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Review

Recombinant β-galactosidases – Past, present and future: A mini review

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ABSTRACT

The present review deals with current developments of novel β-galactosidases derived from recombinant vectors and by protein engineering approaches together with the use of efficient recombinant microbial production systems in order to present the applications of recombinant enzymes as a relevant synthetic tool in biotechnology. The union of specific physical and chemical properties of recombinant proteins with specific recognition of catalytic properties of biomolecules has led to their appearance in myriad novel biotechnological applications. The interest in exploiting recombinant enzymes as biocatalysts is constantly increasing nowadays. The plausible advantages involved with their use include their (1) rigidity and permeability, (2) hydrophobic/hydrophilic character, (3) ease of purification and large-scale production, (4) immediate separation from the reaction mixture after completion of reaction without using any chemicals or heating, (5) regenerability as they impart stability to enzymes by protecting their active sites from deactivation, and (6) recombinant enzymes can be conveniently tailored within utility limits. β-Galactosidase is one of the relatively few enzymes that have been used in large-scale processes to perform lactose hydrolysis and galacto-oligosaccharide production. Thus, the present article gives brief outline of recombinant β-galactosidases obtained from various mesophilic, psychrophilic and thermophilic sources and their potential applications in biotechnology industry.

Contents

1. Introduction

β-Galactosidases (E.C. 3.2.1.23) catalyzes the hydrolysis of lactose to glucose and galactose and transfers the galactose formed from lactose cleavage onto the galactose moiety of another lactose to yield galacto-oligosaccharides (GOS) which are utilized as growth promoting substrates of bifidobacteria in human intestine [1–3]. The parameters and price of β-galactosidases are major attributes which determine the technology and relative costs of lactose hydrolysis and galacto-oligosaccharide production as ascertained by its direct addition to the substrate which is economically unacceptable due to low value of whey as its waste product [4,5]. The enzymatic properties of recombinant β-galactosidases suggest that they bring efficient conversion of lactose in dairy products via their structural modification. Studies are therefore in progress on the large-scale application of recombinant β-galactosidases for degradation of lactose [6]. The drive for cost-cutting efficiencies in the heating/cooling steps of biotechnological processes and for increase in the recovery of the products of enzymatic reactions has led to an increased interest in the use of β-galactosidases isolated from psychrophilic and thermophilic microorganisms [7,8]. Compared to animal and plant sources, β-galactosidases from microorganisms are produced in

Abbreviations: *E. coli*, *Escherichia coli*; GOS, galacto-oligosaccharides; ONPG, o-nitrophenylβ-D-galactopyranoside; ORFs, open reading frames.

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higher yields and thus are more technologically important. The major enzymes of commercial interest are isolated mainly from the yeast *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Kluyveromyces marxianus*, *Candida kefyr*, *Saccharomyces cerevisae* and the fungi *Aspergillus niger*, *Aspergillus oryzae* [9,10]. Considerable knowledge of active sites of such enzymes will enable methods to be chosen that would avoid reaction with their essential groups protected. Thus, this technology protects enzyme inhibition by retaining their tertiary structure and provides enhanced stability to them against physical and chemical denaturants for greater production of lactose and GOS. Moreover, other recombinant enzymes that have been exploited in the recent past for other biotechnological potential include proteinases, lipases, amylases and cellulases (detergent formulations), dehydrogenases (environmental biosensors), peroxidases (bioremediation), and methylases and aminotransferases (biotransformations).

2. Thermostable β-galactosidases

Enzymes from thermophilic microorganisms are particularly attractive for their thermostability and resistance to organic solvents thereby giving favorable equilibrium for trans-glycosylation reactions [11,12]. High temperature resulted in increased initial productivity of enzyme and higher solubility of substrates in aqueous phase. The reduced water activity resulting from high substrate concentration and added organic solvent increases the trans-glycosylation reaction. Thermostable enzymes are thus gaining considerable interest in industries since they give better yields at higher temperatures (Table 1).

A number of thermostable β-galactosidases have been isolated previously from both mesophilic eubacteria and archaeabacteria. They were characterized and employed to hydrolyze lactose and in producing GOS [13–17]. However, most thermostable enzymes are synthesized at very low levels by thermophilic bacteria or archaeabacteria and are therefore cumbersome to purify. Thus, their large scale production was achieved at industrial scale by producing them in mesophilic hosts using recombinant techniques [18,19]. The efficiency of recombinant thermostable β-galactosidases obtained from *Thermus* sp., *Pyrococcus furiosus*, *Thermotoga maritima*, *Sulfolobus solfataricus* and *Geobacillus stearothermophilus* had been reported in pursuit of GOS production at high temperatures. They exhibited several advantages over native enzymes including their ease of purification, large-scale production and improvements in their activity [16,20–23].

LacZ gene from *Thermotoga maritima* β-galactosidase was cloned on 11 kb fragment by complementation of an *Escherichia coli* LacZ deletion strain. The nucleotide sequence of structural gene and two other open reading frames (ORFs) found within a 6317-bp region were investigated. The deduced amino acid sequence of *T. maritima* β-galactosidase exhibited a 1037 amino acid polypeptide with a calculated M_r of 122312. The translated sequence was 30% similar to nine other β-galactosidase sequences from bacteria and yeast. Alignment of *T. maritima* β-galactosidase with other sequences revealed that the residues responsible for Mg^{2+} binding, catalysis and substrate recognition are conserved in thermophilic enzymes. Sequence analysis also exhibited the presence of a divergently transcribed operon containing two other genes 5′ to LacZ. These ORFs encode proteins homologous to a second family of β-galactosidase found in *Bacillus* sp. and to an ATP-dependent family of bacterial oligopeptide transport proteins [24]. BgaA gene for *T. maritima* β-galactosidase was also cloned and characterized by Gabelsberger et al. [25]. However, *T. maritima LacZ* gene (TM1193) was considered as a putative gene since it was not cloned correctly and its product was not characterized due to the difficulty of its expression in an active form in *E. coli* [26]. Another thermostable β-galactosidase gene, BgaA, from *Thermus* sp. was expressed in *E. coli* as a fusion protein of BgaA with a histidine tag [27,28]. A β-galactosidase isoenzyme, β-GalI, from *Bifidobacterium infantis* HL96, was expressed in *E. coli*. It exhibited strong transgalactosylation activity. The optimum temperature and pH for ONPG and lactose were 60 °C, pH 7.5 and 50 °C, pH 7.5, respectively. The enzyme showed inhibition in presence of *p*-chloromercuribenzoic acid, divalent metal cations, Cr^{3+}, EDTA and urea. K_m and V_{max} values with ONPG and lactose as substrate were 2.6 mM, 262 U/mg and 73.8 mM, 1.28 U/mg, respectively. The rate of GOS production from 20% and 30% lactose solution was 120 mg/mL and 190 mg/mL, respectively [29].

A thermostable β-galactosidase gene bgaB from bacterium, *Bacillus stearothermophilus* was cloned and expressed in *Bacillus subtilis* WB600. It exhibited pH and temperature optimum at pH 7.0 and 70 °C, respectively, and K_m and V_{max} values of 2.96 mM and 6.62 μM/min/mg, respectively. Half-life for this thermostable enzyme was 50 h and 9 h at 65 °C and 70 °C, respectively. Findings of the work suggested that this enzyme was suitable for both hydrolysis of lactose as well as in the production of GOS in milk processing [30]. Several recombinant proteins were successfully expressed using δ-integrative systems. Oliveira et al. [31] constructed stable flocculent *Saccharomyces cerevisiae* strains producing *Aspergillus niger* β-galactosidase which was later on employed in a continuous bioreactor for lactose hydrolysis. Moreover, Slepak et al. [32] had shown a mechanism in which production of yeast β-galactosidase was used to measure estrogenic activity of some chemical compounds. They showed that yeast estrogen assay makes use of recombinant yeast cells that have estrogen receptor expression cassette and a reporter construct coding for β-galactosidase. The induction mechanism starts with the binding of estrogenic compounds to the estrogen receptor and this complex activates β-galactosidase production. Moreover, β-galactosidase was expressed in *Pichia pastoris* in a defined medium containing metals where magnesium and zinc ions were required to support their production. The product yield of this recombinant β-galactosidase was significantly influenced by the concentration of trace metals [33].

Di Lauro et al. [34] reported the purification and characterization of bacterial β-galactosidase from thermoacidophilic bacterium *Alicyclobacillus acidocaldarius*. The cloning, expression and the characterization of this recombinant enzyme (Aaβ-gal) were investigated and found to be optically active and stable at 65 °C. Similarly, Nguyen et al. [35,36] showed the cloning of β-galactosidase from *Lactobacillus reuteri* L103 and its expression in *E. coli*. These studies also revealed a significant improvement in lactose hydrolysis which proved useful in dairy industries. Moreover, *Pyrococcus woesei* gene

Table 1
Efficiency of GOS production by recombinant β-galactosidases.

Source of recombinant β-galactosidase	Temperature (°C)	pH	% GOS (g/L)	Productivity (g/L/h)	Reference
Thermus sp.	70	7.0	30 (91)	–	Akiyama et al. [16]
Pyrococcus furiosus	80	5.0	22 (60)	–	Bruins et al. [68]
Thermotoga maritima	80	6.0	19 (97)	18	Ji et al. [21]
Bifidobacterium infantis	60	7.5	63 (190)	13	Jung and Lee [67]
Sulfolobus solfataricus	80	6.0	53 (315)	5.6	Park et al. [22]
Geobacillus stearothermophilus R109W	37	6.5	23 (41)	6.9	Placier et al. [23]

coding thermostable β-galactosidase was cloned into pET30-LIC expression plasmid. The nucleotide sequence revealed that *P. woesei* β-galactosidase consisted of 510 amino acids and M_r of 59 kDa. It showed 99.9% nucleotide homology with *Pyrococcus furiosus* β-galactosidase. It was produced in high yield by *E. coli* strain and was easily separated by thermal precipitation from other bacterial proteins at 85 °C [37]. Here, they exhibited a new expression system for producing *P. woesei* β-galactosidase in *E. coli* and one-step chromatography purification procedure for obtaining pure enzyme (His6-tagged β galactosidase). This recombinant β-galactosidase contained a poly histidine tag at N-terminus (20 additional amino acids) that allowed single-step isolation by affinity chromatography. The enzyme was purified by heat treatment (to denature *E. coli* proteins) followed by metal-affinity chromatography. The enzyme was characterized and displayed high activity and thermostability. This bacterial expression system appeared to be a good technique for the production of thermostable β-galactosidase [38].

A novel heterodimeric β-galactosidase was obtained from *Lactobacillus pentosus* KUB-ST10-1 using ammonium sulfate fractionation followed by hydrophobic interaction and affinity chromatography. The electrophoretically homogenous enzyme showed molecular mass of 105 kDa, temperature optimum at 65 °C and had a specific activity of 97 U (ONPG)/mg protein. The K_m, k_{cat} and k_{cat}/K_m values for lactose and ONPG were 38 mM, 20/s, 530/M/s and 1.67 mM, 540/s, 325,000/M/s, respectively. A maximum yield of 31% GOS of total sugars was obtained at 78% lactose conversion by *Lactobacillus pentosus* β-galactosidase. The enzyme showed a strong preference for the formation of β-(1 → 3) and β-(1 → 6) linkages, and the main transgalactosylation products identified were the disaccharides β-D-Galp-(1 → 6)-D-Glc, β-D-Galp-(1 → 3)-D-Glc, β-D-Galp-(1 → 6)-D-Gal, β-D-Galp-(1 → 3)-D-Gal, and the trisaccharides β-D-Galp-(1 → 3)-D-Lac, β-D-Galp-(1 → 6)-D-Lac [39].

Pessela et al. [40] have developed a strategy to purify and immobilize proteins onto standard epoxy supports with good yields by using poly-His-tagged β-galactosidase from *Thermus* sp. strain T_2 overexpressed in *E. coli* (MC1116) as a model enzyme. These novel epoxy-metal chelate heterofunctional supports contained low concentration of Co^{2+} ions chelated in iminodiacetate groups and high density of epoxy groups. It enabled selective adsorption of poly-His-tagged enzymes, thereby allowing maximum loadings of the target enzyme either by using its pure or crude preparation. Recombinant β-galactosidase thus obtained exhibited higher activity at 70 °C as compared to its soluble counterpart. Moreover, a recombinant poly-His tagged multimeric β-galactosidase from *Thermus* sp. strain T2 was cloned in *E. coli* and used to evaluate healthy prospects of certain novel dextran-coated IMAC support [41]. A recombinant thermostable β-galactosidase from *B. stearothermophilus* was immobilized onto chitosan using Tris(hydroxymethyl)phosphine and glutaraldehyde to hydrolyze milk lactose by packed bed reactor. Immobilized recombinant thermostable β-galactosidase showed greater relative activity in presence of Ca^{2+} than the free enzyme and was stable during storage at 4 °C for 6 weeks whereas the free enzyme lost 31% of initial activity under same storage conditions. It exhibited 80% lactose hydrolysis in milk after 2 h of operation in the reactor [42].

Thermostable β-galactosidase gene bgaB from *B. stearothermophilus* was cloned and expressed in *B. subtilis* WB600 by Chen et al. [42]. The recombinant enzyme exhibited molecular mass and isoelectric point of 70 kDa and 5.1, respectively, while pH and temperature optimum for this enzyme were at pH 7.0 and 70 °C, respectively. Kinetics of thermal inactivation and half-life for recombinant thermostable β-galactosidase at 65 and 70 °C were 50 and 9 h, respectively, and K_m and V_{max} values were 2.96 mM and 6.62 μM/min/mg. The results suggested that this recombinant thermostable enzyme may be suitable for both hydrolysis of lactose and production of GOS in milk processing. Xia et al. [43] reported the extracellular secretion of a cytoplasmic thermostable β-galactosidase from *Geobacillus stearothermophilus* IAM11001 in *B. subtilis*. Defined and rich culture media were used for recombinant enzyme production and the extracellular target enzymatic activity reached about 44% of the total enzymatic activity synthesized at 18 h of cultivation in LB medium. This study revealed that co-expression of the *B. subtilis* proteins, TatAd and TatCd, were indispensable for the secretion of target enzyme.

Volkov et al. [44] have determined the nucleotide sequence of a 4936-bp genomic DNA fragment from the thermophilic bacterium *Thermoanaerobacter ethanolicus*. The fragment contained three ORFs. One of the ORFs corresponded to the *LacA* gene for a thermostable β-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. Thus, thermostable β-galactosidases play a vital role in hydrolysis of lactose and GOS production.

3. Mesophilic β galactosidases

The biotechnological interest of mesophilic enzymes was motivated by their ability to function under conditions that normally denature thermophilic enzymes. *Kluyveromyces lactis* β-galactosidase *lac4* gene was expressed in *E. coli* as soluble His-tagged recombinant enzyme under optimized culture conditions. The expressed protein was multimeric with a subunit molecular mass of 118 kDa. The purified enzyme required Mn^{2+} ions for activity and was inactivated irreversibly by imidazole above 50 mM. The activity was optimal at 37 °C and 40 °C for ONPG and lactose, respectively. It exhibited K_m and V_{max} values of 1.5 mM and 560 μM/min/mg, and 20 mM and 570 μM/min/mg with ONPG and lactose as substrate, respectively [45]. The utilization of such *K. lactis* β-galactosidase had increased lactose hydrolysis and prevented the formation of non-enzymatic browning products which are generally formed at lower temperatures.

A flocculent *S. cerevisiae* strain secreting *A. niger* β-galactosidase activity was constructed by transforming *S. cerevisiae* NCYC869-A3 strain with plasmid pVK1 harboring *A. niger* β-galactosidase gene, *LacA* [46]. It exhibited higher level of extracellular β-galactosidase activity and allowed enzyme production with high productivity in continuous fermentation systems with downstream processing. Moreover, it provided an alternative method to produce fungal β-galactosidases since the enzyme was produced in pure form. In another study, cloning and expression of genes encoding heterodimeric β galactosidase from *L. reuteri* L103, *Lactobacillus acidophilus* R22, *Lactobacillus plantarum* WCFS1 and *Lactobacillus sakei* Lb790 was investigated. These enzymes consist of two subunits of approximately 73 and 35 kDa which are encoded by two overlapping genes, lacL and lacM, respectively. The presented results lead the way to efficient overproduction of β-galactosidase in a food-grade expression system which is of high interest for applications in food industry [47].

The recombinant β-galactosidase from *K. lactis* was expressed as a C-terminal His-tagged fusion protein in *E. coli* and purified in order to obtain highly efficient model for both hydrolysis and transgalactosylation reactions with glucose and lactose as acceptors. It was found that galactose acted as an inhibitor at its lower concentration. Moreover, the addition of glucose at lower concentrations (≤50 mM) resulted in an increased reaction rate [48]. *Candida famata* mutant *lac4* gene which was not able to utilize lactose as recipient strain was later on employed in recombinant system for the hydrolysis of lactose. It was transformed with the plasmid containing analyzable promoters fused with the promoterless *lac4* gene. The resulting transformants were able to hydrolyze lactose

successfully [49]. In another study, recombinant β-galactosidase was produced from human isolate *Bifidobacterium breve* B24 and characterized for the glycoside transferase (GT) and glycoside hydrolase (GH) activities on lactose [50]. The recombinant enzyme shown by activity staining and gel-filtration chromatography was composed of a homodimer of 75 kDa with a total molecular mass of 150 kDa. The K_m value for lactose (95.58 mM) was 52.5-fold higher than corresponding K_m values for the synthetic substrate ONPG (1.82 mM). It showed its pH and temperature optimum at pH 7.0 and 45 °C. About 97.00% of lactose in milk was hydrolyzed by this enzyme (50 units) at 45 °C for 5 h to produce 46.30% of glucose, 46.60% of galactose and 7.10% of GOS. The results suggest that this recombinant β-galactosidase may be suitable for both the hydrolysis and synthesis of GOS in milk and lactose processing.

Samoylova et al. [51] reported the cloning of a feline β-galactosidase cDNA into a mammalian expression vector and its subsequent expression in Chinese hamster ovary (CHO-K1) cells. The enzyme exhibited specific activity on two synthetic substrates as well as on the native β-galactosidase substrate, GM1 ganglioside. The enzyme was purified from transfected CHO-K1 cell culture medium by chromatography on PATG-agarose. The affinity-purified enzyme preparation consisted mainly of the protein with approximate molecular weight of 94 kDa and displayed immunoreactivity with antibodies raised against a 16-mer synthetic peptide corresponding to C-terminal amino acid sequence deduced from the feline β-galactosidase cDNA. Moreover, Hu et al. [52] investigated the gene encoding an acid-stable β-galactosidase from *A. niger* van Tiegh and expressed in *P. pastoris*. The purified recombinant protein exhibited increased potential to rationally engineer *A. niger* van Tiegh enzyme to relieve product inhibition and create mutants with improved application-relevant kinetic properties for treatment of lactose intolerance as compared to that of native enzyme.

A putative recombinant β-galactosidase from *Deinococcus geothermalis* was purified as a single 79 kDa band with 42 U/mg activity by using His-Trap affinity chromatography. The native enzyme was a 158 kDa dimer. The catalytic residues E151 and E325 of β-galactosidase from *D. geothermalis* were conserved in all aligned GH family 42 β-galactosidases, indicating that this enzyme is also a GH family 42 β galactosidase. Maximal activity of the enzyme was at pH 6.5 and 60 °C. It has a unique hydrolytic activity for *p*-nitrophenyl (*p*-NP)-β-D-galactopyranoside (k_{cat}/K_m = 69/s/mM), *p*-NP-β-D-fucopyranoside (13), *o*-NP-β-D-galactopyranoside (9.5), *o*-NP-β-D-fucopyranoside (2.6), lactose (0.97), and *p*-NP-α-L-arabinopyranoside (0.78), whereas no activity or less than 2% of the *p*-NP-β-D-galactopyranoside activity for other *p*-NP- and *o*-NP- glycosides [53].

4. Psychrophilic β 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 and cheese whey bioremediation at low temperatures. 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 [54].

The drive for cost cutting efficiency in heating and cooling steps of biotechnological processes in improving the recovery of the products of enzymatic reactions has led to an increasing interest in using enzymes isolated from psychrophilic microorganisms. Psychrophilic enzymes demonstrated huge biotechnological potential in detergent formulations (*e.g.* proteinases, lipases, amylases and cellulases), dairy industry (*e.g.* β galactosidase), environmental biosensors (*e.g.* dehydrogenases), bioremediation (*e.g.* oxidases) and for biotransformations (*e.g.* methylases and aminotransferases). They have developed adaptive mechanisms in bringing efficient enzymatic reactions even at 0 °C but the major drawback of these cold active enzymes is their thermolability, *i.e.* they are inactivated at moderate temperatures at which mesophiles and thermophiles remained functional [55,56].

Psychrophilic β-galactosidases are potentially useful (i) for fast lactose digestion at low temperatures to produce lactose-free milk products, (ii) to avoid lactose crystallization in dairy products, and (iii) to degrade their bulk pollutants in dairy sewage thereby reducing environmental pollution. Thus, an attention was drawn in recent years to study cold active β-galactosidases from different sources like *Arthrobacter psychrolactophilus*, *B. subtilis* KL88, *Carnobacterium piscicola* BA, *Planococcus* and *Pseudoalteromonas haloplanktis*, *Pseudoalteromonas* sp. 22b, etc. The optimal properties and kinetic parameters of β-galactosidases from these sources were investigated and employed for the production of GOS and lactose hydrolysis. It was observed that these enzymes exhibited greater activity in temperature range from 0 to 20 °C [57–64].

Ciemlijskia et al. [65] isolated gram-negative antarctic bacterium *Pseudoalteromonas* sp. 22b from the alimentary tract of krill *Thyssanoessa macrura* which produced an intracellular cold adapted β galactosidase. The enzyme displayed temperature optimum at 40 °C and exhibited broad pH optimum from pH 6.0 to 8.0. It retained 20% activity at 10 °C. Moreover, the enzyme lost its entire activity within 10 min at 50 °C. It hydrolyzed 90% milk lactose at 30 °C in 6 h and at 15 °C in 28 h. Thus, it could be applied at refrigeration temperatures for producing lactose-reduced dairy products. Nakagawa et al. [66] have overexpressed a cold-stable β-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 °C, its most favorable temperature was 10 °C, and it was achievable to swiftly inactivate the rBglAp at 50 °C 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 °C. rBglAp is a cold-active and extremely heat labile enzyme and has major possible application to the food industry.

5. Structural modification of β-galactosidases by site-directed mutagenesis

In vitro directed evolution through DNA shuffling is a powerful molecular tool for creating new biological phenotypes. *E. coli* β-galactosidase is an efficient model system for studying enzyme thermostability and for optimizing a strategy for deducing structure-function relationships of directed evolution *in vitro* [67,68]. Thus, *E. coli* β-galactosidase was characterized to study its biological function, catalytic mechanism and molecular structures. Bader et al. [69] replaced Glu-461 of *E. coli* β-galactosidase by Gln using site-directed mutagenesis. Kinetic studies on the purified Q461-β-galactosidase showed its less wild type activity with ONPG as substrate and more susceptible to thermal denaturation than wild type enzyme. K_m values of wild and mutant enzyme remains same thereby indicating that binding of substrate was not decreased by this change. Similar studies were carried out by replacing Tyr-503 of *E. coli* β-galactosidase with Phe. The kinetic and stability properties of the mutant enzyme showed that this site directed mutagenesis resulted in decrease in activity due to

loss of a catalytic group rather than a detrimental change in overall structure/binding capacity of the enzyme [70].

It was observed that an *in vitro* directed evolution strategy through DNA shuffling resulted in five mutants named YG6764, YG6768, YG6769, YG6770 and YG6771 after two rounds of DNA shuffling and screening, which exhibited more β-glucuronidase activity than wild-type β-galactosidase. These variants had mutations at fourteen nucleic acid sites and exhibited changes in ten amino acids: S193N, T266A, Q267R, V411A, D448G, G466A, L527I, M543I, Q626R and Q951R [71]. Moreover, *E. coli* β-galactosidase variants with enhanced β-fucosidase activity were also evolved by DNA shuffling and site saturation mutagenesis [72,73]. In another study, Glu-416 of E. coli β-galactosidase was replaced with Gln and Val using site-directed mutagenesis. The substituted enzyme displayed greatly decreased sensitivity to Mg^{2+}. Equilibrium dialysis studies showed that wild type β-galactosidase bound Mg^{2+} tightly as compared to E416V β-galactosidase. pH–activity profile of E416V β-galactosidase was unaffected by the presence or absence of 1 mM Mg^{2+} along with the inactivation of substituted enzymes by Mg^{2+}. Moreover, the substituted enzymes displayed dramatically lowered affinity for ONPG and Isopropyl β-D-1-thiogalactopyranoside (a substrate analog inhibitor) in both the presence and absence of Mg^{2+} ions [74]. Xiong et al. [75] have investigated directed evolution on a chemically synthesized 1533 bp recombinant β-galactosidase gene from *P. woesei*. Twenty thousand variant colonies in each round of directed evolution were screened using pYPX251 vector and host strain Rosetta-Blue. One mutant (named YG6762) was obtained by shifting β-galactosidase to β-glucuronidase after four rounds of directed evolution and screening. This mutant had eight mutated amino acid residues. T29A, V213I, L217M, N277H, I387V, R491C, and N496D were key mutations for high β-glucuronidase activity while E414D was not essential because the mutation did not lead to a change in β-glucuronidase activity. The amino acid site 277 was most essential because mutating H back to N resulted in 50% decrease in β-glucuronidase activity at 37 °C. Any change in YG6762 variant exhibited greater β-glucuronidase activity than the wild type enzyme at different temperatures.

6. Conclusion

This review provides an overview of the general properties of recombinant β-galactosidases and the multiple techniques involving their biotechnological utilization along with the current possibilities of their transfer into alternative organisms through genetic engineering. Emphasis is also made on the potential that some of the latest technologies such as generation of transgenics will benefit in using the related genes (*e.g.* Lac genes). Research and development in β-galactosidases will help to address the problems faced in food and allied industries that look for enzymes with novel properties like cold-stability and thermo-stability. Enzymes from thermophilic microorganisms are particularly attractive due to their ability to function under conditions that normally denature mesophilic enzymes. The sequence alignment of recombinant β-galactosidases studied by several workers revealed that conservation of amino acids residues is involved in their improved catalysis. It was found that Pro and Arg residue content and Arg/Arg + Lys ratio was lower for cold-adapted β galactosidase as compared to their mesophilic counterpart which might be the possible reason for adaptation of these enzymes in bringing efficient catalysis at lower temperatures. Besides, several recombinant β-galactosidases with improved lactose hydrolysis and GOS production by reduced product inhibition via different procedures and their structural modification by site-directed mutagenesis have also been reviewed. Therefore, recombinant β-galactosidases would constitute an excellent tool in near future for improving the productivity of GOS as well as in hydrolyzing lactose in batch as well as in continuous systems. Thus, research and development of β-galactosidases finds application in several industries.

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