E. coli* beta-galactosidase in muscle fibers and concluded that repeated intramuscular injections of beta-galactosidase can encourage strong immune responses in immune-competent animals and cause abolition of transduced muscle fibers by inflammatory cells [55].

Recently, a beta-galactosidase from the mesoacidophilic fungus *Bispora sp.* MEY-1 under simulated gastric conditions, has shown greater stability (100%) and hydrolysis ratio (>80 %) toward milk lactose than the commercially available beta-galactosidase from *A. oryzae* ATCC 20423. Thus, this beta-galactosidase may be a superior digestive supplement for alleviating symptoms associated with lactase deficiency [56]. Sanchez-Aparicio et al. (2009) have developed recombinant β-D-galactosidases accommodating one or two different peptides from the foot-and-mouth disease virus nonstructural protein 3B per enzyme monomer that allowed differentiation between sera of FMDV-infected pigs, cattle, and sheep and those of naïve and conventionally vaccinated animals. These FMDV infection-

specific biosensors can provide an effective and versatile alternative for the serological distinction of FMDV-infected animals [57].

5. Individual molecule studies of beta-galactosidase

Individual molecule analysis of beta-galactosidase of *E. coli* give in-sights into its kinetic properties. Craig and Dovichi (1998) have incubated *E. coli* beta-galactosidase molecules with fluorogenic substrate. Individual molecules were found to differ with respect to their activities. Molecules showed a 23-fold distribution of activities with the bulk of molecules within a 4-fold distribution [58]. In 2000, it was observed that when *E. coli* ß-galactosidase stored at 25 ºC in buffer containing no added $MgCl_2$, over half the enzyme molecules became inactive. However, those molecules which retained activity remained dynamic for the duration of the experiment. This indicated that there exist two populations of *E. coli* ß-galactosidase, one which requires storage in the presence of the higher concentration of Mg^{2+} in order to remain active. [59].

Craig et al. (2003) have performed single enzyme molecule assays on *E. coli* beta-galactosidase from three different sets of samples. The first set consisted of lysates of induced cells from 5 diverse strains of the bacteria, as well as 2 dissimilar commercial preparations of the enzyme. These samples were found to have considerably different distributions of single molecule activities. For the second set of samples, beta-galactosidase expression was induced for 1.5 h, followed by further incubation where expression was subdued. Assays were performed on the lysates of the preinduction and on the lysates from aliquots taken set times post induction. The freshly induced enzyme had a 25 % higher average single molecule activity than the basally expressed enzyme. This average activity returned to the basal value 3.5 h post induction and remained unaffected thereafter. Finally, beta-galactosidase was induced at 26 and 42°C. The samples were found to be indistinguishable with respect to their average single molecule activities [60].

In 2003, it was found that individual molecules of beta-galactosidase molecules from the crystallized enzyme as well as the original enzyme preparation displayed a range of activity of 20-fold or greater. Molecules obtained from two diverse crystals had identical activity distributions of 31600 ± 1100 and 31800 ±1100 reactions min^{-1} (enzyme $molecule)^{-1}$. This activity was found to be drastically different from that of the enzyme used to grow the crystals, which showed an activity distribution of 38500 ±900 reactions min^{-1} (enzyme $molecule)^{-1}$[61].

Nichols et al. (2007) have induced beta-galactosidase in *E. coli* wild type strains ATCC 8677 and 35321 in the presence of various protease inhibitors. The presence of the protease inhibitors had a least effect on the average and distribution of single molecule activities. Assays performed on beta-galactosidase from strains 8677 and 35321 demonstrated that the relative activities of the enzyme for the diverse substrates differed between the strains. These two strains differ in their primary sequence by a single amino acid substitution in position 280, which is in the region of the active site [62]. Nichols and Craig (2008) have performed the single enzyme molecule assays of *in vivo* and *in vitro* synthesized beta-galactosidase from wild-type *E. coli* strains ATCC 35321 and 8677. The average combined turnover numbers of the enzyme from wild-type *E. coli* strains ATCC 35321 and 8677 synthesized *in vivo* and *in vitro* and with the presence and absence of a His_6 tag were found to differ considerably. This indicates that the *in vivo* and *in vitro* produced enzymes are not alike and the presence of a C-terminal His_6 tag alters the activity of ß-galactosidase [63].

Nichols and Craig (2008) have calculated the electrophoretic mobility and catalytic activity of individual molecules of *E. coli* beta-galactosidase. Together the mobility and activity were reproducible for each molecule but differed between individual molecules. There was no experimental relationship between the experimental activities and electrophoretic mobilities. If the finding that single beta-galactosidase molecules have heterogeneous electrophoretic mobility can be extended to other proteins, this may prevent the resolution possible for capillary zone electrophoresis protein separations [64]. In 2008, CE-LIF detection-based assay for the study of individual molecules of *E. coli* ß-galactosidase was developed. The assay allows for the real-time measurement of the electrophoretic mobility, catalytic activity and the difference in activity over time of single enzyme molecules. In adding together to showing the microheterogeneity of the enzyme molecules with respect to mobility and activity, it was established that at superior temperatures the enzyme activity changed over time. Incubation at different temperatures showed that single beta-galactosidase molecules exhibited differences in their change in activity upon a modification in incubation temperature. In addition, thermal denaturation was found to cause a quick and absolute loss of activity [65]. Thus, study of individual molecules of beta-galactosidase has shown the previously unstudied features of the enzyme.

6. Conclusion

Research and development in the beta-galactosidase will help to address the problems faced in the food and allied industries that look for enzymes with novel properties like cold-stability and thermo-active. Immobilization of beta-galactosidase will reduce the cost of production of food products and allow for reusage of the enzyme. Novel galactooligosaccharides production by beta-galactosidase will pave the way for development of prebiotics that can be used as food supplement. Whey utilization by beta-galactosidase will help to reduce the water pollution caused by lack of downstream-processing and lead to production of products like bioethanol and lactose-hydrolysed milk. Beta-

galactosidase's capability has been realised by the pleothera of products like biosensors, digestive supplements. Individual molecule study of beta-galactosidase has shown the various unknown kinetic properties of beta-galactosidase. Thus, research and development of beta-galactosidase finds application in several industries.

Acknowledgements: Authors thank Department of Science and Technology, New Delhi (SR/SO/BB-50/2007) for the Junior Research Fellowship to SSA through the Centre for excellence in Genomic Sciences. Authors thank University Grants Commission, New Delhi for providing facility and financial support through Networking Resource Centre in Biological Sciences, School of Biological Sciences, Madurai Kamaraj University. SSA thanks all the authors listed in references for their generous help by sending their publication.

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