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Biochemical and Thermal properties of β-Galactosidase produced by Whey Utilizing Actinomycetes isolate

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Abstract : This study investigates the kinetics of β-galactosidase enzyme produced from *Streptomyces thermocarboxydus (strain NBRC 16323)* on acid whey based growth medium. Maximum enzymatic activity was observed at pH 6 when investigated from pH 5 to 9.The enzyme's peak activity was found at 41^{0}C when studied at a wide temperature range 10-70^{0}C when incubated for 50mins. Thermostability study of β-galactosidase showed 58% residual activity after 180 mins of incubation at 41°C and 38% residual activity after 180mins at 50°C. Presence of Mg^{2+}, K^+ enhanced β-galactosidase activity at 1mM while other ions (Mn^{2+} ,Zn^{2+} Fe^{2+} and Cu^{2+}) showed inhibitory effect. Enzyme activity was retained in the presence of Lactose at 1mM, inhibited by Galactose and Glucose but not greatly affected by Maltose and Sucrose. Enzyme retained its activity till 1mM of BME (Beta-Mercaptoethanol) while loss in activity was observed in the presence of other additives (Urea, Sodium Dodecyl Sulphate, Ethylene diamine tetra acetic acid and TritonX-100). With ONPG as substrate, Km and Vmax are determined from Lineweaver -Burk Plot as 3.27mg/ml and 45µM/min respectively. Specific activity of the enzyme was found to be 10.3 µmol/min/mg.

IndexTerms **- β-galactosidase, Enzyme kinetics, Thermostability ,Additives, Inhibitors, Metal ions, Km-Vmax**

Abbreviations

BME	-	Beta Mercapto ethanol
SDS	-	Sodium Ddecyl Sulphate
EDTA	-	Ethylenediamine tetra acetic acid
GOS	-	Galacto Oligo Saccharides
BSA	-	Bovine Serum Albumin
ONPG	-	*O*-Nitrophenyl-β-D-Galactoside

I. INTRODUCTION

β-galactosidase (EC 3.2.1 to 3.2.3) cleaves the β-1,4 D-galactosidic linkage of lactose, as well as related chromogens, o-nitrophenyl-β-D-galactopyranoside (ONPG), p-nitrophenyl-β-D-galactopyranoside (PNPG) and 6-bromo-2-naphthyl-galactopyranoside (BNG). The enzyme is widely applied in dairy industry in the production of lactose hydrolyzed milk and whey to benefit lactose-intolerant people, detection of lactose from pollutants and production of prebiotics i.e. galacto-oligosaccharides for use in probiotic foods (Panesar etal, 2006; Rani V etal, 2019; Chakraborti S etal, 2000; Aaron Gosling etal, 2011).

Whey is the supernatant that remains after coagulation of casein from milk in dairy industry with 4-5% Lactose as its major component besides considerable presence of soluble proteins, lipids and minerals. It is usually discarded as industrial effluent on commercial scale into soil and environment. Therefore discarding whey not only results in wastage of potential bionutrients but also generates substantial pollution to the ecosystem. On the other hand whey has the potential to be used in bioprocess to harvest lactose hydrolyzed whey and GOS production(Sabrina Gabardo etal, 2014). β-galactosidase catalyzes the hydrolysis in the presence of various other additives, chelators and inhibitors that may interfere with its catalytic activity (Park, AR., Oh DK. 2010; M.Portaccio etal., 1998).

The characteristics of the enzyme and rate of catalytic reaction depend not only on the source of the enzyme but also on several process parameters like pH ,temperature and incubation time of enzyme reaction, substrate concentration and thermostability of enzyme to remain unaffected resisting the unfolding of conformation . In order to maximize product output from enzyme activity the process parameters require to be modified and standardized (Bernal C etal,2012., Escobar S etal., 2015).

It is observed that the enzyme from yeasts and bacteria show optimum activity at pH 6.5-7 whereas the same enzyme from molds works better at a pH of 3-5 (Frank V K etal., 1973). Most of the beta galactosidases are found to be dependent, either activated or inhibited, on divalent metal ions for maximum efficiency and Mg 2+ is predominant among them (Gopinath Sutendra etal,2007., Martinez bilbao etal.,2011).

As the group of Actinomycetes has not been explored for β-galactosidase production and utilization so far, this study is aimed to characterize the enzyme kinetics of β-galactosidase produced from the newly isolated Streptomyces thermocarboxydus (strain NBRC 16323) using whey based growth medium. Functional stability of the crude enzyme at various temperatures and pH for different time intervals and the effects of reaction products -glucose, galactose and other sugars are studied. Also the effect of divalent and monovalent cations and different additives like EDTA, SDS, Urea, Beta Mercaptoethanol, Triton X-100 on enzyme activity are evaluated at varying concentrations. The optimization of these different chemical and physical parameters adds to enhance the enzyme performance to achieve highest product turnover and also helps to reduce operating costs where enzyme is utilized for lactose hydrolysis.

Materials and Methods

Enzyme Production & Extraction Newly isolated ***Streptomyces thermocarboxydus (strain NBRC 16323)*** was inoculated on modified Casein Broth with addition of 7.3% of Whey and 0.05% of $MgSO_4.7H_2O$ at 41^0C for 3days. Crude enzyme was prepared by lysing the cell with 0.2% SDS and chloroform and suspended in an optimum quantity of Z buffer and stored at 4^0C till further use.

Enzyme Assay 0.3 mL of substrate ONPG (from HiMedia) was added to equal quantity of crude enzyme extract in 2ml Z buffer and incubated at 37°C for 30-45mins till yellow color develops. The reaction was stopped by adding 0.5 mL of 1M Na_2CO_3 and centrifuged for 5 mins at 10000rpm to remove cell debris and chloroform. OD of the ONP-o-nitro phenol released from ONPG was recorded at 420 nm .The enzyme activity was calculated from the formula-(Dahal etal ,2020)

$$EA = \frac{OD_{420} \times RV}{0.0045 \times EV \times T}$$ where

OD_{420} -- optical density of the product ONP at 420nm
RV -- reaction volume (total of lysate, buffer, substrate, stop solution) in ml
0.0045 -- optical density of 1µmole/ml solution of ONP at pH 7
EV -- extract (lysate)volume in ml
T --reaction time in mins
EA --enzyme activity in µmoles /min/ml of lysate

Determination of Protein Concentration Protein concentration was estimated by Biuret method. Known concentrations of BSA were incubated with Biuret reagent in ascending order (1-10mg/ml) for 10 mins and absorbance taken at 540nm. A calibration curve was plotted for protein concentration vs absorbance.The absorbance of the test sample was also determined by the same method and the concentration of the sample calculated from the calibration curve.

Effect of Reaction time Crude lysate was incubated with buffered substrate at 41 ^{0}C and enzyme assay performed every 10mins till 70 mins of incubation . The relative activity was expressed as the ratio of the enzyme activity at the given time to maximum activity observed in the total time taken as control and expressed as percentage.

Effect of temperature on β -galactosidase activity Crude lysate was incubated with buffered substrate at different temperatures ranging from 4 ^{0}C to 70 ^{0}C and assayed for enzyme activity after the incubation period. The relative activity was expressed as the ratio of the enzyme activity at a certain temperature to the maximum activity obtained in the given temperature range taken as control.

Effect of pH Crude lysate was incubated with substrate buffered separately with buffers of pH ranging from 5.0 to 9.0 (0.05 M) - acetate (pH 5.0, 5.5,6.0), phosphate (pH 6.5,7.0 and 7.5) and Tris–HCl (pH 8.0, 8.5 and 9.0). Relative activity expressed as percentage with respect to control.

Thermostability of enzyme Crude enzyme was incubated with sodium acetate buffer pH6 separately at 41^0C and 50^0C for a period of 3hrs and enzyme assay performed for every 30 mins till 180 mins of incubation. Residual activity of enzyme estimated with respect to control activity at 0 time of incubation taken as 100%.

Effect of Metal ions on activity of β -galactosidase Metal ions (Cu^{2+},Zn^{2+},Cu^{2+},Mg^{2+}, Mn^{2+},Fe^{2+}, K^+) were added to buffered substrate as their salts in four concentrations ranging from 0.1mM to 10mM and incubated for 15 minutes prior to crude enzyme addition. Enzyme activity was assayed after incubation period with standard procedure and relative activities are estimated with respect to control without any additions.

Effect of Inhibitors on activity of β -galactosidase Mono and Disaccharides namely
Galactose, Glucose, Lactose,Sucrose and Maltose were added to buffered substrate at four concentrations ranging from 0.1mM to 10mM and equilibrated for 10 mins before incubation with crude enzyme. β-galactosidase activity was determined after incubation and relative activities estimated with respect to control without any additions.

Effect of Additives on activity of β -galactosidase Various additives and chelating agents namely EDTA, SDS, β-ME, Urea, Triton X-100 were added to buffered substrate in varying concentrations (0.1mM to 10mM) and incubated for 10 minutes before adding the crude enzyme. Enzyme activity was assayed after incubation period and relative activities are determined with respect to control without any additions.

Effect of Substrate Concentration on activity of β -galactosidase From a stock solution of ONPG(10mg/ml) in phosphate buffer pH7, working solutions were prepared in increasing concentrations from 1mg/ml to 10mg/ml . Crude enzyme was incubated with each concentration of ONPG and enzyme assay was performed after incubation period and absorbance at 420nm was recorded for each concentration.

Determination of Km-Vmax Stock solution of ONPG was prepared with the concentration that yielded highest enzyme activity from the above assay (8mg/ml) in phosphate buffer pH7. From the stock solution ten increasing concentrations were prepared from 0.8mg/ml to 8mg/ml. Crude enzyme was incubated with each concentration of ONPG and enzyme assay was performed after incubation period and absorbance at 420nm was recorded for each concentration. 1/[s], v, 1/[v] of the enzyme were calculated and plotted on the graph.

Results and Discussion

Effect of incubation time, temperature and pH on enzyme activity From Fig-1 β-galactosidase activity found to be highest at 50minsof incubation period with 93% and 97% activity at 40 and 60mins respectively . Enzyme activity was highest when incubated at 41^{0}C with 92% and 95% activity at 37°C and 50°C resp. (Fig-2 & 3). 22%-95% enzyme activity was observed at temperatures ranging from 10^{0}C -70^{0}C, even at 4^{0}C showed slight activity of 16%. These observations indicate that the enzyme has potential application at normal temperatures. Reduction in the enzyme activity at higher temperatures can be attributed to possible denaturation of protein conformation. β -galactosidase showed maximum activity at a slightly acidic pH 6. However, the enzyme showed 58%- 45% activity over a pH range of 5-8.5 . The temperature optima of 55 ^{0}C and 65 ^{0}C were reported earlier for two extracellular β -galactosidases from *Aspergillus carbonicus* (O'Connell, S., & Walsh, G. 2008) and an optimum temperature and pH of 50^{0}C and 5 respectively for β -galactosidase from *Asp. Niger* (Martarello R.D, etal.,2019) optimum pH 7.2 and temperature 40^{0}C was reported for β-galactosidase from *Streptococcus thermophilus* grown in whey (S. Princely etal, 2013).

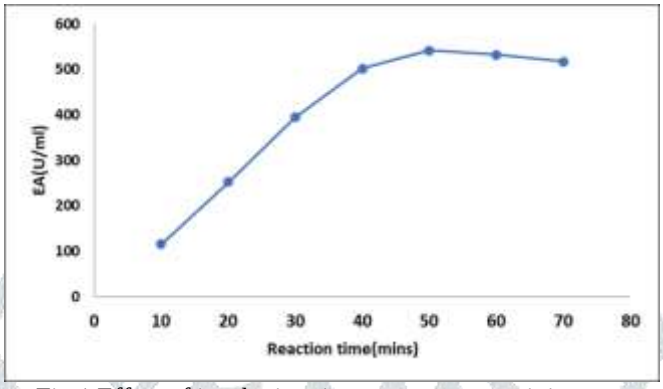

Fig.1 Effect of incubation time on enzyme activity

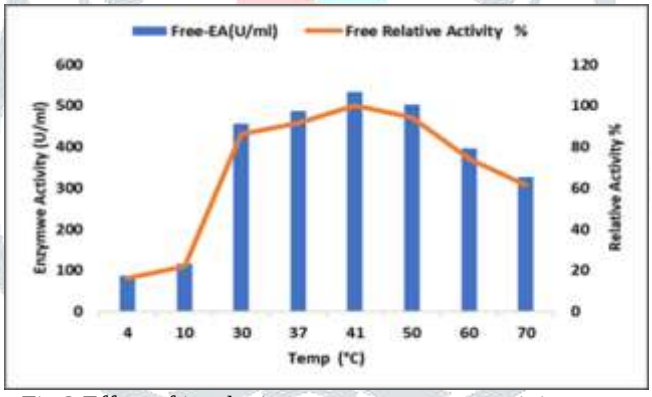

Fig.2 Effect of incubation temp on enzyme activity

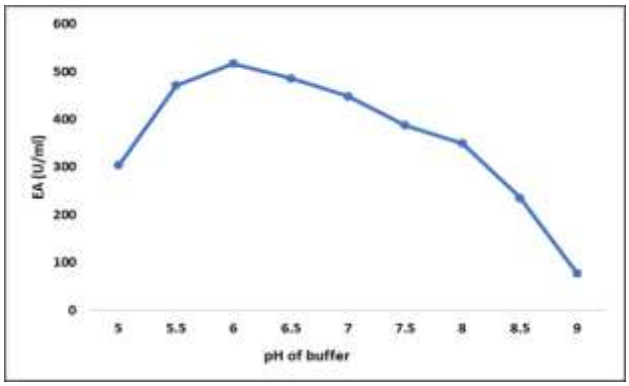

Fig.3 Effect of pH on enzyme activity

Thermostability as function of time β-galactosidase retained 58% activity after 3hrs of incubation at 41^{0}C and the activity reduced to 38% at 50^{0}C after 3 hrs(Fig-4). These observations suggest enzyme has the ability to remain active for longer duration at optimum temperatures resisting unfolding in the absence of substrates. Similar study of thermostability was Kamran A., Bibi Z., etal reported for *Aspergillus nidulans*–derived β -galactosidase retaining100% activity even after 2 h of incubation at 40°C but 40% and 95% reduction was observed at 50°C and 60°C after 2 h of incubation respectively (Kamran, A etal., 2019), a recombinant β-galactosidase from *Bacillus licheniformis* KG9 exhibited high stability at 60°C even after 120 min of incubation (Matpan Bekler, etal., 2015).

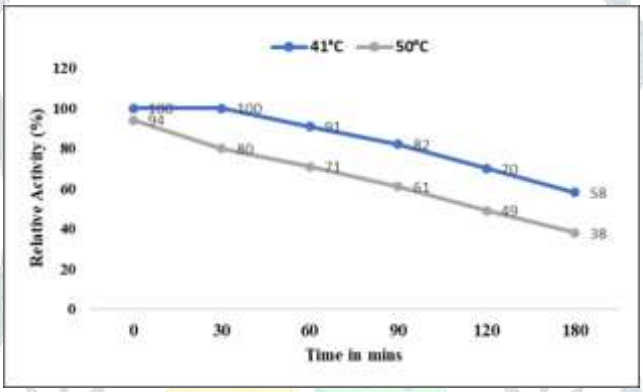

Fig.4 Thermostability of β-galactosidase

Effect of Metal ions From Fig-5 Mg^{2+} found to be enhancing the enzyme activity by 7% at 1mM conc while K^+ retained the enzyme activity at same conc. Presence of $Cu2^+$, $Zn2^+$ and Fe^{2+} found to be inhibiting β -galactosidase activity at all tested concentrations. Mn^{2+} retained 96% activity at 1mM but inhibited at further higher concentrations. This observation shows the potential of β -galactosidase for varying degrees of lactose hydrolysis in the presence of metal ions that are naturally available with milk and whey .Similar results were reported on *Erwinia sps* where cations Li^+, K^+, and Mg^{++} enhanced enzyme activity whereas Cu^{++}, Fe^{++}, and Pb^{++} showed inhibition(Yu Xia, etal., 2018), β-galactosidase from *Bacillus sps* was activated by divalent metal ions Co^{++},Mn^{++} and Mg^{++} (Zhou Z etal.,2021; Maksimainen, M etal., 2012) .

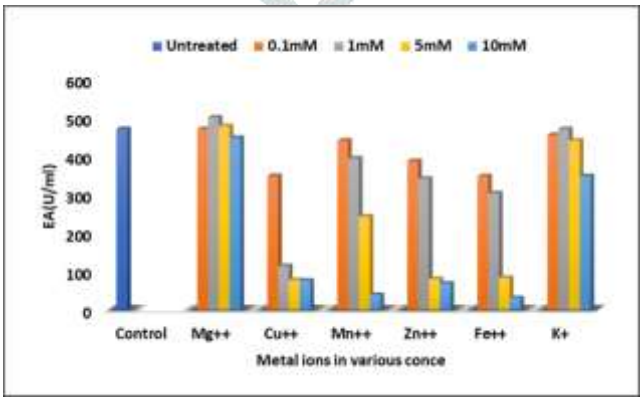

Fig-5 Effect of metal ions von enzyme activity

Effect of inhibitors From Fig-6 β -galactosidase is inhibited strongly by Galactose (65% activity retained at 1mM and 45% at 10mM) followed by Glucose. Maltose and Sucrose did not show great reduction in the activity (83% activity retained at 10mM) while Lactose enhanced the enzyme activity marginally (2%) at 1mM but retained 90% activity at 10mM. These observations indicate the feedback inhibitions of glucose and galactose as reaction products of lactose hydrolysis.Positive effect of Lactose shows possible competition with ONPG as substrate. Similar observations were reported by with Galactose and Glucose while

working on β -galactosidase from *A. oryzae* (Carlos Vera etal.,2012), and β-galactosidases purified from *C. saccharolyticus* (Park, AR., Oh DK., 2010).

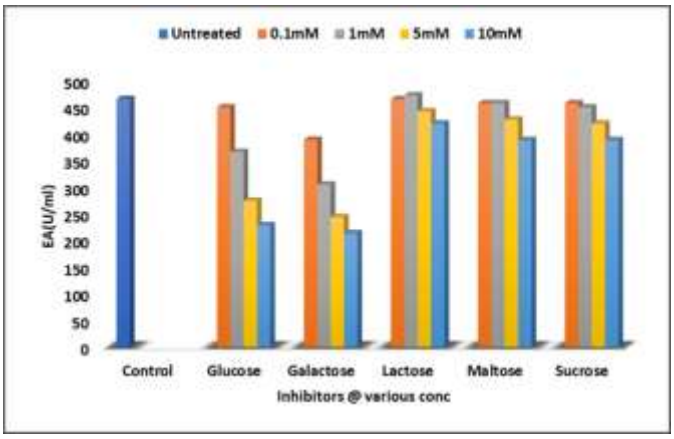

Fig-6 Effect of inhibitors on enzyme activity

Effect of Additives From Fig -7 Urea and SDS were found to inhibit the enzyme activity suggesting they form complexes with protein interacting with catalytic sites. BME at 0.1mM resulted in marginal increase of 2% in β-galactosidase activity, at 1mM retained 95% activity but found to be inhibitory at higher concentrations. Non-ionic detergent Triton X-100 retained 92% and 65% β-galactosidase activity at 0.1mM and 1mM. This observation indicates that it delays enzyme deactivation at certain concentrations. At 1mM and 10mM of EDTA relative activity of 75% and 45% was observed indicating destabilization of β-galactosidase by EDTA by chelating Mg^{++}. Similar results of enzyme inhibition in the presence of DTT, Urea, EDTA were reported with β-galactosidase (Azra Shafi, Qayyum Husain ., 2022), enhanced β-galactosidase activity indicating a sulfhydryl group in the active site of the enzyme from psychrotrophic *B. subtilis* KL88 in the presence of 2-mercaptoethanol and other reducing compounds (Rahim, K.A & Lee, B. H., 1991), thermal stabilization of β-galactosidase from *Bacillus circulans* in the presence of TritonX-100 (Soto, Dayana etal., 2016).

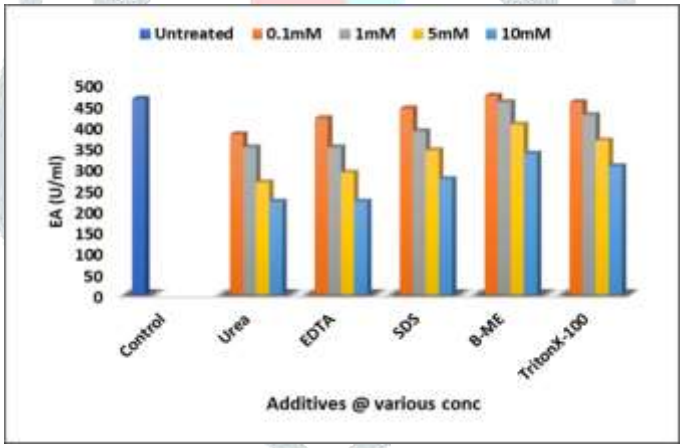

Fig-7 Effect of additives on enzyme activity

Effect of Substrate Concentration and Determination of Km-V_{max} From Fig-8 β-galactosidase activity found to be increased with increase in the conc of ONPG, highest at 8mg/ml of ONPG and the activity did not rise at further increase in ONPG. Fig-9 represents the Lineweaver Burk plot for Km and Vmax. Km was found to be 3.27mg/ml and Vmax 45µmoles/min. Km indicates that the enzyme has an affinity for ONPG. Literature study reports varied Km values for β-galactosidases isolated from different sources: a Km of 1.32 mM for ONPG for *Rhizomucor* derived β-galactosidase (Shaikh S etal., 1999), Km of 1.74 mM and Vmax of 137 UI/mL for ONPG in a study on *A. niger* β-galactosidase (O'Connell S, Walsh G ., 2010) , Vmax for *Rhizomucor* as 4.45 mmol.min-1.mg prot-1 and for *Kluyveromyces lactis* as 5.40 ± 0.86 x10-2 mmol.min-1.mg-1(Adalberto P etal., 2010).

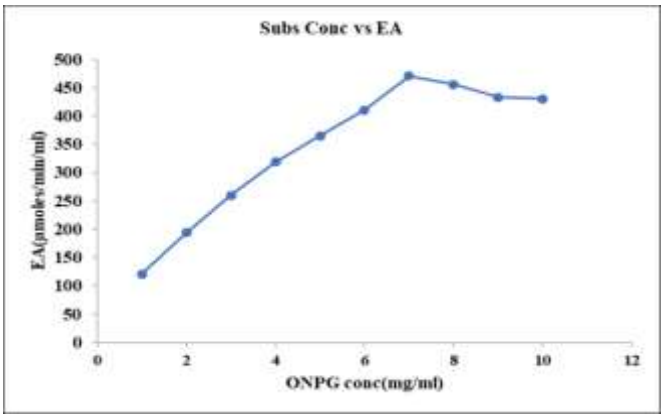

Fig-8 Effect of ONPG concentration on enzyme activity

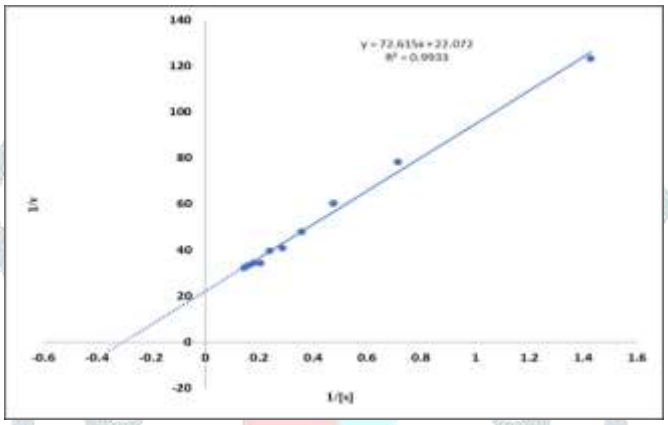

Fig-9 Km-Vmax of β-galactosidase

Conclusion Table 1 summarizes the kinetics and characteristics of β-galactosidase produced from the newly isolated Actinomycetes strain *Streptomyces thermocarboxydus* using whey in growth medium to achieve sustainability. As milk gets often adulterated with urea and other compounds for a better shelf life, the chances of these contaminants to get detected in whey also increase . This study helps to optimize the stability of enzyme in the presence of such various interactive compounds and additives at industrial level that interfere with lactose hydrolysis of whey.

Table-1 Summary of Enzyme Characteristics

Enzyme parameters	Values
Protein concentration of enzyme extract	6 mg/ml
Volume of enzyme extract	40 ml
Total protein of enzyme extract	240 mg
Enzyme activity	471 U/ml
Total enzyme activity	18840U
Specific enzyme activity	78.5U/mg
Optimum incubation time	50min
Optimum pH	6
Optimum temperature	41ºC
Km	3.27 mM of ONPG
Vmax	45nmol/min/ml

The findings of this study provide new insight into the kinetics of free β-galactosidase in the presence of different physical parameters, inhibitors and additives. Considering that so far there are no studies reported on the characterisation of β-galactosidase from Streptomyces strains, this study provides valuable information to scaleup to industrial level of enzyme production. This study also offers a strong potential to further investigate enzyme purification and immobilization to allow its reuse in order to further lower the process costs

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Conflict of interest
The authors declare that they have no conflict of interest

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