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Purification and characterization of β-galactosidase from *Aspergillus fumigatus* PCSIR-2013

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Abstract: Extra cellular β-galactosidase enzyme was purified and characterized from *Aspergillus fumigatus* PCSIR-2013. Estimated molecular mass of the enzyme was approximately 95 kDa. by native polyacrylamide gel electrophoresis. Initially, different fermentation parameters were optimized for maximum production of β-galactosidase. The kinetic study of the partially purified enzyme exhibited that it remained active in broad range of temperature from 25°C to 70°C with an optimum of 60°C. The Km and Vmax were calculated as 9.95mmol/l and 51.78 U/ml/min, respectively. The optimum pH was 5.0, when reaction mixture was incubated for 30 min. The enzyme was very stable in the presence of different metal ions, although Na^+ (16%) stimulates the activity at 10mM concentration. In contrast, Ba^{+2} and Hg^{+2} have negative effect on enzyme activity and activity decreased to 54% and 19%, respectively. Thermo stability study was revealed that the enzyme retained 72% of its activity at 50°C. Whereas, when enzyme was incubated at 60°C for 120 min, its residual activity was decreased to 42.0%. However, the enzyme was completely inactivated at 80°C after 120 min of pre-incubation. Among different surfactant which incorporated with enzyme, Tween 20 and Triton X-100 both have stimulatory effect and activity increased to 29% and 17%, respectively.

Keywords: Extra cellular β-galactosidase, enzyme activity, fermentation, chromatography, purification.

INTRODUCTION

β-galactosidase (E.C. 3.2.1.23) also referred as lactase, classified as hydrolases. The Lactase enzyme-produce glucose and galactose by the hydrolysis of lactose (Galβ1-4Glc). β-galactosidase act on the terminal residue of β-lactose galactopiranosil (Galb1-4Glc) and produce glucose and galactose (Carminatti 2001, Ansari & Satar 2012). The β-galactosidase widely found in nature, in plants, particularly peaches, almonds and certain species of untamed roses; studies available for presence in animal organs like intestine, brain, placenta, testis and skin tissues; also found in yeasts, such as *Kluyveromyces lactis, K. fragilis* and *Candida pseudotropicalis* and many bacteria, such as *Escherichia coli*, *Lactobacillus bulgaricus*, *Streptococcus lactis* and *Bacillus* sp. The huge fungus population, such as *Aspergillus foetidus*, *A. niger*, *A. oryzae* and *A. Phoenecia* produces the lactase. (Holsinger *et al*., 1991, Husain, 2010, Kazemi *et al*. 2016). This enzyme has industrial importance especially in dairy industry for the hydrolysis of lactose in milk or products derived from whey. Additionally, the enzyme is clinically important for patients with lactose intolerance for the preparation of lactose-free milk and milk products such as ice cream and confectionery industries, to assist patients with lactose intolerance (Shaikh *et al*. 1999, Husain 2010, Saqib S *et al*. 2017). The enzyme β-galactosidase as non- conventional applications arose in recent decades based on its transgalactosylation activity. The enzyme can be used now as a catalyst for the upgrading of readily available and cheap lactose into high added-value of glycosides in synthesis of valuable organic compounds. (Vera *et al*. 2020). Furthermore, the ability of this enzyme to produce a colored product during a chemical reaction has gained its importance in molecular biology (Ianiro *et al*. 2017). In present study, β-galactosidase was isolated and purified from a new strain of *A. fumigatus* PCSIR-2013.There were few reports regarding this strain.

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MATERIALS AND METHODS

Screening of β-Galactosidase producing fungal strain
Nutrient agar plates were infused with 0.05ml of 20mg/ml X-Gal(5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) for the selection of colonies, with lactose fermenting ability. The plates were then incubated at RT for 48 hrs. Blue colonies were observed on the plates, indicating the presence of β-galactosidase producing fungal strain. In another screening procedure, crude enzyme (100µl) was poured in the well of X-Gal plate and incubated for 18 hours. Appearance of blue color outside the well confirmed the presence β-galactosidase enzyme (fig. 1).

Fungal strain and culture conditions
The fungal strain used in this study was isolated from environment and identified as *A. fumigates* PCSIR-2013. Isolated fungal strain was grown at 35°C in modified Vogel's medium consisting of (g/L): glucose 50.0; peptone 1.0; $MgSO_4$ 0.2; NH_4NO_3 2.0; $(NH_4)_2SO_4$ 4.0; Tri–sodium citrate 5.0 and yeast extract 2.0. The pH of the medium was adjusted to 6.0 before autoclaving.

Purification of β-Galactosidase
In the crude enzyme (cell free filtrate) solid Ammonium

sulphate ($(NH_4)_2$ SO_4) was added slowly with constant stirring at 4°C to give a final saturation of 60% and then kept for 18 hours at 4°C. After 18 hour's centrifuge at 35,000×g for 15 minutes at 0°C to separate the precipitated proteins. The proteins dissolved in minimal volume of 0.1M sodium acetate buffer pH 5.0). The enzyme solution was dialyzed (using Visking dialysis tubing 36/32, Serva.). The gel filtration chromatography was carried out to complete the purification of extracellular β-galactosidase. For this purpose, sephadex G-200 column was used for gel filtration chromatography, then dialyzed sample was applied to column and fractions were collected. Collected fractions were checked for protein and enzyme activity. Active fractions were pooled and concentrated. Then purity of concentrated enzyme was checked and its zymography or in-situ electrophoresis was performed. Enzyme was purified to homogeneity. Purified sample was run on electrophoresis and molecular weight of enzyme was calculated by using molecular weight markers.

β galactosidase assay and protein determination
Substrate
Ortho nitro phenyl beta D-galactopyranoside (ONPG) 40 mM solution in 0.1M sodium acetate buffer pH 5.0.

Assay
Substrate (1.0 ml) was mixed with 0.2 ml cell free filtrate (CFF). Reaction mixture was incubated at 60°C for 30 min. Then reaction was stopped by 1.0 ml of 10% Na_2CO_3 solution. Absorbance was noted at 420 nm (Nagy *et al*. 2001). One unit of enzyme activity was defined as amount of enzyme which liberates 1 micro mole of ortho nitro phenol per min. under standard assay conditions.

Enzyme samples were estimated for total protein concentrations by the method of Lowry *et al*, 1951 with the standard of bovine serum albumin.

Zymography of β-Galactosidase
Non-denaturing polyacrylamide gel (10%) was used in electrophoresis. After running the gel on electrophoresis, the gel was incubated in an assay buffer. The assay buffer contains X-Gal (10 mg/ml) was substitute for ONPG to detect β-galactosidase activity in situ. Blue band formed within gel in results of X-gal hydrolysis (O'Connell and Walsh 2008).

Effect of temperature and Thermal stability on β-Galactosidase activity
Extra cellular β-galactosidase activity was performed at different temperatures (30°C to 70°C). Thermal stability of the enzyme was determined by pre-incubation at various temperatures (from 30°C to 70°C) in the absence of substrate for different period of time (0 to 120 min).

Effect of substrate concentration on β-Galactosidase activity
Concentration of substrate for the optimum activity of extra cellular β-galactosidase was varied from 4.0 mM to 48.0 mM in sodium acetate buffer (0.1M sodium acetate buffer, pH 5.0) and enzyme activity was performed. The kinetic parameters were estimated according to Line weaver and Burk (1934) by linear regression from double reciprocal plots by Microsoft excel worksheet.

Effect of metal ions on β-Galactosidase activity
The extracellular β-galactosidase was incubated with the chloride salt of the different ions at 30°C for 15 min and the enzyme activity was performed. The activity of enzyme in the absence of ions was taken as control 100%.

Effect of surfactant on β-Galactosidase activity
5mM and 10 mM concentration of Tween-20, Tween 80, Brij-35, Triton X-100, sodium dodecyl sulphate (SDS) and sodium tauroglycocholate were added to the enzyme solution. The incubation time was 30 min at 30°C. To determined the Relative enzyme activity control taken as 100%.

Effect of protein denaturing agents on β-Galactosidase activity
The final concentration of 5mM and 10 mM enzyme solution was prepared after addition of EDTA, DTT, β-mercaptoethanol, PMSF and urea. The incubation time was 30 minutes at 37°C. The control was taken as 100% to determine relative enzyme activities.

STATISTICAL ANALYSIS

Statistical analyses were performed using the Microsoft Office Excel 2003 software.

RESULTS

Selection of medium and time course study for β-Galactosidase production
Two different media were used for the production of β-galactosidase. Maximum β-galactosidase production was obtained in Vogel's medium (data not shown). For optimization purpose, different components of the media were varied and it was found that glucose in a concentration of 5.0%, medium pH (6.0) increased enzyme production. In addition, the time course study was revealed that maximum enzyme production was determined after 14 days of fermentation (fig. 1, 2, 3). The decrease in enzyme production after 14 days might be due to decrease in the amount of nutrients in the medium or due to denaturation of enzyme.

Effect of temperature on β-Galactosidase production
The present study suggests that highest production of β-galactosidase was determined at 35°C. Then there was a

gradual decrease was observed. In the same way, fungal growth was also observed well from 25°C to 45°C, but maximum production was determined when fungal growth occurred with black spores (fig. 4).

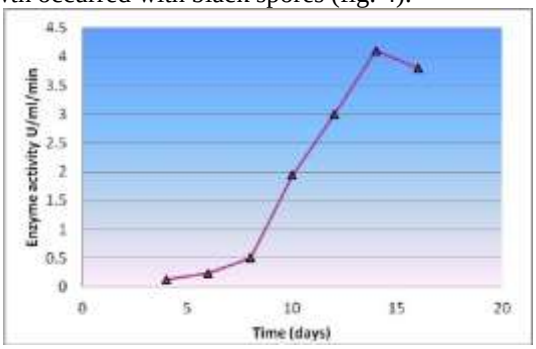


Fig. 1: Screening of β-galactosidase producing strain.

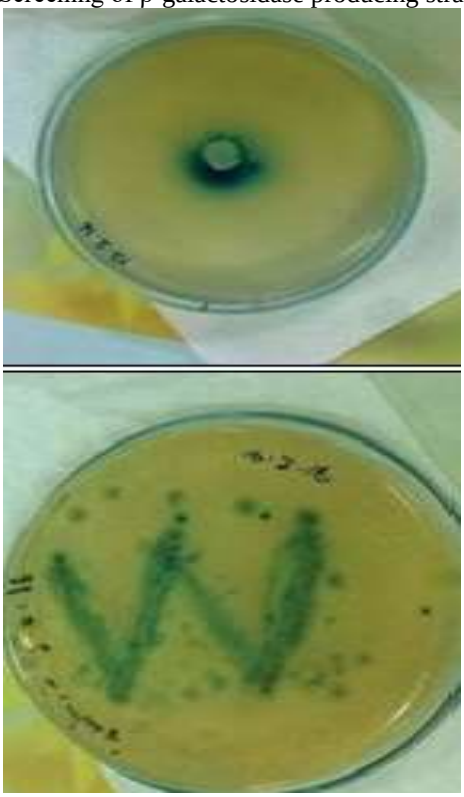


Fig. 2: Time course study for β-galactosidase production from *A. fumigatus* PCSIR-2013

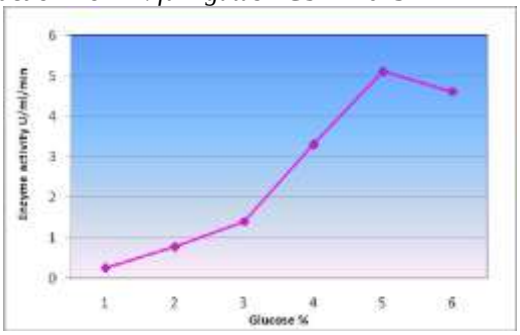


Fig. 3: Effect of glucose concentration on β-galactosidase production *A. fumigatus* PCSIR-2013

Effect of medium pH on β-Galactosidase production

Enzyme production was observed in a broad range of pH (4 to 9) even at extreme acidic and basic medium pH. However, maximum enzyme production was recorded at pH 6 (fig. 5).

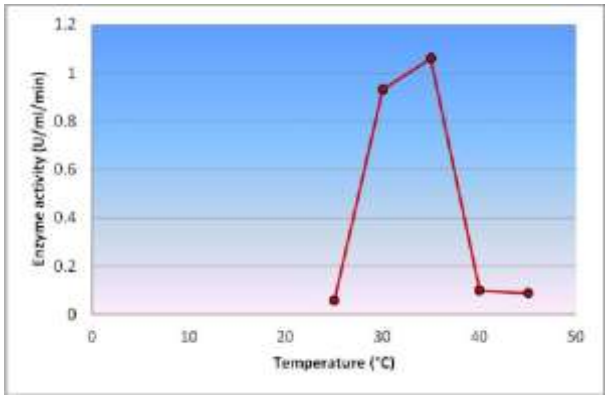


Fig. 4: Effect of temperature on β-galactosidase production from *A. fumigatus* PCSIR-2013

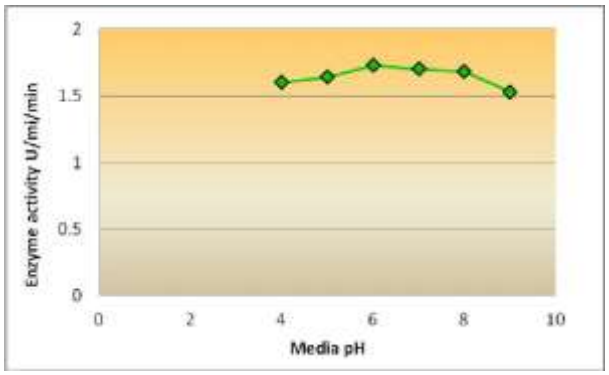
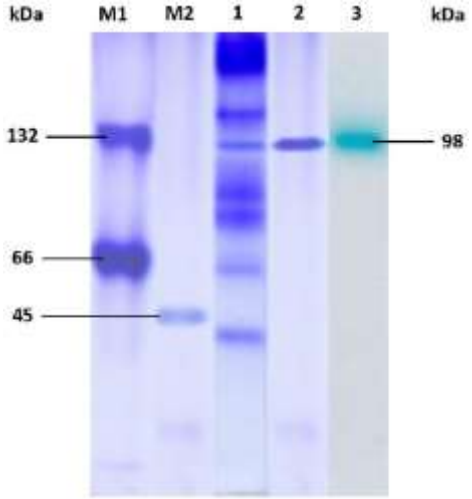


Fig. 5: Effect of medium pH on β-galactosidase production from *A. fumigatus* PCSIR-2013



Lane 1- activity staining of β-galactosidase Lane 2 - purified β-galactosidase (Coomassie blue staining) Lane 3 - partially purified β-galactosidase. M_1&M_2: molecular weight standard

Fig. 6: Native-PAGE of β-galactosidase from *A. fumigatus* PCSIR-2013

Table 1: purification steps of β-galactosidase from *A. fumigates* PCSIR-2013

Purification Step	Enzyme Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification
Crude	732	1380	0.53	1
Partially Purified	501	89	5.6	10.5
Dialysis	477	73	6.4	12.0
Gel chromatography	10.1	1.1	9.0	17.1

Table 2: Effect of Metal Ions on β-galactosidase Activity *A. fumigatus* PCSIR-2013

Metal Ions*	Relative activity (%) Concentration (mM)	
	5	10
Control	100	
Al^{3+}	93	91
Ni^{2+}	91	99
Na^{2+}	100	116
Ca^{2+}	94	94
Hg^{2+}	22	19
K^{2+}	105	103
Ba^{2+}	90	54
Mn^{2+}	99	100
Co^{2+}	92	95
Mg^{2+}	99	101
Cu^{2+}	103	88

*Metal ions used as chloride salts

Then there was a slight decrease was observed but still 88% activity was obtained at 9 pH. Many kinds of fungi have more acidic pH optima during submerged fermentation.

Purification of β-Galactosidase enzyme & molecular mass determination

Some strategies for purifying β-galactosidases from filamentous fungi have been revealed. Some methodologies involve several chromatography steps resulting in multiple yield and purification factor levels.

The purification steps for β-galactosidase was summarized in table 1. The partially purified β-galactosidase enzyme showed specific activity of 6.4 U/mg of protein. The fold purification was increased up to 12.0 fold after dialysis. Native-PAGE showed a single protein band with molecular weight of 95 kDa, which was quite similar to molecular mass of 92 kDa as reported from *A. fumigatus* Z-5 by SDS-PAGE (Liu *et al*. 2012). While its activity staining by X-Gal showed a blue colour band (fig. 6).

Effect of pH on β-Galactosidase activity

It is well known that pH of the buffer influence the rate of enzyme-substrate reaction. Therefore, effect of pH on β-galactosidase activity was determined and it was found that enzyme was fairly stable in a pH range of 3.0 to70.

The Optimum pH for *A. fumigatus* PCSIR-2013 is 5.0 but β-galactosidase can function in acidic to neutral environment, which corresponds to the typical pH of this range, particularly to high basic level of 10 to 12, which can cause lactase enzyme to become denatured (fig. 7).

While at pH 8, when Tris-HCl buffer was used, the enzyme activity drastically decreased, which may be due to the fact that Tris (hydroxymethyl amino methane) completely inhibit some glycoside hydrolases (Saishin *etal*, 2009; Vasseur *et al*. 1990 & Ghalanbor *et al*. 2008). β-Galactosidase activity was inhibited 27% and 46% in the presence of 50 and 100mM Tris, respectively from *Bifidobacterium longum* (Siashin *et al*. 2010).

In general, at extreme pH, the main force, unfolding the protein is the repulsion between charged groups on the protein molecule.

Effect of temperature and Thermal stability of β-Galactosidase

Although enzyme was remains active in wide range of temperature (25-70°C) with an optimum temperature of 60°C.Whereas, at 70°C the enzyme activity decrease only 23%. Further increase in temperature beyond 60°C resulted in reduction in enzyme activity (fig. 8). The loss of activity of the enzyme at higher temperatures could be attributed to its unfolding and subsequent loss of active site (Haider *et al*. 2007). Thermostability is the ability of an enzyme to resist against thermal unfolding in the absence of substrate (Bhatti *et al*. 2006). As shown in fig. after pre-incubation of enzyme at 60°C for 120 min, the β-galactosidase activity reduced to 42%. In contrast, the

enzyme lost 93% of its residual activity at 80°C, after 30 min of incubation (fig. 9). At temperature beyond 70°C, protein most likely undergoes denaturation.

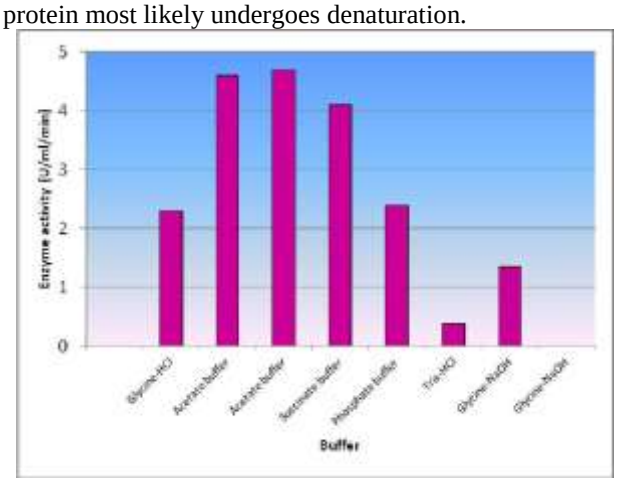


Fig. 7: Effect of buffer pH on β-galactosidase Activity *A. fumigatus* PCSIR-2013.

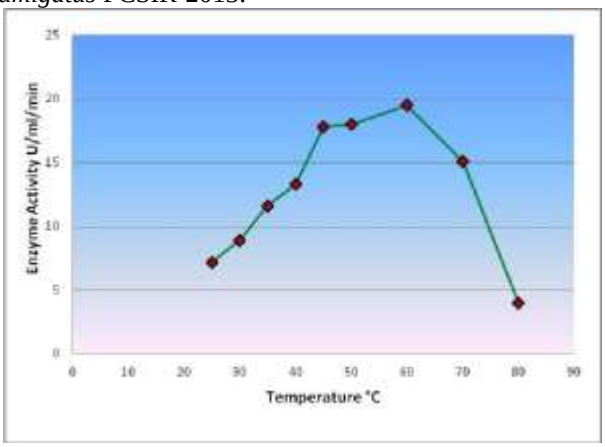


Fig. 8: Effect of temperature on β-galactosidase Activity *A. fumigatus* PCSIR-2013

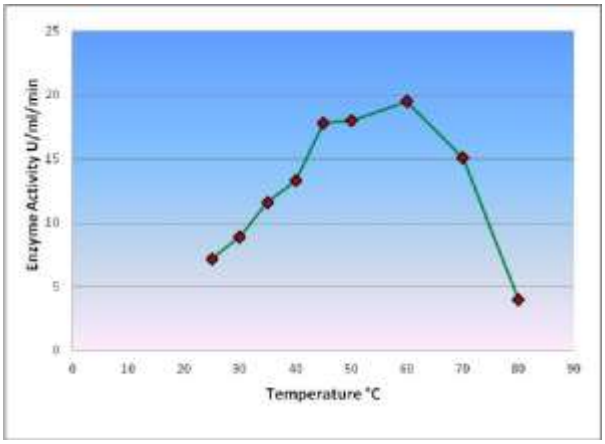


Fig. 9: Thermal stability of β-galactosidase *A. fumigatus* PCSIR-2013

The concomitant aggregation that may leads to intrinsic irreversibility and precluded thermo-dynamic analysis of unfolding. The aggregation might be due to the exposures of the hydrophobic clusters in the protein. The thermal unfolding transitions mostly for multi-domain proteins are accompanied by an irreversible step, often associated with aggregation at elevated temperatures as chemical modifications such as deamidation, cysteine oxidation, or peptide bond hydrolysis take place once the protein is unfolded.

Effect of substrate concentration on β-Galactosidase activity

Specificity of the enzyme was evaluated by a hydrolysis assay involving several substrates. The enzyme was active in the hydrolysis of ONPG and lactose. Toward the other substrates, the enzyme showed little or no activity. The Km & Vmax for β-galactosidase was calculated as 9.95 and 51.78, respectively (fig. 10).

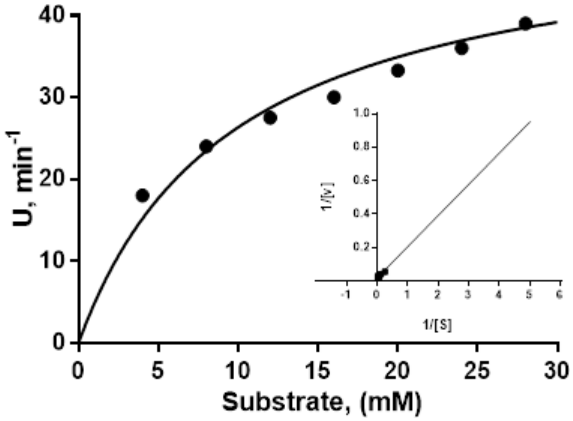


Fig. 10: Effect of substrate concentration on β-galactosidase activity *A. fumigatus* PCSIR-2013.

Effect of metal ions on β-Galactosidase activity

Presence of various cat ions in solution may have effect on the activation or inhibition of enzyme during the reaction. For this reason different metal ion as chloride salts were incorporated with enzyme solution separately and β-galactosidase activity was assayed (table 2). It was observed from table that enzyme was quite stable in the presence of different metal ions, although some metal ions cause slight activation of enzyme like Na^{+} (16%).

While both Ba^{2+} and Hg^{2+} had a concentration dependent inhibitory effect on β-galactosidase activity and at 10 mM concentration residual activity decreased to 52% and 19%, respectively. At the molecular level, Mercury which can exist in 11 different chemical states or compounds forms bonds with sulfhydryl groups on an enzyme, which are parts of the enzyme that contain a sulfur atom that is attached to a hydrogen atom (SH).

Effect of surfactant on β-Galactosidase Activity

In the presence of different surfactant, enzyme activity was also checked and it was found that both Tween-20 and Triton X-100 have stimulatory effect and catalytic activity increased to 29% and 17%, respectively (fig. 11). All other surfactants have stabilizing effect.

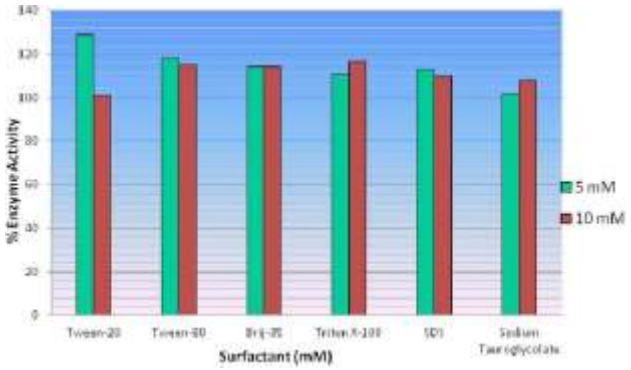


Fig. 11: Effect of surfactant on β-galactosidase Activity *A. fumigatus* PCSIR-2013

Effect of protein denaturing agents on β-Galactosidase activity

The enzyme activity was not significantly altered by chelating agent EDTA up to 10mM, indicating that β-galactosidase is not a metallo enzyme. PMSF was found to enhance 26% enzyme activity at 5mM concentration, while urea has a stabilizing effect. Different reducing agents are extensively used for disulfide bond reduction. The protein denaturing reducing agents effects on beta galactosidase activity was determined (fig. 12).

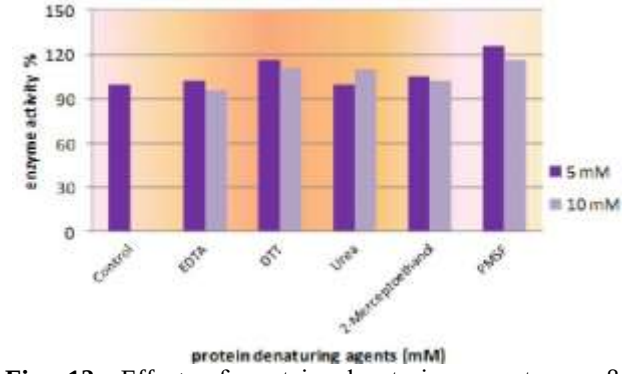


Fig. 12: Effect of protein denaturing agents on β-galactosidase activity

Results indicated that both DTT and 2-Merceptoethanol, at a concentration of 5mM in reaction mixture, activated the enzyme activity up to 16 and 5%, respectively. The β-galactosidase activity stimulation by reducing agents indicates that there is a relationship between the reduced form of the cysteine residues and the activity of the enzyme.

DISCUSSION

β-galactosidases is important for the biotechnology industry. Scientist are making efforts in searching the source for abundant low-cost enzymatic production. For this reason, the study evaluates the production of β-galactosidases from environmentally isolated *A. fumigates* and identified as *A. fumigates* PCSIR-2013. Several β-galactosidases secreted by the *Aspergillus* have been purified and characterized. The occurrence of this enzyme in multiple molecular forms has been suggested by previous work (O'Connell and Walsh 2008; Hu et al. 2010). In this study, different culture conditions were optimized by using modified Vogel's medium for enzyme production.

Literature data reveal some strategies for purifying β-galactosidases from filamentous fungi. Some methodologies involve several chromatography steps resulting in multiple yield and purification factor levels (Nagy *et al.,* 2001 O'Connell and Walsh 2008). In this study we applied the smart technique of gel filtration chromatography to complete the purification of extracellular β-galactosidase and purity is checked by in-situ electrophoresis (Isobe *et al.* 2013b). It has been reported in many studies that acidic pH is ideal for beta-glucosidase activity and also for beta-glucosidases from many sources such as A. oryzae, A. phoenicis, A. carbonarius, A. *aculeatus*, A. *foetidus*, A. *japonicus*, *A. niger* and *A. tubingensis*. The optimum range for those enzymes ranging between pH 4-5, and almost no activity at alkaline conditions (Decker *et al*. 2000, Jager *et al*. 2001, Korotkova 2009, Riou *et al*, 1998) and the enzymatic activity decreased significantly for higher pH values (Cardoso *et al*. 2017). The optimium pH for *A. fumigatus* PCSIR-2013 is 5.0. Thermostability is the ability of an enzyme to resist against thermal unfolding. At higher temperatures, tryptophan residues might also remain buried within protein which limits their further exposure to solvent as a consequence of protein aggregation (Jäger *et al*. 2001, Fitter & Haber-Pohlmeier 2004, Duy & Fitter 2005, 2006, Kumari *et al*., 2010). As previously studied beta-glucosidases from *A. phoenicis*, *A. niger*, *A. carbonarius*, reported all to be stable at 2 hours incubation at 50°C, while activities of 87%, 64% and 53%,respectively, remained after 2 hours incubation at 60°C, and total inactivation was observed after 2 hours at 70°C. It was observed that in an *A. niger* strain studied by Niu *et al* (2017) the enzyme produced has an optimum pH between 4 and 5 and an optimum temperature of 50°C. Compared to this, A. saccharolyticus BGL1 showed approximately the same stability as *A. phoenicis* expect for the total inactivation at 70°C. The studied beta-glucosidases from *A. fumigatus* PCSIR-2013 shows maximum activity at 60°C.

The enzyme activity was found to be stimulate by the metal ions, it may acting as a binding link between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme (Afifi *et al.* 2008). The binding of mercury to enzyme blocks its activities due to the change in shape (Boadi *et al.,* 1992). Inhibition by Hg^{+} may be due to fact that enzyme might posses thiol groups that cause sensitivity to inhibit by Hg^{+} (Ajsuvakova *et al.,* 2020). The surfactants Triton X-100

and SDS caused positive impact on the catalytic activity of enzyme at 1.0mM concentration (Kamran *et al*., 2019). Poly sorbate act as stabilizers for pepsin. It is believed that such surfactants prevent the protein to be adsorbed to it and its subsequent unfolding are preferentially adsorbed at the interface. We observed stabilizing effects on enzymes activity by surfactants. The experimental data for beta-glucosidases from *A. fumigatus* PCSIR-2013 suggest the enzyme has good potential for research and development.

CONCLUSION

In this paper, we present the extensive work on purification and characterization of a thermostable β-galactosidase from *A. fumigatus* PCSIR-2013. The time course study was revealed that maximum enzyme production was determined after 14 days of fermentation and kinetic study of the partially purified enzyme exhibited that it remained active in broad range of temperature from 25°C to 70°C with an optimum of 60°C. The Km and V_{max} were calculated as 9.95mmol/l and 51.78U/ml/min, respectively. The enzyme was very stable in the presence of different metal ions. This study shows that *A. fumigatus* PCSIR-2013 can be used as a potential source of β-galactosidase enzyme in the food industry because of its stable properties.

REFERENCES

Afifi AF, Kamel EM, Khalil AA, Foaad MA, Fawziand EM and Houseny M (2008). Purification and characterization of amylase from penicillium olsonii under the effect of some antioxidant vitamins. *Glob. J. Biotechnol and Biochem.,* **3**(1): 14-12.

Ajsuvakova OP, Tinkov AA, Aschner M, Rocha J, Michalke B, Skalnaya MG, Skalny AV, Butnariu M, Dadar M, Sarac I, Aaseth J and Bjørklund G (2020). Sulfhydryl groups as targets of mercury toxicity. *Coord. Chem. Rev.*, **417**(15) 213343.

Ansari SA and Satar R (2012). Recombinant β-galactosidases-past, present and future: A mini Review. *J. Mol. Catal. B: Enzym.*, **81**: 1-6.

Banga AK (2006). Therapeutic Peptides and Proteins Formulation, Processing and Delivery Systems, 2nd ed., Taylor & Francis., New York.

Boadi WY, Urbach J, Brandes JM, Yannai S. (1992). *In vitro* effect of mercury on enzyme activities and its accumulation in the first-trimester human placenta. *Environ Res.*, **57**(1): 96-106.

Cardoso BB, Silverio SC, Abrunhosa L, Teixeira JA and Rodrigues LR (2017). β-galactosidase from *Aspergillus lacticoffeatus*: A promising biocatalyst for the synthesis of novel prebiotics. *Int. J. Food Microbiol.*, **257**(4): 67-74.

Decker CH, Visser J and Schreier P (2000). Beta-glucosidases from five black *Aspergillus* species: Study of their physico-chemical and biocatalytic properties. *J. Agric. Food Chem.*, **48**(10): 4929-4936.

Duy C and Fitter J (2005). Thermostability of irreversible unfolding α-amylases analyzed by unfolding kinetics. *J. Biol. Chem.,* **280**(45): 37360-37365.

Duy C and Fitter J (2006). How aggregation and conformational scrambling of unfolded states govern fluorescence emission spectra? *Biophys J.,* **90**(10): 3704-3711.

Fitter J, Haber-Pohlmeier S (2004). Structural stability and unfolding properties of thermostable bacterial α-amylases: A comparative study of homologous enzymes. *Biochemistry,* **43**(30): 9589-9599.

Ghalanbor Z, Ghaemi N, Marashi SA, Amanlou M, Habibi-Rezaei M, Khajeh K and Ranjbar B (2008). Binding of Tris to *Bacillus licheniformis* α-amylase can affect its starch hydrolysis activity. *Prot. Peptide Lett.*, **15**(2): 212-214.

Haider T and Husain Q (2007). Preparation of lactose-free milk by using salt-fractionated almond (*Amygdalus communis*) beta -galactosidase, *J. Sci. Food Agric.*, **87**(Issue): 1278-1283.

Holsinger VH and Kilgerman KH (1991). Application of lactose in dairy foods and other foods containing lactose. *Food Technology,* **45**(1): 94-95.

Husain Q. (2010). β Galactosidases and their potential applications. *Crit. Rev. Biotechnology,* **30**(1): 41-62.

Ianiro G, Pecere S, Giorgio V, Gasbarrini A, Cammarota G (2017). Digestive enzyme supplementation in gastrointestinal diseases. *Curr. Drug Metab.,* **17**(2): 187-193.

Isobe K, Yamashita M, Chiba S, Takahashi N and Koyama T (2013b). Characterization of new β-galactosidase from acidophilic fungus, *Teratosphaeria acidotherma* AIU BGA-1. *J. Biosci. Bioeng.,* **116**(3): 293-297.

Jäger S, Brumbauer A, Feher E, Reczey K and Kiss L (2001). Production and characterization of β-glucosidases from different *Aspergillus* strains. *World J. Microbiol. Biotechnol.,* **17**(5): 455-461.

Jahan SN (2009). Characterization and stabilization of Pepsin - A systematic approach in formulating therapeutic proteins (Master of Science), Uppsala University, (Degree Project in Applied Biotechnology).

Kamran A, Bibi Z, Aman A and Qader SAU (2019). Purification and catalytic behavior optimization of lactose degrading β-galactosidase from *Aspergillus nidulans*. *J. Food Sci. Technol.,* **56**(1): 167-176.

Kazemi S, Khayati G and Faezi-Ghasemi M (2016). B-Galactosidase production by *Aspergillus niger* ATCC 9142 using inexpensive substrates in solid-state fermentation: Optimization by orthogonal arrays design. *Iran Biomed J.*, **20**(5): 287-294.

Kong F, Wang Y, Cao S, Gao R and Xie G (2014). Cloning, purification and characterization of a thermostable β-galactosidase from *Thermotoga naphthophila* RUK-10. *Process Biochem.,* **49**(5): 775-

782.

Korotkova OG, Semenova MV, Morozova VV, Zorov IN, Sokolova LM, Bubnova TM, Okunev ON and Sinitsyn AP (2009). Isolation and properties of fungal beta-glucosidases. *Biochemistry-Moscow*, **74**(5): 569-577.

Kumari A, Rosenkranz T, Kayastha AM and Fitter J (2010). The effect of calcium binding on the unfolding barrier:a kinetic study on homologous α-amylases. *Biophys Chem.,* **151**(1-2): 54-60.

Lineweaver H and Burk D (1934). The determination of enzyme dissociation constants. *J. Chem. Soc.*, **56**: 658-66.

Liu D, Zhang R, Yang X. Zhang Z, Song S, Miao Y and Shen Q (2012). Characterization of a thermostable β-glucosidase from *Aspergillus fumigatus* Z5 and its functional expression in Pichia pastoris X33. *Microbial Cell Factories.,* **11**(1): 25.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951). Protein measurement with the Folin-Phenol reagents. *J. Biol. Chem.,* **193**(1): 265-275.

Maria L Guzman, Margareth R Marques, Maria E Olivera ME and Erika S Stippler (2016). Activity in the presence of surfactants commonly used in dissolution media, Part1: *Pepsin. Results in Pharma Sciences,* **6**(3): 15-19.

Nagy Z, Kiss T, Szentirmai A and Biro S (2001) β-Galactosidase of *Penicillium chrysogenum*: Production, purification and characterization of the enzyme. *Protein. Expr. Purif.,* **21**(1): 24-29.

Niu D, Tian X, Mchunu NP, Jia C, Singh S, Liu X, Prior BA and Lu F (2017). Biochemical characterization of three *Aspergillus niger*β-galactosidases. *Electron J Biotechnol.,* **27**: 37-43.

O'Connell S and Walsh G (2008) Application relevant studies of fungal β-galactosidases with potential application in the alleviation of lactose intolerance. *Appl. Biochem. Biotechnol.,* **149**(2): 129-138.

Riou C, Salmon JM, Vallier MJ, Günata Z and Barre P (1998). Purification, characterization, and substrate specificity of a novel highly glucose-tolerant beta-glucosidase from *Aspergillus oryzae*. *Appl. Environ. Microbiol.,* **64**: 3607-3614.

Saishin N and Yamamoto I (2009). α-Galactosidase purified from Bifidobacterium longum JCM 7052 grown on gum arabic. *J. Biol. Macromol.,* **9**(2): 73-81.

Saqib S, Akram A, Halim S and Tassaduq R (2017). Sources of β-galactosidase and its applications in food industry. 3 *Biotech.,* **7**(79): 10.

Shaikh SA, Khire JM and Khan MI (1999). Characterization of a thermostable extra cellular β-galactosidase from a thermophilic fungus Rhizomucor sp. *Biochim. Biophys. Acta.,* **1472**(1-2): 314-322.

Vasseur M, Frange R, Cauzac M, Mahmood A and Alvarado F (1990). pH-dependent inhibitory effects of Tris and lithium ion on intestinal brush-border sucrase. *J. Enz. Inhibit. Med. Chem.,* **4**(1): 15-26.

Vera C, Guerrero C, Aburto C, Cordova A and Illanes A (2020). Conventional and non-conventional applications of β-galactosidases. *Biochim. Biophys. Acta Proteins Proteom.,* **1868**(1): 140271.

Zappone M, Kaziska A and Bogush G (2009). Application of detergents in laundering, Handbook of Detergents, Part E: Applications, CRC Press., Boca Raton, FL, pp.69-82.