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# Cloning, High-level Expression and Characterization of a β-Galactosidase from *Bacillus subtilis* G1

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**Abstract:** A gene (*lacA*) coding for β-galactosidase from *Bacillus subtilis* strain G1 was cloned, expressed in *E. coli* and the β-galactosidase LacA was purified and characterized. The *lacA* gene consisted of an open reading frame of 2061 nucleotides, encoding a 686-aa protein, with a predicted molecular mass of 79 kDa and pI 6.16. The β-galactosidase showed an identity of 97.8% with the corresponding amino acid sequences from *B. subtilis* strains (NP_391293, CAB08008, and CAB15418). LacA was over-expressed in *E. coli* with a level of 40% of the total proteins and purified to homogeneity with specific activity of 72.7 U/mg and a molecular mass of 79 kDa determined by SDS-PAGE. The purified enzyme showed an optimal temperature of 55°C and optimal pH of 7. The enzyme was stable at temperature range of 20-37°C and at pH range of 7-8. Most tested solvents, metal ions and detergents showed a slight effect on the β-galactosidase activity except for n-butanol, $Zn^{2+}$, $Ag^{+}$, and $Cu^{2+}$, and SDS which inhibited the enzyme. This β-galactosidase is a good candidate for food industry after further application study.

**Key words:** *Bacillus subtilis* G1. β-galactosidase gene. Cloning. Expression. Characterization.

## INTRODUCTION

β-Galactosidase (β-D-galactoside galactohydrolase, E.C 3.2.1.23), a commercially important enzyme, catalyses the hydrolysis of β-D-galactopyranosides such as lactose to glucose and galactose. Because of this property, β-galactosidase (lactase) is frequently employed in the food industry to alleviate the problems associated with lactose crystallization in frozen concentrated desserts, whey disposal and milk consumption by lactose-intolerant individuals (Kim and Rajagopal 2000; Rhimi *et al*. 2010). Thus, identification of β-galactosidases could greatly benefit the dairy industry. β-Galactosidase genes were used as reporter system in the DNA technology, transgenic animals (Mozdziak *et al*. 2006), cancer study (Roigas *et al*. 1997), as a tracer in immunocytochemistry (Bondi *et al*. 1982) and as drug to supplement enzymes for digestion system. β-Galactosidases show a high transgalactosylation activity, so that they are used for the synthesis of prebiotic galactooligosaccharides (Iqbal *et al*. 2010), novel galactosides (Irazoqui *et al*. 2009). The beta-galactosidase activity also contributes to glycoprotein degradation (Terra *et al*. 2010), the degradation of GM1 ganglioside and other glycolipids and glycoproteins with a terminal galactose moiety (Samoylova *et al*. 2008).

Galactosidases are widely distributed in microorganisms (yeasts, fungi, bacteria, and archaea), plants (Biswas *et al*. 2003; Kaneko and Kobayashi 2003), and animals (Sopelsa *et al*. 2000; Ferreira *et al*. 2003). For application in the dairy industry, many bacterial β-galactosidase genes have been isolated, cloned, analyzed, expressed and characterized. Kim *et al*. (2004) characterized a thermostable recombinant β-galactosidase from *Thermotoga maritime* for the hydrolysis of lactose and the production of galacto-oligosaccharides. Yuan *et al*. (2008) expressed a gene (2067 bp) coding a thermostable β-galactosidase from *Alicyclobacillus acidocaldarius* ATCC 27009 in *Pichia pastoris*. Nakagawa *et al*. (2006) purified and molecularly characterized a cold-active β-galactosidase from *Arthrobacter psychrolactophilus* strain F2. So far, the beta-galactosidase-encoding gene from psychotrophic *Bacillus subtilis* KL88 (Torres and Lee 1995), *B. circulans* ATCC 31382 (Ito and Sasaki 1997), *B. licheniformis* (Trân *et al*. 1998) and *B. megaterium* ATCC 14581 (Shaw *et al*. 1998) was cloned and expressed in *Escherichia coli*. However, there was no report about expression level of β-galactosidase from *B. subtilis* in *E. coli*.

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In this study, we reported cloning, analysis and expression of a β-galactosidase-encoding gene from *Bacillus subtilis* strain G1 isolated in Vietnam soil and purification and characterization of the recombinant enzyme.

## MATERIALS AND METHODS

***Chemicals and Agents:***

DNA cloning kit, RNase A, and restriction enzymes (*Bam*HI, *Not*I, *Eco*RI), T4-ligase, Proteinase K were supplied by Fermentas (Germany); DNA Extraction Kit and Protein Extraction Kit were from QIAgen; ONPG (ortho-nitrophenyl-β-D-galactopyranoside) from Wako (Japan); Probond$^{TM}$ resin from Invitrogen (USA).

***Plasmids, Bacterial Strains and Culture Conditions:***

The bacterial strain *Bacillus subtilis* G1 from the Laboratory of Molecular Microbiology (Institute of Biotechnology, Hanoi) was used as the source of β-galactosidase gene. *Escherichia coli* DH5α and pJET1/blunt (Fermentas) was used for DNA manipulations and amplification. *Escherichia coli* BL21 and pET22b+ (Invitrogen) was used for expression of the β-galactosidase. LB medium (Luria-Bertani) containing 1% (w/v) bacto tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl; pH 7-7.5 was used for cultivation of *E. coli* and *B. subtilis*. The LB agar plates contained additionally 2% (w/v) agar and 100 mg ampicillin/ml.

***DNA Manipulations:***

Genomic and plasmid DNA isolation was carried as described previously (Quyen *et al*. 2007). DNA fragments and PCR products were excised from a 0.8% agarose gel and purified as described by Qiagen. DNA sequencing was performed on ABI PRISM 3100 Avant Genetic Analyzer. *E. coli* BL21 and DH5α were transformed using heat shock method as described previously (Quyen *et al*. 2007).

***DNA Amplification and Plasmid Construction:***

The putative *lacA*-coding DNA fragment was amplified from *B. subtilis* G1 genomic DNA by PCR with *Taq* DNA polymerase. Based on the DNA sequence of the putative *lacA* gene from the complete genome of *Bacillus subtilis* subsp. subtilis str. 168 (GenBank: NC_000964), two oligonucleotides, LacAF (5'-GCG GTG ATG TCA AAG CTT GAA AAA ACG C-3') and LacAR (5'-GCAT GTG TGT TTA CGA CAA T-3') were designed as primers for introduction of the underlined *Bam*HI and *Not*I restriction sites, respectively.

The PCR mixture contained 2.5 µl 10x PCR buffer; 2 µl of 2.5 mM dNTP; 2 µl of 25 mM $MgCl_2$; 1 µl genomic DNA; 0.5 µl 5 unit *Taq* polymerase and 1 µl each primer, supplemented with 11.5 µl distillated water to fulfill 25 µl. The thermocycler conditions were as follows: 94°C/3'; 35 cycles of 94°C/1', 54°C/1¢, 72°C/1'; 72°C/10'. The PCR products amplified from the genomic DNA with both primer LacAF and LacAR were inserted into the cloning vector pJET1/blunt, resulting in pJLacA and then sequenced. It was followed by ligation of the *Bam*HI-*Not*I digested pJLacA products with pET22b+ linearized by the same enzymes, resulting in pELacA under the control of the T7-promoter induced by IPTG and possessing the ampicillin marker. The galactosidase LacAhis encoded by the plasmid pELacA contains the pelB leader, the mature galactosidase and the 6x histidine-tag.

***Gene Expression:***

The transformant *E. coli* BL21/pELacA was cultivated in 5 ml of LB medium containing 5 µl of 100 mg ampicillin/ml at 37°C with agitation at 220 rpm. Two ml of the overnight culture were transferred into 200 ml of LB medium containing 200 µl of 100 mg ampicillin/ml. The culture was cultivated at 37°C with agitation at 220 rpm until an optical density (OD) at 600 nm of 0.6 was reached (for approximately 3 hours), then 200 µl of 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. The culture was continuously incubated at 37°C with agitation of 220 rpm for 3-6 hours of induction. Cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C. Wet cells were used immediately for protein purification or stored at -20°C for later purification.

***Purification of β-Galactosidase:***

The fusion form LacAhis carrying a C-termial 6xHis tag was expressed in *E. coli* BL21. To purify the β-galactosidase LacAhis, 100 mg wet cells from a 120-ml culture in LB medium were harvested by centrifugation, and suspended in 10 ml of the non-denaturalized buffer Z (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 50 mM β-mercaptoethanol (BME), 1 mM $MgSO_4$, adjusted to pH 7). The cell lysate was sonificated (three bursts of 1 min each at 1-min intervals) and then 100 µl of 1% (w/v) lysozyme

was added to the cell suspension. After 30-60 min incubation in ice with slight shaking, the cell suspension was centrifuged at 13000 rpm at 4°C for 25 min to remove cell debris. The cell supernatant was applied to Ni-NTA column (Invitrogen) according to the manufacturer's recommendation. The LacAhis solution was used for characterization study. Protein concentrations were determined by Bradford method.

***DNA and Amino Acid Sequence Alignments:***

Sequence alignments were constructed and analyzed using the program Megalign DNAStar.

***β-Galactosidase Assay:***

To estimate the activity of the purified β-galactosidase, 20 µl purified enzyme solution was added to 480 µl 22 mM ONPG in 50 mM buffer Z pH 7, incubated at 30°C for 10 minutes. Then the reaction was stopped by addition of 500 µl 1 M $Na_2CO_3$. The absorbance was read at 420 nm against a blank containing ONPG, buffer Z without enzyme solution. The amount of OPNG hydrolyzed was calculated using the following formula according to the Invitrogen's introduction.

Specific activity = nmoles of ONPG hydrolyzed/t/mg protein

nmoles of ONPG hydrolyzed = $(OD_{420})$ (8 x $10^5$ nanoliters)/[(4500 nl/nmoles-cm)](1 cm) (β-Gal Assay Kit, Invitrogen Catalog no. K1455-01); where 4500 is the extinction coefficient, t = the time of incubation in minutes at 30°C (i.e. 10 minutes), and mg protein is the amount of protein assayed, which can be determined using Bradford method. The background activity of the untransfected cell lysate was subtracted. All measurements were carried out three times and from these values the average value was taken.

***Gel Electrophoresis:***

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970) with Biometra equipment. SDS-PAGE was usually performed with gels of 12.5% (w/v) of acrylamide according to the manufacturer's recommendations.

***Temperature and pH Optima:***

The temperature and pH optima of LacA were determined by measuring the activity as described above using 50 mM buffer Z (pH 7) at the temperature range of 20-80°C for 10 min and using different buffers pH 4-10 (pH 4-5: acetate buffer, pH 6-8: phosphate buffer, pH 7: buffer Z, pH 9-10: Tris buffer) at 30°C for 30 min, respectively.

***Temperature and pH Stability:***

For the determination of temperature and pH stability, purified enzyme (2 µg protein for each reaction) was preincubated in 50 mM buffer Z at different temperatures 20-60°C for 0-96 h, and in 50 mM buffer pH range 5-9 (acetate pH 5-5, phosphate pH 6-8, and Tris-HCl, pH 9) at 30°C for 0-96 h, respectively. The residual activity was then determined.

***Effect of Metal Ions, Organic Solvents and Detergents:***

Purified LacA (2 µg protein) was incubated in 5 mM of various metal ions ($Ca^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Ni^{2+}$, $Zn^{2+}$, $Ag^{+}$, $K^{+}$) and EDTA for , in 10-30% (v/v) of different solvents (acetone, methanol, ethanol, isopropanol, n-butanol), and in 0.5-2% (w/v) of different detergents (Tween 80, Tween 20, Triton X-100, and SDS) at 30°C for 3 h. The residual activity was then determined as described above.

## RESULTS AND DISCUSSIONS

***Cloning and Sequence Analysis of a Putative β-Galactosidase Gene from B. subtilis:***

The recombinant plasmid pJLacA containing the insert was sequenced and the putative β-galactosidase gene was aligned with sequences from the GenBank using DNAstar. The *lacA* gene consisted of an open reading frame of 2061 nucleotides, encoding a 686-aa protein, with a predicted molecular mass of 79 kDa and pI 6.16. The sequence of gene *lacA* from *B. subtilis* G1 showed an identity of 98% with corresponding sequences from *B. subtilis* strains (NC_000964, Z94043, and Z99121), and 73.3% with sequences from *B. licheniformis* strains (AE017333 and CP000002). Whereas, the putative amino acid sequence of G1 had an identity of 97.8% with the corresponding amino acid sequences from *B. subtilis* strains (NP_391293,

CAB08008, and CAB15418) and 76.9% that from *B. licheniformis* strains (AAU43090, AAU25712, YP_081350, and YP_093783). The whole genome from *Bacillus subtilis* subsp. subtilis str. 168 (NZ_CM000487) contain two different beta-galactosidase genes encoding the beta-galactosidase (ZP_03593211) and a hypothetical protein Bsubs1_03978 (ZP_03590391) which showed 98% and 21% identities with the LacA from *B. subtilis* G1.

The sequence was deposited in the GenBank with an accession number EU585783.

***Expression and Purification of LacA:***

To over-express the recombinant β-galactosidase in *E. coli*, the expression plasmid pELacA, containing the β-galactosidase gene under the control of T7 promoter was constructed. The β-galactosidase LacA was expressed from this plasmid in *E. coli* BL21 at a level of 40% of total cell proteins (Fig. 1, lane 5). LacA was purified by Ni-NTA chromatography with a yield of 18% (data not shown) and a specific activity of 72.7 U/mg. SDS-PAGE analysis showed only a single band (Fig. 1, lane 3). The molecular mass determined by SDS-PAGE (Fig. 1, lane 2 and 3) was in good agreement with that calculated (79 kDa). So far, there was no report about over-expression of β-galactosidase from *B. subtilis* in *E. coli*.

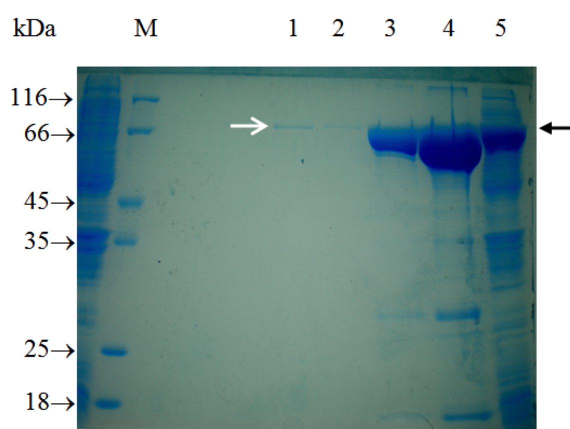



**Fig. 1:** SDS-PAGE: total *E. coli* BL21 proteins (5), Ni-NTA purified fractions 1, 2, 3, and 4 (4, 3, 2, and 1), protein standard (M).

***Temperature and pH Optima:***

The β-galactosidase had an optimum temperature of 55°C and optimum pH of 7 (Fig. 2). This result was coincident with the optimum temperature of the β-galactosidase from *B. subtilis* KL88 (Torres and Lee 1995). The recombinant β-galactosidase from *Alicyclobacillus acidocaldarius* (Yuan *et al*. 2008), *B. stearothermophilus* (Chen *et al*. 2008), from *Thermotoga maritime* (Kim *et al*. 2004) shown a higher optimum temperature of 70°C and 80-85°C, respectively, whereas that from the cold-adapted organism *Arthrobacter psychrolactophilus* strain F2 shown an even much lower optimum temperature of 10°C (Nakagawa *et al*. 2006). The β-galactosidases from *B. stearothermophilus* (Chen *et al*. 2008) shown also a pH optimum at 7. The optimum pH for the β-galactosidase from *T. maritime* (Kim *et al*. 2004), *B. subtilis* KL88 (Torres and Lee 1995), and *A. acidocaldarius* ATCC 27009 (Yuan *et al*. 2008) were little lower at 5.8-6.5. Whereas the purified cold-active β-galactosidase from *A. psychrolactophilus* strain F2 (Nakagawa *et al*. 2006) was optimally active at pH 8.

***Temperature and pH Stability:***

The galactosidase was stable at 20°C to 37°C. The galactosidase activity remained ≥84% at lower incubation temperature 20°C and 30°C, and ≥54% at 37°C for 96 h incubation. For 24 h incubation, the residual galactosidase activity was ≥86% at the temperature range from 20°C to 37°C. The relative residual galactosidase activity decreased strongly to 2% when incubated at 50-70°C just for 3 hours (Fig. 3A). The thermostability of the β-galactosidase from *B. megaterium* 2-37-4-1 was reported also beneath 40°C (Li *et al*. 2009). Other β-galactosidases were reported to be very thermostable, half-life times for the thermostable β-galactosidase from *B. stearothermophilus* at 65 and 70°C were 50 and 9 h, respectively (Chen *et al*. 2008) and that from *T. maritime* at 80-90°C were 16 h and 16 min, respectively (Kim *et al*. 2004).

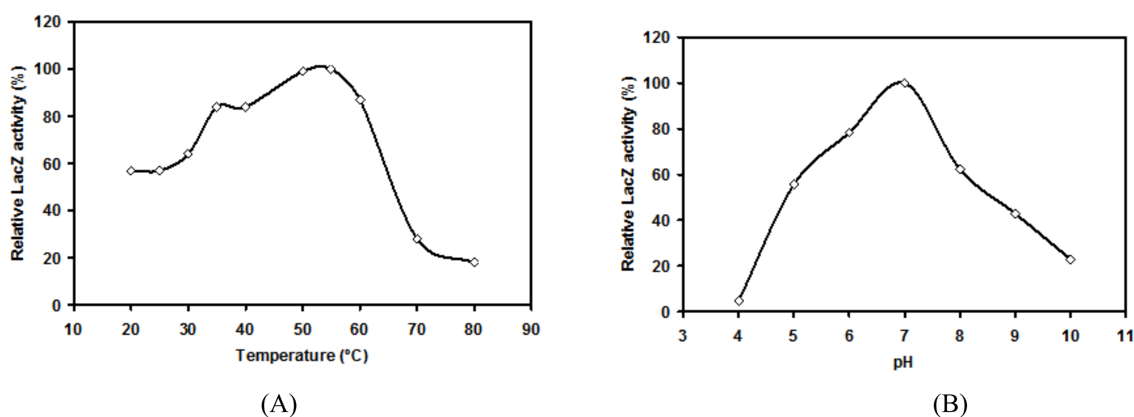



**Fig. 2:** Temperature (A) and pH (B) optimum curves of purified β-galactosidase from *B. subtilis* G1.

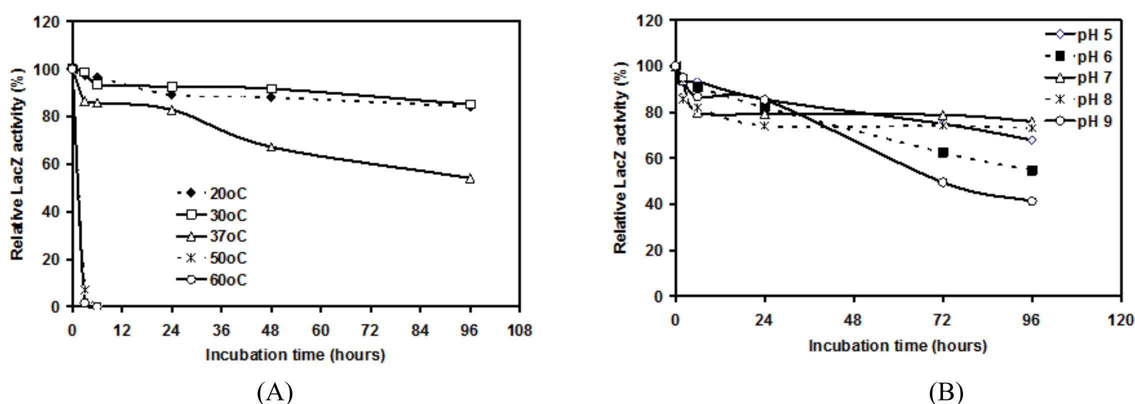



**Fig. 3:** Temperature (A) and pH (B) stability curves of purified β-galactosidase from *B. subtilis* G1.

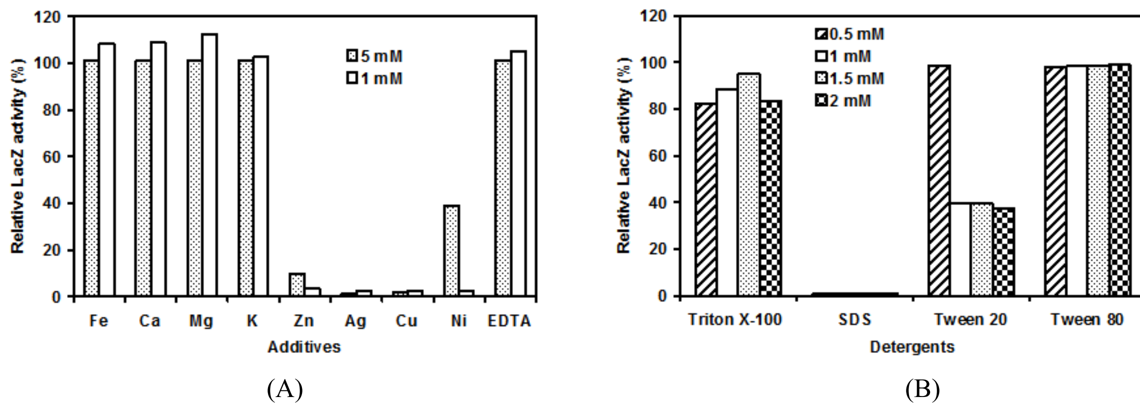
The β-galactosidase was stable at pH 7-8. After 24-h incubation, the residual activity was very high (around 80%) at all tested pH 5-9 (Fig. 3B). After 96 h incubation, the residual β-galactosidase activity was around 75% at pH 7-8 and over 40% at pH 9. Other β-galactosidases from *B. coagulans* RCS3 (Batra *et al.* 2002) and *B. megaterium* 2-37-4-1 (Li *et al.* 2009) were also reported to be stable at a neutral pH range: pH 6-9.

***Effect of Metal Ions on the β-Galactosidase Activity:***

In general, the addition of 1 and 5 mM of the metal ions $Fe^{2+}$, $Ca^{2+}$, $K^{+}$, $Mg^{2+}$ and EDTA showed no significant effect on β-galactosidase LacA activity, a slight change in β-galactosidase LacA activity was up to ±13% (Fig. 4A). Other metal ions including $Ni^{2+}$, $Zn^{2+}$, $Ag^{+}$, and $Cu^{2+}$ showed an obvious inhibitory effect on the β-galactosidase activity, $Zn^{2+}$, $Ag^{+}$, and $Cu^{2+}$ except for $Ni^{2+}$ inhibited completely or decreased the β-galactosidase activity to 1-13%. Batra *et al.* (2002) reported that $Cu^{2+}$ and $Ni^{2+}$ at the concentration of 0.5-2 mM inhibited the activity of the β-galactosidase from *B. coagulans* RCS3. Several divalent metal ions and EDTA did not decrease the activity, however $Cu^{2+}$ and $Zn^{2+}$ decreased strongly the activity of the β-galactosidase from *B. stearothermophilus* (Chen *et al.* 2008).

***Effect of Detergents on the β-Galactosidase Activity:***

The addition of 0.5-2 (w/v) of Tween 80 did not show any effect after 3 hours of incubation (Fig. 4B). The addition of 0.5-2 (w/v) of Triton X-100 decreased the β-galactosidase slightly by up to 18%. Tween 20 did not influence the β-galactosidase activity at 0.5% (w/v) but the addition of 1-2% (w/v) of Tween 20 decreased the β-galactosidase activity to around 40% (Fig. 4B). In contrast to other detergents, SDS showed a complete inhibition on the β-galactosidase activity. The sheep liver's β-galactosidase activity decreased slightly by Triton X-100 and inhibited strongly by SDS.




(A) (B)

**Fig. 4:** Effect of metal ions (A) and detergents (B) on β-galactosidase activity.

**Table 1:** Effect of organic solvents on β-galactosidase activity

| | Remaining activity (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Incubation times (hours) | 2 | | | 5 | | | 24 | | |
| Concentration (%) of solvents | 10 | 20 | 30 | 10 | 20 | 30 | 10 | 20 | 30 |
| Methanol | 97 | 93 | 97 | 94 | 91 | 84 | 59 | 54 | 61 |
| Ethanol | 86 | 94 | 93 | 75 | 81 | 91 | 52 | 55 | 39 |
| Acetone | 97 | 100 | 57 | 90 | 96 | 50 | 56 | 54 | 30 |
| n-Butanol | 29 | 3 | 1 | 3 | 2 | 2 | 0 | 0 | 0 |
| Iso-propanol | 99 | 96 | 83 | 98 | 92 | 79 | 69 | 40 | 15 |

***Effect of Organic Solvents on the β-Galactosidase Activity:***

Most organic solvents tested, except for n-butanol, decreased slightly the β-galactosidase LacA activity, most residual activity remained around 90% after 2-5 hours of incubation and around 50% after 24 hours of incubation (Table 1). However, n-butanol inhibited the β-galactosidase LacA activity very strongly. The residual activity remained only 29% after 2 hours of incubation at 10% of n-butanol, 1-3% after 2-5 hours of incubation at 20-30% of n-butanol. After 24 hours of incubation, the β-galactosidase LacA was completely inhibited by n-butanol (Table 1).

## ACKNOWLEDGMENT

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