Purification of new β-galactosidase from *Enterococcus faecium* MTCC 5153 with transgalactosylation activity

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Abstract

A new β-galactosidase (β-gal) was purified from a lactic acid bacterial strain of *Enterococcus faecium* MTCC5153 by chromatographic techniques. The purified enzyme had a specific activity of 24.06 U/mg of protein with k_m and V_{max} values of 2 mM and 18.2 mM/min/mg of protein, respectively. The yield of purified β-gal was 10.65% and estimated molecular weight found to be ~90 kDa, consisting of two homodimeric subunits of 43kDa. The enzyme was stable in pH range of 8.0-9.0 with an optimum pH of 8 and the optimum temperature of 40°C. In presence of metal ions such as Mg^{+2}, Mn^{+2}, Ca^{+2}, K^{+} and Na^{+} enzyme was activated and was inhibited by Zn^{+2}, Co^{+2} and Cu^{+2}. Chemical modifiers viz. N-bromosuccinamide and Diethylpyro carbonate, inactivated the enzyme indicating the role of tryptophan and histidine moieties for activity. The purified β-gal was able to synthesize oligosaccharides from lactose. This study assume that the β-gal of *Enterococcus faecium* MTCC5153 could be applied in dairy industry for hydrolysis of lactose and to improve its digestibility. β-gal of probiotic cultures are of particular interest due to their transgalactosylation properties.

Key words: *E. faecium*, β-galactosidase, Prebiotics and transgalactosylation

Introduction

β-galactosidase (β-D-galactoside-galactohydrolase; β-gal; E.C 3.1.2.23) catalyzes hydrolysis reactions that have wide applications in dairy industries. Low lactose containing dairy products generally decrease the symptoms associated with lactose intolerance (Nakayama and Amachi, 1999). Hydrolysis of lactose is important for the production of refrigerated dairy products to overcome the technological difficulties that occur with lactose crystallization (Vasiljevic and Jelen, 2001). Enzymatic hydrolysis of lactose in milk has been considered as best alternative in food industry as it can be done in mild temperature and pH conditions when compared to acid hydrolysis (Sener et al, 2001). Other interesting property of β-gal is transgalactosylation activity, that has enormous potential in synthesizing novel oligosaccharides (Sako et al, 1999, Toba et al, 1980). In addition, the β–Gal encoding gene can be used as a reporter in molecular biology for the construction of food-grade vectors (Schenborn and Groskreutz, 1999). β-gal is reported to be present in diverse sources, such as plants, animal, microbial sources etc (Gekas and Lopez-leiva, 1985). Among them lactic acid bacteria (LAB) is one of the important microbial sources of β-gal due to their GRAS status (Vinderola and Reinheimer, 2003).

Enterococci constitute major group of LAB and also a natural flora of different fermented foods (Girraffa, 2002). Some species of entercocci are being used as adjunct and as non-starter cultures in dairy product and fermented sausages (Girraffa, 2003). These bacterial cultures apart from their role in development of organoleptic character also produce bacteriocins in the fermented foods (Girraffa, 2002). Reports on purification and characterization of β-gal from LAB such as *Lactobacillus* sp., *Bifidobacterium* sp and *Streptococcus* sp. etc. are known (Table 1). However to our knowledge, no information is available about characterization of β-gal from

enterococci and *Enterococcus faecium* (*E. faecium*) in particular. We have previously reported some of the probiotic properties of *E. faecium* MTCC5153 (Badarinath and Halami, 2009). In this communication, we report the purification and partial characterization of β-gal produced by *E. faecium* MTCC5153.

Materials and methods

Bacterial strain, culture conditions and fine chemicals

The LAB strain used in this study was a native isolate of *E. faecium* known to produce a potent antilisterial bacteriocin. An Indian patent has been obtained on the process (Halami and Chandrashekar, 2010*)*. de Man, Rogosa and Sharpe (MRS) medium, M17 medium, yeast extract, beef extract and sugars like lactose, glucose, raffinose and galactose were procured from Hi-media laboratories, Mumbai (India). Acrylamide, bis-acrylamide, Urea, 1,4dithiothreitol (DTT), Sodium dodecyl sulphate, TEMED and 4-methyl β-D-umbellyferyl β-D-galactopyranoside were procured from SRL laboratory chemicals Pvt. Ltd., Mumbai. Molecular weight marker kit (14.5-99 kDa) was procured from Bangalore Genei Pvt, Ltd., Bangalore, India. 2-mercaptoethanol, N-bromosuccinimide (NBS), Phenyl methyl sulfonyl fluoride (PMSF), Dithio-bis 2-nitro-benzoicacid (DTNB) and Diethylpyro carbonate (DEPC) were purchased from Sigma-Aldrich, St. Louis, USA. DEAE cellulose and Sephadex G-150 was purchased from Amersham biosciences. All the chemicals used in this study were of analytical grade. For the cultivation and maintenance of *E. faecium* MTCC5153, MRS medium was used and it was grown at 37ºC under static condition.

β-gal extraction

The MRS medium containing 2% lactose (MRS-L) as a carbon source, was used for β-gal production. Freshly grown culture (1 % (v/v), ~10^8 CFU/ml) was inoculated into 1000 ml of MRS-L medium and incubated at 37°C for 12 h under static condition. After incubation period, the cells were harvested by centrifugation (Remi CL-30, India) at 6000 X g for 10 min at 4°C. The precipitate was washed twice with 0.05M Buffer A (Sodium phosphate buffer (pH 7.0) containing 1 mM Mg^{+2} ions). The cell pellet was grounded using mortar and pestle with liquid nitrogen in Buffer A. The resulting suspension was collected and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was used as a crude β-gal preparation.

Determination of β-galactosidase activity

β-gal activity assay was carried out according to the method described by Nuygen et al. (Nguyen et al, 2007). Specific activity of β-gal was calculated as micromolar of *o*NP released per microgram of protein per min at a given temperature. Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard.

Enzyme purification

The enzyme portion was precipitated by 60% ammonium sulphate saturation and the pellet was collected by centrifugation at 10,000 rpm for 30 min at 4°C. The pellet was dissolved in 1/10th volume of Buffer A and dialyzed against the same buffer. The resulted sample was applied to DEAE cellulose ion-exchange column (30 X 2 cm, 20 ml) equilibrated with Buffer A and eluted with a salt gradient of 0 to 0.5M NaCl concentration. The active fractions were collected and pooled. It was further purified by gel permeation chromatography on Sephadex G-

150 (90 X 1.3 cm) column. The sample was eluted at a flow rate of 6 ml/h. The active fraction was pooled and purity was confirmed by SDS-PAGE.

Molecular weight determination

Molecular weight of the β-galactosidase was determined by gel permeation chromatography on Sephadex G-150 column using standard marker proteins (Sigma Aldrich, Bangalore).

Denaturing and native polyacrylamide gel electrophoresis (PAGE) was performed according to the methods described by Nuygen et al (2006). Native PAGE was performed on 10% (w/v) acrylamide gel in the absence of SDS and β-mercaptoethanol. After electrophoresis, half of the gel was used for activity staining using 4-methyl umbelleferyl β-D galactopyranoside (4 mg/ml in Buffer A) as chromogenic substrate.

MALDI-TOF analysis

The β-gal protein spots obtained on non-denaturing gel (corresponding to fluorescent band) was excised and sent for MALDI TOF (PMF) analysis at Vimta labs, Hyderabad India.

Determination of kinetic constants

To determine the effect of substrate concentration on enzyme activity, *o*-nitrophenyl β-D galactopyranoside (*o*NPG) concentrations ranging from 0.05 to 5mM in buffer A was used. The *Km*, *Vmax* were calculated from Lineweaver-Burk Plot.

Optimum pH and temperature

Effect of pH on enzyme activity was measured at varying pH in 50mM citrate buffer (pH 4.0-5.5), 50mM phosphate buffer (pH 6.0-8.0), and 50mM carbonate buffer (pH 9.0). All the buffers contained 1mM Mg^{2+} ions. Stability of the enzyme at different pH (3.0 to 8.0) was determined by incubating 10 µl of enzyme sample with 30 µl of each 50 mM of respective pH buffer (4.0-9.0) and kept for incubation at 4°C for 12 h. After incubation, 20 µl of reaction mixture was analyzed for residual activity under standard assay conditions. The relative activity at different pH was calculated by taking the maximum activity obtained as 100%.

The optimum temperature for the β-gal activity was measured by assaying the enzyme activity over a range of temperatures (20-60°C) in buffer-A. Buffer A was pre-equilibrated to the set temperature before addition of the enzyme solution. The temperature stability of enzyme was obtained by incubating the enzyme sample at different temperatures for 15 min in phosphate buffer (50 mM, pH 7.0) in presence of Mg^{+2} ions, at a final concentration of 0-10 mM. After rapid cooling of the sample on ice, the residual activity was determined under standard assay conditions.

Effect of reagents and cations on enzyme activity

The enzyme activity was determined with 22mM of *o*NPG in 50mM phosphate buffer (pH7.0) in presence of cations (10mM each of Mg^{+2}, Mn^{+2}, K^{+}, Na^{+}, Ca^{+2}, Zn^{+2}, Co^{+2} and Cu^{+2}) and reagents (1 to 10 mM of 2-mercaptoethanol, EDTA, DTT and Urea) in reaction mixture and the reaction was carried out under standard assay conditions. Reaction carried out without addition of cations was used as control.

Chemical modification studies

The assay was performed according to the method described by Kestwal and Bhide (2007). Briefly, 0.2 ml of the purified enzyme (2 μg/ml) was incubated along with 0.2 ml of 10 mM NBS (acetate buffer pH 4.0), 10mM DTNB (phosphate buffer, pH8.0), 10mM PMSF (in methanol) and 10mM DEPC individually for 30 min at room temperature. After incubation, 20 μl of the sample was taken and the residual activity was determined using standard assay conditions.

Transgalactosylation activity

Transgalactosylation activity was determined according to the method described by Toba et al. (1980). Briefly, the reaction mixture containing 0.1 ml of purified enzyme and 1.5 ml of lactose (33% w/v in phosphate buffer pH 7.0) was kept for incubation at 37ºC under shaking condition for 12 h. After incubation, enzyme activity was terminated by keeping the reaction mixture in a boiling water bath for 10 min. The oligosaccharides formed were analyzed on paper chromatography using Whatmann No-1 filter paper (40 cm X 30 cm) and also using High Performance Liquid Chromatography (HPLC) (Hsu et al, 2007). The HPLC system consists of Supelcosil LC-NH2 column, (4.6. 25 cm, Supelco Co., USA) with refractive index detector (Shimadzu, Japan). Mobile phase for elution was acetonitrile and water (75:25, v/v), at a flow rate of 1.0 ml/min.

Results

Optimization of the culture conditions for the maximum production of β-gal by *E. faecium* was evident. The highest enzyme activity was observed in MRS medium with 2% lactose (w/v) as carbon source at 37ºC under controlled pH (7.0) conditions.

Purification and molecular weight estimation of β-galactosidase

β-gal was isolated from the cell extract of *E. faecium* MTCC5153 using following steps. The first purification step involved ammonium sulphate precipitation (60% saturation) of crude β-gal preparation resulted in 1.17 fold purification. Followed by DEAE cellulose column chromatography resulted in 2.62 fold purification of enzyme activity with a yield of 14.51 %. Further purification of 5.23 fold with a specific activity of 24.06 U/mg was achieved upon Sephadex- G-150 column chromatography (Table 2).

Purified β-gal eluted as a single peak after gel filtration chromatography. The native molecular weight was found to be 90 kDa as assessed by elution profile of standard proteins in the range of 14.5 to 150 kDa (Figure 1). Whereas purified β-gal moved as a single band on SDS-PAGE and molecular weight corresponding to 43 kDa was observed (fig 2A) suggesting that the β-gal appears to be a homodimer. However, upon native PAGE analysis, two active bands migrating closely were observed (Fig 2B). To investigate the nature of these two bands, MALDI TOF (PMF) analysis was performed. Based on the MASCOT results, two active bands were found to exhibit sequence similarity with that of small subunit of β-galactosidase from *E. faecium* DO (Acc no.Q3XWY6_ENTFC). These results suggest that the two bands appeared on the native PAGE was probably derived from a single polypeptide; where in smaller band may be a degraded product of intact enzyme without losing its activity.

Properties of β-galactosidase from E. faecium MTCC5153

Determination of k_m and V_{max}

Steady state kinetics of purified β-gal was determined for the hydrolysis of *o*NPG under standard assay conditions. The *km* and *Vmax* values were calculated using Sigmaplot (SPSS Inc.) and was found to be 2 mM and 18 mM/min/mg, respectively.

Effect of pH and temperature on the activity and stability of β-gal

Optimum pH for β-gal activity was found to be 8.0 and the enzyme was stable in the pH range of 8.0 to 9.0, retaining 90% of its activity at 4°C for 12h (Fig 3). The Optimum temperature for β-gal activity was 40ºC (Fig 4).

Thermal stability of β-gal was improved in presence of $MgSo_4$. Figure 5 represent the concentration of Mg^{+2}(1mM to 10mM) on thermal stability of β-gal. Improved enzyme stability was observed with increasing Mg^{+2} ions concentrations (0, 1 and 10mM). In presence of 10mM Mg^{+2} ions, the β-gal retained 80% of activity after incubation for 15min at 40°C. However loss of activity without Mg^{+2} was observed in above storage condition.

Effect of different reagents and cations on the β-gal activity

Stimulating or inhibitory effect of various cations and reagents on β-gal activity was determined. Enzyme was slightly activated by 1 mM urea, whereas at higher concentration of urea (10mM) was found to inhibit the enzyme activity, whereas no change in enzyme activity was observed for 2-mercaptoethanol and DTT (1 and 10 mM). EDTA at a concentration tested (1

and 10 mM) was found to inhibit the enzyme activity and retained only about 10% of residual activity (Table 3).

The activity of β-gal was improved significantly by Mg^{+2}, Mn^{+2}, Ca^{+2}, Na^{+} and K^{+}, showing relative activity in the range of 109-255%. Other cations such as Zn^{+2}, Cu^{+2} and Co^{+2} markedly inhibited the enzyme activity (Table 4) and the inhibitory effect of these cations are similar, in that β-gal retaining less than 35% of its activity.

Chemical modification study

This study was carried out to ascertain the role of specific amino acid residues involved in the β-gal activity. Upon treatment with chemical modifiers such as NBS and DEPC resulted in loss of about 97% of activity suggesting involvement of tryptophan and histidine residues in the catalytic activity of enzyme (Table 5). However, treatment with DTNB showed no effect on enzyme activity and PMSF marginally inhibited the enzyme activity by 12% suggesting amino acids such as cysteine, serine residues are not associated with catalytic activity of β-gal.

Transgalactosylation activity of β-gal

Oligosaccharides can be synthesized by β-gal due to its transgalactosylation activity. Variety of oligosaccharides with variable linkages can be manipulated in the final product using novel β-gal, Fig 6 (In set) shows presentation of typical paper chromatography results of oligosaccharides formation with β-gal during 0, 12 and 24h of incubation. As it can be seen that, decrease in the concentration of lactose resulted in increase in oligosaccharide formation during 24h of incubation. In order to provide better evidences for the formation of oligosaccharides

HPLC analysis was performed. Results indicated that elution profile at 5 min was found to associate with trisaccharide.

Discussion

The enzymatic hydrolysis of lactose using β-gal is of interest from both the nutritional and technological view points. The resulting hydrolyzed monosaccharides are sweeter and are easily digested in the intestine. This has led to the development of sweeteners from whey and its incorporation into other foods (Mahoney, 1998). Other interesting properties of β-gal is its transgalactosylation activity by which the enzyme produces a series of oligosaccharides containing galactose. It is well established that probiotic bacteria exerts an influence on health. Similarly prebiotic oligosaccharides are also recognized as an important dietary tool to modulate the intestinal microflora and also reported some health benefits (Gibson, 1998). Even though there are several prebiotic oligosaccharides are available in the market, there is great interest in development of novel prebiotics to support the growth of specific group of bacteria or probiotics (Nguyen et al, 2006). One potential approach is the use of β-galactosidase from a probiotic strain for the synthesis of oligosaccharides (Sako et al, 1999).

We have characterized *E. faecium* MTCC5153 for bacteriocin production and for its beneficial properties and reported that, culture is known to produce heat stable enterocin and offers several probiotic properties (Badarinath and Halami, 2009; Halami and Chandrashekar, 2010). Since this culture was found to produce inducible β-gal enzyme, the studies were undertaken for purification of enzyme with respect to its properties.

Upon purification the molecular weight of β-gal in denaturing gel was found to be 43 kDa. A native molecular weight of 100kDa was observed and it was probably due to

dimerisation of identical subunits. Formation of multimeric complexes are commonly reported in microbial β-galactosidases (Adams et al, 1994; Onishi and Tanaka, 1995). Subunit masses of β-gal from several LAB such as, *Lactobacillus delbruckii* sub sp. *bulgaricus* (110 kDa subunit, homodimer), *Lactobacillus sakei*, *L. acidophilus* and *L. helveticus* are known. In contrast β-gal having single peptide with low molecular weight (64 kDa) was observed for *Pediococcus pentosaseus* upon SDS PAGE analysis (Bhowmik and Marth, 1990). Since we have found two active bands on native PAGE, we have conducted MALDI TOF peptide figure printing to study the nature of these bands observed. Based on the results obtained by MASCOT analysis of trypsin digested peptide mass data, it can be postulated that intact high molecular weight β-gal (Fig 2B Gal 1) partially degraded during purification of enzyme resulted in low molecular weight of active band (Fig 2B Gal 2). This phenomenon was also observed with β-gal from *Lactobacillus reuteri* L103 and L461 strains (Nguyen et al, 2006).

Optimum temperature and pH for β-gal activity was found to be 40°C and 8.0 respectively. Similar temperature & pH optimum has been reported with other bacterial β-galactosidases (Bhowmik and Marth, 1990; Nguyen et al, 2006). β-gal activity was stimulated by incorporation 10mM of Na^{+}, K^{+}, Mg^{+2}, Mn^{+2}, Ca^{+2} and inhibited by Zn^{+2}, Cu^{+2} and Co^{+2}. Similar pattern of metal activation was reported previously (Garman et al, 1996; Hung and Lee, 2002). In contrast, presence of Ca^{+2} was found to inhibit β-gal activity in *Lactobacillus reuteri* (Nguyen et al, 2007) and β-gal from *Bifidobacterium infantis* HL96 (Hung and Lee, 2002) were also inhibited by Mg^{+2} and Mn^{+2}. Thus application of β-gal from *E. faecium* MTCC5153 in fluid milk will not be affected by Ca^{+2}. The activation mechanism of β-gal enzyme with metal ions are not yet fully understood. In case of *E.coli*, the binding of magnesium ion at the active site of

LacZ is known. It was proposed that these interactions might involve in stabilizing the enzyme (Huber et al, 2001).

Chemical modification of β-gal was performed to study the role of specific amino acid on enzyme activity. The result revealed the involvement of tryptophan and histidine residues for enzyme activity. Since loss of 97% of enzyme activity on treatment with NBS and DEPC was observed. Involvement of tryptophan and histidine residues at active site or their possible role on stability of enzyme has been reported in *Rhizomucor* sp, *Lactococcus lactis*, *E.coli* and *Kluyveromyces lactis* (Kestwal and Bhide, 2007; Jures et al, 2000; Sheikh et al, 1999; Wiesmann et al, 1997; Kim et al, 2003).

We have subsequently studied Transgalactosylation (TOS) activity of purified enzyme. TOS activity is one of the properties of β-gal, in which, instead of transferring of galactose unit to the hydroxyl group of water, it transfers to the other carbohydrate, such as, lactose resulting in the formation of oligosaccharides (Tzortzis et al, 2005). Several reports showed the formation of 3 to 7% oligosaccharides using lactose by LAB (Toba et al, 1980). TOS activity of β-gal, from probiotic LAB have great advantage in industrial application, as they have GRAS status (Vinderola and Reinheimer, 2003).

Conclusion

In conclusion, a new β-gal reported in this study has a molecular weight of 90kDa. Properties of purified β-gal such as activity at neutral pH (7.0-7.5), temperature (40°C) and activation by Ca^{+2} ions finds better applications for milk and sweet whey hydrolysis. This enzyme found to posses transgalactosylation activity. However, additional analysis is required for optimum conditions for formation and characterization of oligosaccharides. β-gal producing

317 strain of *E. faecium* MTCC5153 has additional probiotic properties. Since species of *E. faecium*
318 are known to grow in extreme environment conditions and in simpler medium as compared to
319 other LAB, use of *E. faecium* with probiotic properties in combination with its ability to
320 synthesize prebiotics are found to have enormous application in food industry.
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Table 1: β-galactosidases of lactic acid bacteria

Lactic acid bacteria	Optimum pH	Optimum temperature (°C)	Molecular weight (kDa)	Reference
L. bulgaricus	-	42-45	-	(Adams et al, 1994)
K. lactis	7.2	35	135	(Kim et al, 2003)
L. reuteri L103 and L461	6.5 and 8.0	50	105	(Nguyen et al, 2006)
Bifidobacterium infantis HL96	7.5	60	470 (Tetramer of 115 kDa subunits)	(Hung and Lee, 2002)
Lactobacillus acidophilus R22	6.5-8.0	55	105 (35 & 72 kDa subunits)	(Nguyen et al, 2007)
Pediococcus pentosaseus	6.0-7.0	30	64	(Bhowmik and Marth, 1999)
E. faecium MTCC5153	8.0	40	43	This study

Table 2: Purification of β-galactosidase from *Enterococcus faecium* MTCC5153

Preparation	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Fold purity	Yield (%)
Crude	14.98	3.25	4.60	1.00	100.00
AS*(After dialysis)	7.41	1.37	5.40	1.17	49.48
DEAE-Cellulose	2.17	0.18	12.07	2.62	14.51
Sephadex G-150	0.23	0.01	24.06	5.23	10.65

*Ammonium sulphate precipitate

Table 3: Effect of various reagents of the activity of β-gal

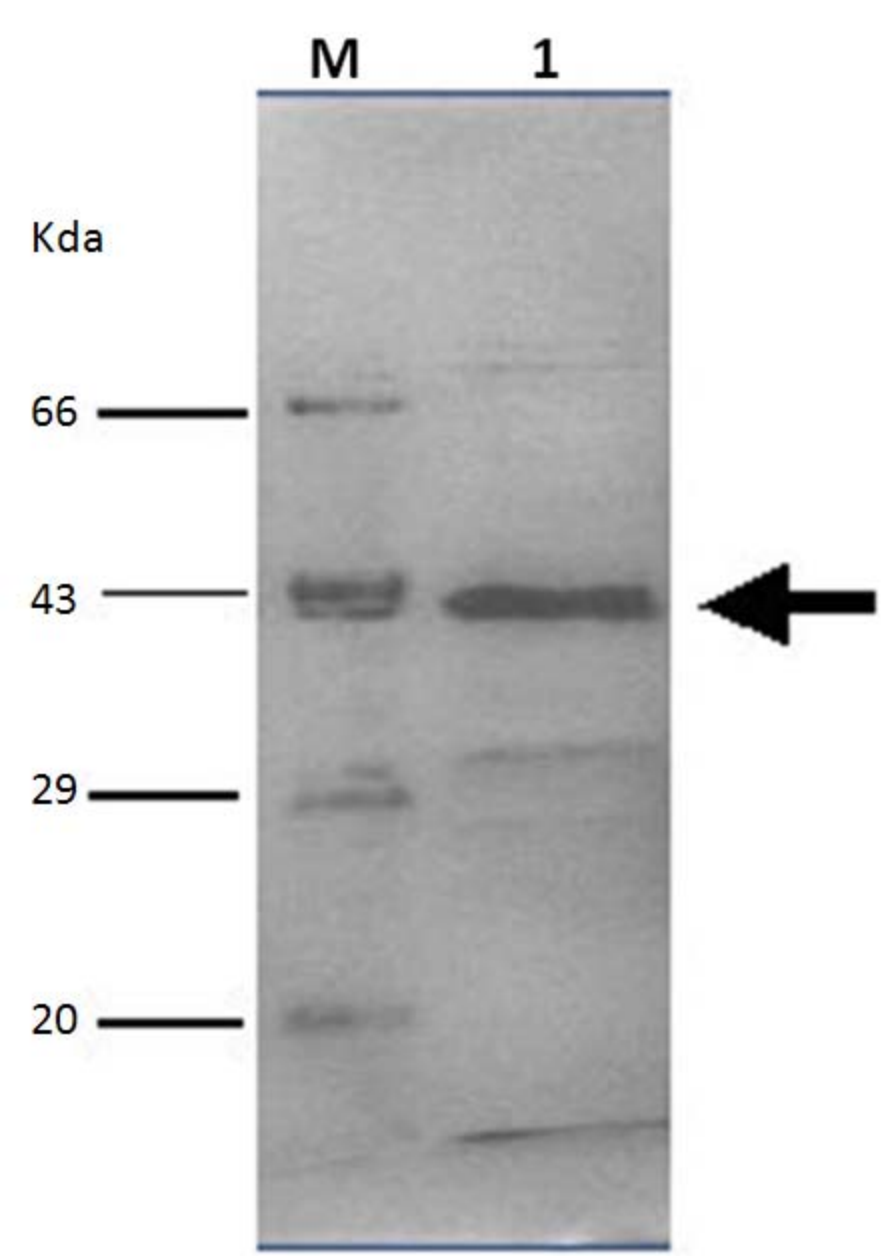
Reagents	**Concentration (mM)**	**Residual activity (%)**
2-mercaptoethanol	100	100
	10	97
DTT	100	98
	10	97
Urea	100	115
	10	127
EDTA	100	13
	10	15

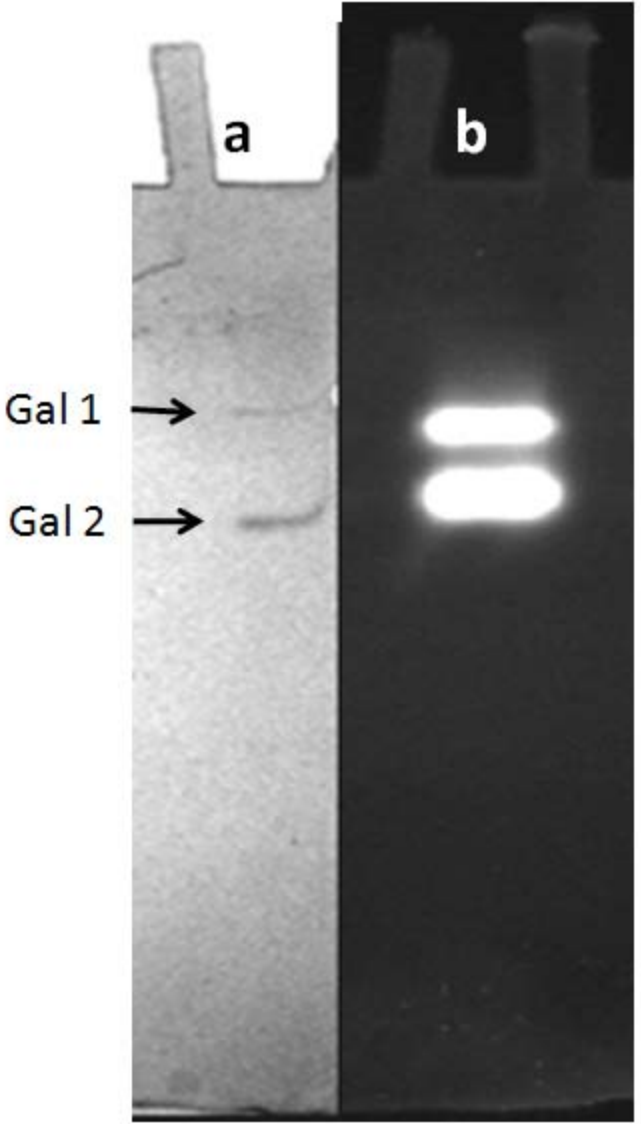
Table 4: Effect of different cations on enzyme activity

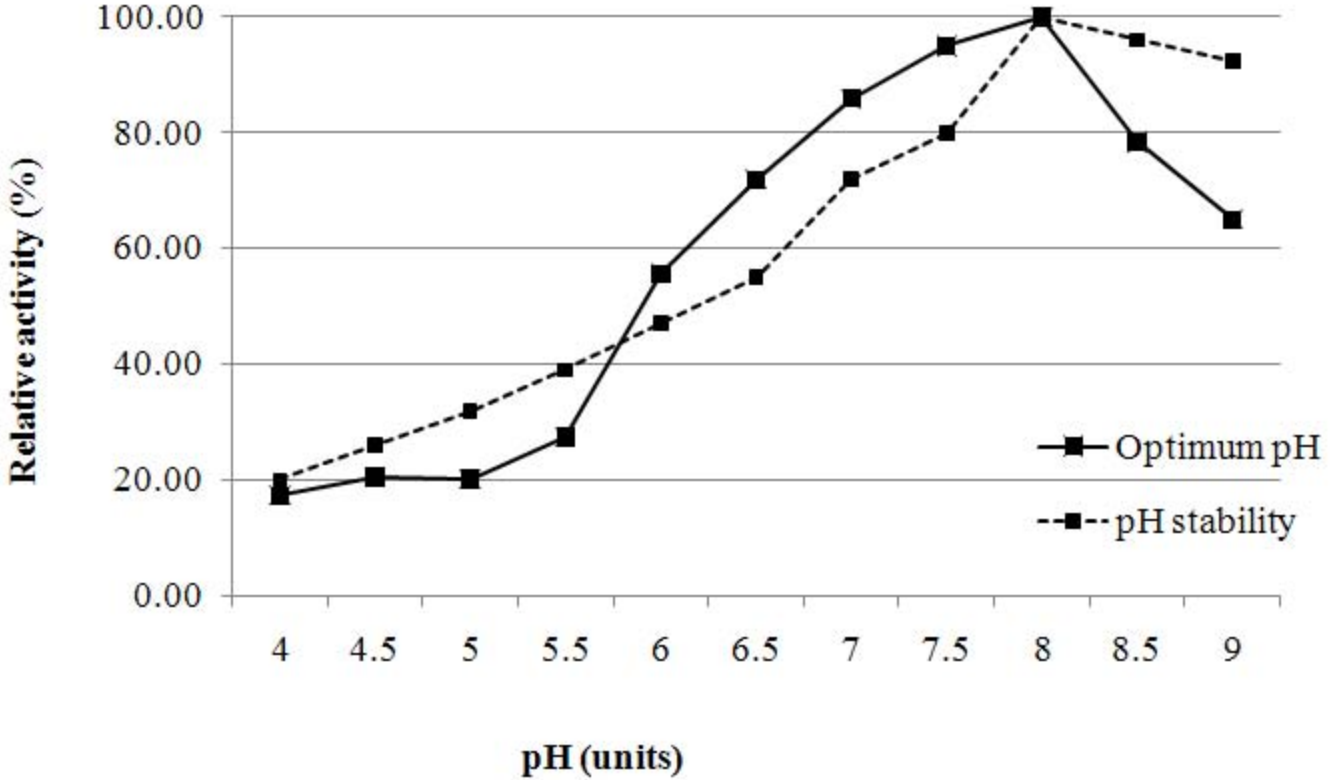
Cations (10mM)	**Relative activity (%)**
Control	100
Mg^{+2}	255
Mn^{+2}	215
K^{+}	141
Na^{+}	109
Ca^{+2}	208
Zn^{+2}	35
Co^{+2}	28
Cu^{+2}	24

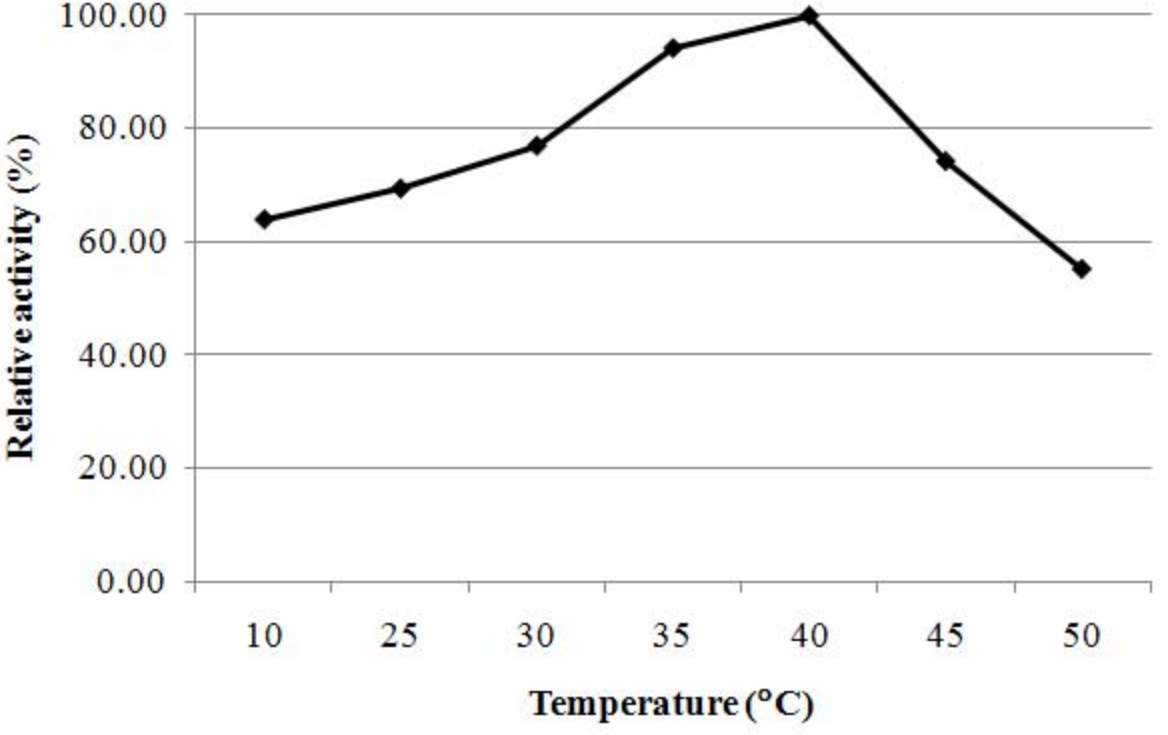
Table 5: Residual activity of β-gal after treatment with chemical modifiers

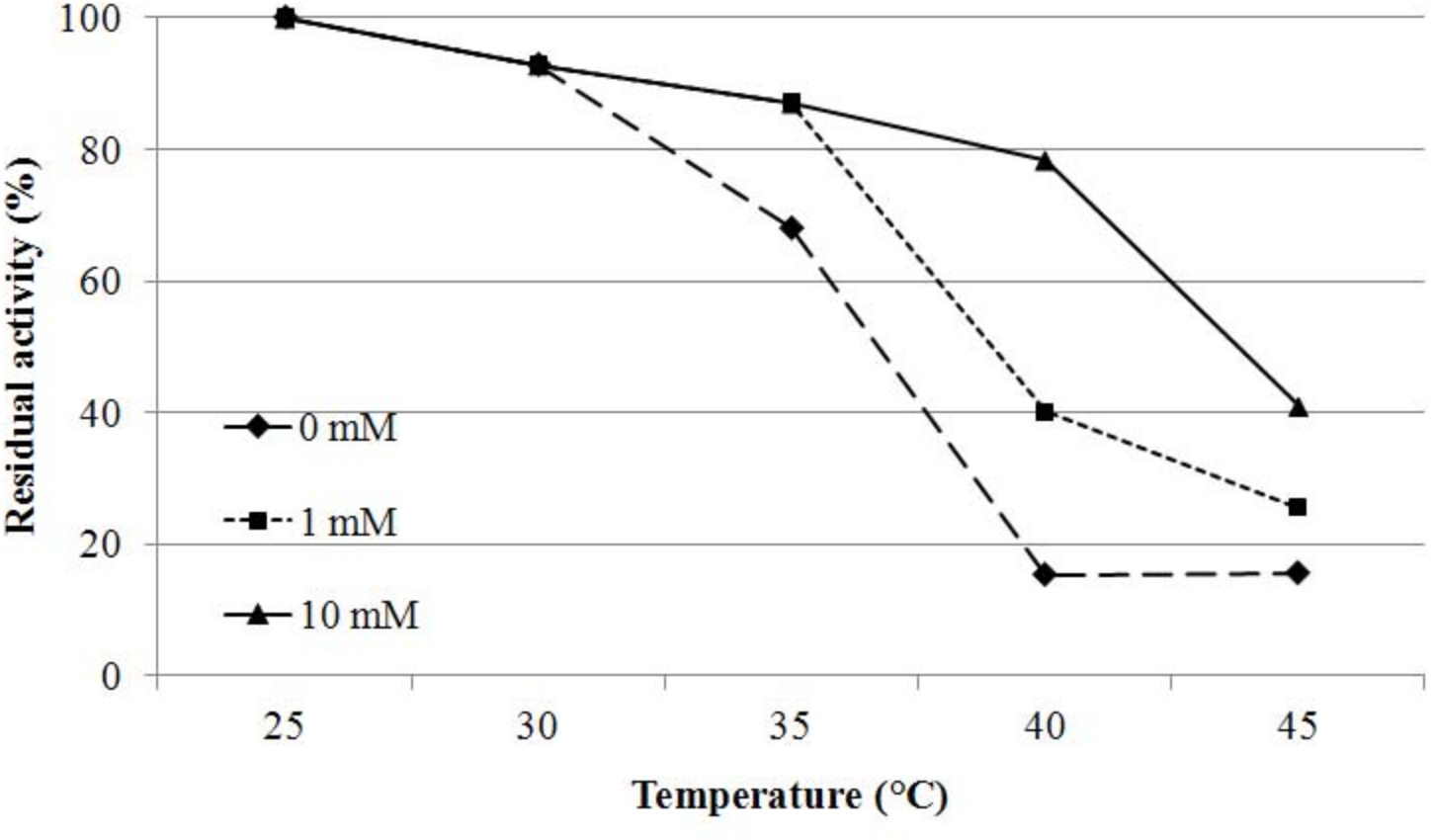
Chemicals (10mM)	**Residual activity (%)**
NBS	3
PMSF	88
DTNB	100
DEPC	3
Control	100

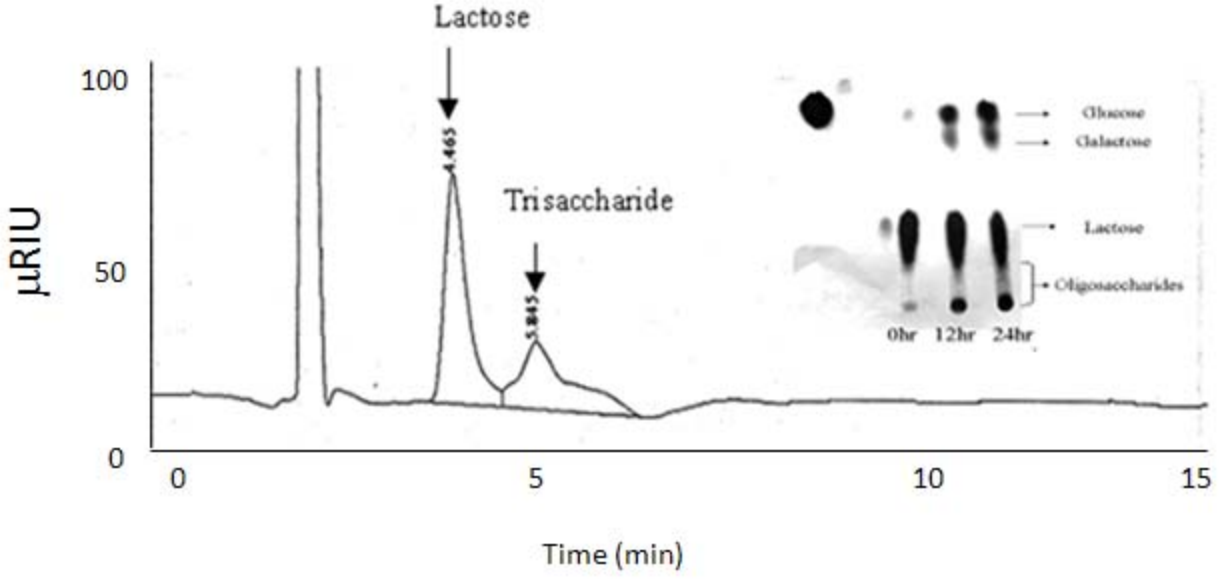

M
1
Kda
66
43
29
20


a
b
Gal 1
Gal 2


100.00
80.00
60.00
40.00
20.00
0.00
Relative activity (%)
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
pH (units)
Optimum pH
pH stability


100.00
80.00
60.00
40.00
20.00
0.00
Relative activity (%)
10
25
30
35
40
45
50
Temperature (°C)


100
80
60
40
20
0
Residual activity (%)
0 mM
1 mM
10 mM
25
30
35
40
45
Temperature (°C)


Lactose
Trisaccharide
4.465
5.845
100
50
0
µRIU
0
5
10
15
Time (min)
Glucose
Galactose
Lactose
Oligosaccharides
0hr
12hr
24hr