Macrothink Institute™

Journal of Applied Biotechnology
ISSN 2327-0640
2017, Vol. 5, No. 1

Influence of pH, Temperature and Various Heavy Metals on β-galactosidase Activity in the Crude Extract of *Pleurotus ostreatus*

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Received: December 19, 2016 Accepted: January 15, 2017 Published: January 18, 2017

Doi: 10.5296/jab.v5i1.10473 URL: http://dx.doi.org/10.5296/ jab.v5i1.10473

Abstract

The aim of this study was to determine the activity of β-galactosidase in the crude extracts of *Pleurotus ostreatus* in the presence and absence of various heavy metals. β-galactosidase (EC 3.2.1.23), is a hydrolase enzyme which helps in the hydrolysis of lactose into monosaccharides. Characterization of β-galactosidase from *Pleurotus ostreatus* was achieved using the substrate 2-nitrophenyl β-D-galactopyranoside (ONPG). The pH and temperature profiles of β-galactosidase showed maximum activity at pH 3.0 and at 50°C, respectively. The V_{max} and K_m values of β-galactosidase using ONPG as a substrate was found to be 0.571 μmol/min and 0.307 mM, respectively. These results revealed that the β-galactosidase activity in the crude extracts of *Pleurotus ostreatus* was changed in the presence of different heavy metals. The results indicated that Hg^{2+} and Mo^{2+} have an uncompetitive inhibition on

the β- galactosidase activity in the extract of *Pleurotus ostreatus* by decreasing both Km and V_{max} values. while Al^{3+}, Cu^{2+}, Cr^{3+}, Zn^{2+} and Ni^{2+} showed mixed inhibition activity by decreasing V_{max} values and by increasing K_m values. However, Pb^{2+} was found to act as a non-competitive inhibition by decreasing V_{max} value. The findings suggested that crude extract of *Pleurotus ostreatus* can be used as a source of β-galactosidase for medical and industrial purposes.

Keywords: β-galactosidase, *Pleurotus ostreatus*, Enzyme activity, Inhibition, Heavy metals

1. Introduction

β-galactosidases (EC 3.2.1.23, galactohydrolase, lactase) are hydrolase enzymes that catalyze the hydrolysis of the glycosidic bonds of terminal nonreducing β-D-galactosyl residues of oligosaccharides and β-D-galactopyranosides (Seddigh & Darabi, 2014; Chandrasekar & van der Hoorn, 2016). β-galactosidases are well known biocatalyst to catalyze hydrolytic and transgalactosylation reactions (Princely et al., 2013). In addition, β-galactosidase enzyme is mainly used in the food industry to reduce the lactose concentration in milk products, with the aim of overcoming lactose intolerance (Dutra Rosolen et al., 2015). It has many medical and industrial applications include treatment of lactose malabsorption and production of lactose hydrolyzed milk (Haider & Husain, 2008; Jokar & Karbassi, 2011; Nath et al., 2014). β-Galactosidases have been obtained from microorganisms (bacteria, fungi and yeasts), plants (almonds, peaches, apricots, and apples) and animal sources (Haider & Husain, 2007; O'connell & Walsh, 2007; Panesar et al., 2010).

Microorganisms are considered potentially to be the most suitable source of β-D-galactosidase for industrial applications. However, they differ in their optimum conditions for the production of enzyme (Panesar et al., 2010; Carević et al., 2015). Microorganisms produce enzymes at higher yields compared to animal and plant sources of enzymes (Jokar & Karbassi, 2011). In fungi, two principal strategies for catabolism of lactose are realized: (i) extracellular hydrolysis and subsequent uptake of resulting monomers and (ii) uptake of disaccharides (Juers et al., 2012; Miguel, 2015). One of major industrial β-Galactosidase sources is isolated from *Kluyveromyces lactis* and it is one of the most used enzymes for manufacturing milk and dairy products (Kim et al., 2004; Kim et al., 2006; Klewicki, 2007).

The problem of environmental pollution due to toxic metals is of major concern in the environment. The toxic metals entering the ecosystem may lead to accumulation, bioaccumulation and biomagnifications (Jaishankar et al., 2014). Heavy metals play an extremely important role in biochemical reactions which are significant for the growth and development of microorganisms, plants and animals (Kavamura & Esposito, 2010). At excess concentrations, these metal ions can become detrimental to living organisms, including fungi (Emamverdian et al., 2015). The presence of toxic compounds, such as heavy metals, is one important factor that can cause damage to organisms by altering major organisms physiological and metabolic processes (Aldoobie & Beltagi, 2013). Several heavy metals such as iron (Fe^{2+}), manganese (Mn^{2+}), zinc (Zn^{2+}), copper (Cu^{2+}), cobalt (Co^{2+}), or molybdenum (Mo^{2+}) are essential for the growth of organisms (Gaur and Adholeya, 2004). Fungi are one of the most important group of organisms that have revealed different values of

sensitivity towards metal ions (Jaeckel et al., 2005; Tong et al., 2016).

Pleurotus ostreatus, also known as the oyster mushroom, is a Basidiomycetes belonging to the family Pleurotaceae (Hibbett, 2007). Interest in this species has increased considerably in the last decade because of its gastronomic value and its nutraceutical properties (Barros et al., 2007; Papaspyridi et al., 2012). The medicinal beneficial effects of *P. ostreatus*, such as antioxidant, antitumor and cholesterol-lowering activities, have been investigated intensively (Horincar et al., 2014). It has been revealed that an extract of *P. ostreatus* was able to alleviate the hepatotoxicity induced by CCl_4 in rats (Jayakumar et al., 2008). It has been also reported that the extract from *P. ostreatus* appeared to protect major organs such as the liver, heart, and brain of aged rats against oxidative stress (Jayakumar et al., 2008).

However, to our knowledge, no study has determined the activity and kinetics of β-galactosidase in the crude extract of *Pleurotus ostreatus*. Therefore, this study was conducted to characterize the enzyme in the term of pH, temperature and enzyme kinetic using the substrate 2-nitrophenyl β-D-galactopyranoside (ONPG) and to identify the effects of various heavy metals on its activity in order to use this enzyme in medical and food industrial purposes.

2. Materials and Methods

2.1 Fungi Sample

Pleurotus ostreatus was cultivated at Muta'h University in the biochemistry research lab at room temperature, Fungus grew through pores of bag (Kong, 2004). Samples have been collected from cultivated organism between April-August, 2015.

2.2 Chemicals

ONPG was purchased from Sigma Chemicals (St. Louis, MO, USA). Sodium potassium tartrate, Sodium carbonate anhydrous, Sodium acetate and Sodium dihydrogen phosphate were provided by Pharmacos (Birmingham, England), FLUKA (Madrid, Spain), Riedel-De Haen. Sigma-Aldrich Laborchemikalien Chemik (Seelze, Germany) and Panreac (Barcelona, Spain), respectively. All other chemicals were of the highest grade.

2.3 Crude Enzyme Preparation

Samples of *Pleurotus ostreatus* were homogenized in 100 mL sodium acetate buffer (100 mM, pH 5) in a blender for 4 min. The crude enzyme extract was filtered using filter paper and then was centrifuged at 10,000 rpm for 15 min (Luz et al., 2012). The extract was stored in a refrigerator at 4°C until use.

2.4 Enzyme Activity Assay

The activity of β-galactosidase was assayed using ONPG as a substrate. The assay mixture (1mL) was prepared by mixing 0.4 ml of 5.0 mM ONPG in 0.5 ml of 0.1 M acetate buffer (pH 4.0) and 0.1 mL of enzyme extract (Sekimata et al., 1989). After incubation for 15 min at 37°C, the reaction was terminated by addition of 1 mL of 0.1 mM Na_2CO_3 and monitored at 420 nm. O-Nitrophenol (ONP) was the product of the enzyme catalyzed reaction and it contributed a certain yellow color in the assay mixture. One unit of enzyme activity is defined

as the amount of enzyme that liberates 1.0 μmol of ONP per minute under the assay condition (Gulzar & Amin, 2012; Carević et al., 2015).

2.5 Kinetic Parameters Determination

The maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) of β-galactosidase for ONGP as substrate were determined. The effect of substrate concentration on enzyme activity was using optimum reaction pH and temperature. Keeping the amount of enzyme constant in assay mixture, the concentration of ONGP was increased from 1 mM to 8 mM. The enzyme activity was assayed by monitoring the absorbance at 420 nm. Lineweaver-Burk Plot (Reciprocal plots) were used to determine V_{max} , K_m values (Lineweaver & Burk, 1934).

2.6 Effect of pH on β -galactosidase Activity

The enzyme-substrate reaction was performed using various buffer systems with different pH ranges from 2.0- 9.0 in various buffer systems (Sodium acetate buffer and sodium phosphate buffer) (Lee et al., 2003; Pal et al., 2013). The enzyme assay was performed separately in each buffer system. The relative activities were calculated by dividing the velocity value at certain pH point by V_{max} value and then multiplied by 100 (Meghdari et al., 2015).

2.7 Effect of Temperature on β -galactosidase Activity

The enzyme-substrate reaction was carried out at various temperatures ranging from 20°C to 90°C using optimum reaction pH (Pal et al., 2013). The β-galactosidase activity was measured as previously described in the enzyme assay, and the relative activities were calculated by dividing the velocity value at certain temperature point by V_{max} value and then multiplied by 100 (Meghdari et al., 2015).

2.8 Effect of Different Heavy Metals on Enzyme Activity

In order to determine the effect of various metal ions on β -galactosidase activity, these metal ions were incorporated in the standard assay mixture at different concentrations (200-800μM). The activity was expressed as relative activity (%) compared to control (Nweke & Okpokwasili, 2011).

2.9 Statistical Analysis

All the experiments were done in triplicate and the results are expressed as mean values±standard deviations (S.D.) using Microsoft excel 2010.

3. Results and Discussion

3.1 Effect of pH on β-galactosidase Activity

Enzymes are affected by changes in pH. The most favorable pH value the point where the enzyme is most active is known as the optimum pH (Talley & Alexov, 2010; Salwanee et al., 2013). The optimal pH value depends on various factors such as nature of buffer system, presence of activators or inhibitors, age of the cell and nature of the substrates (Bisswanger, 2014). The changes in ionization of prototropic groups in the active site of an enzyme at lower acid and higher alkali pH values may prevent proper conformation of the active site,

binding of substrates, and/or catalysis of the reaction (Robinson, 2015).

In the present study, it was observed that the maximum enzyme activity was measured at pH 3.0 (Figure 1). As the pH was increased above 3.0 the activities gradually decreased. The relative activities were based on the ratio of the activity obtained at certain pH to the maximum activity obtained at that range and expressed as percentage. The relative activity of β-galactosidases at pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 was 40.1%, 100%, 63.3%, 48.3%, 38.2%, 28.9%, 21.3% and 16.5%, respectively.

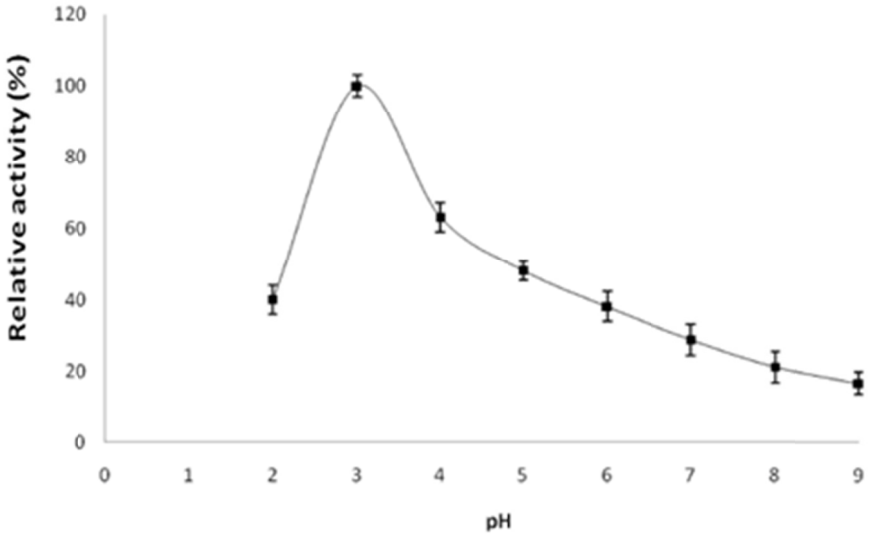

Figure 1. Relative activity (%) of β-galactosidase in the crude extracts of *Pleurotus ostreatus* at different pH values using ONPG as a substrate. Mean±SD (n=3).

Previous studies have shown that fungal β-galactosidases generally have acidic pH-optima in the range of 2.5-5.5 thus they are most effective for the hydrolysis of lactose present in acidic products such as whey (Husain, 2010). β-galactosidase from *Teratosphaeria acidotherma* showed optimum pH between 2.5- 4.0 using ONPG (Isobe et al., 2013). In addition, optimum pH value of β-galactosidase from *Aspergillus fonsecaeus* was found 2.6 to 4.5 using ONPG as a substrate (Gonzalez & Monsan, 1991). Another study showed that the optimum pH value of β-galactosidase from *Penicillium chrysogenum* was found at pH 4.0 (Nagy et al., 2001).

3.2 Effect of Temperature on β-galactosidase Activity

Temperature is another important factor that significantly influences the catalytic activity of enzymes. Each enzyme has an optimum temperature at which it performs best. It is well known that a decrease in the kinetic energy of the reactant molecules at low temperatures corresponds to a slower reaction (Salwanee et al., 2013). Temperature can affect an enzyme in two ways. One is a direct influence on the reaction rate constant, and the other is in thermal

denaturation of the enzyme at elevated temperatures (Peterson et al., 2007).

However, the effect of temperature on the activity of β-galactosidase was determined by performing the standard assay procedure at different temperatures ranging from 20 to 90ºC. In the present findings, the optimum temperature for β -galactosidase activity was found to be 40°C. The enzyme activity was gradually increased with increasing temperature up to 40°C, and thereafter declined. The relative activities (as percentages) were expressed as the ratio of the β -galactosidase activity obtained at certain temperature to the maximum activity obtained at the given temperature range (Figure 2). The decrease in the activity of the enzyme at high temperatures may be attributed to temperature-induced conformational change at the active site and thus loss of active site (Haider & Husain, 2007; Daniel et al., 2010).

However, Optimum temperature value of β-galactosidase from *Aspergillus terreus*, *Aspergillus nidulans* and *Penicillium chrysogenum* using ONPG as a substrate was found to be 30ºC (Diaz et al., 1996; Nagy et al., 2001; Vidya et al., 2014). In addition, optimum temperature of β-galactosidase from *Aspergillus fonsecaeus*, *Thermomyces lanuginosus* and *Rhizomucor* sp. was found to be at 50ºC (Gonzalez & Monsan, 1991; Fischer et al., 1995; Shaikh et al., 1999). Also, β-galactosidase from *Aspergillus oryzae*, *Klumeromyces marxians*, *Aspergillus niger* and *Penicillium simplicissimum* showed optimum temperature between 55-60ºC (Cruz et al., 1999; Rajoka et al., 2003; Domingues et al., 2005; Nizamuddin et al., 2008).

These observations from the pH and temperature-activity relationships indicate that the enzyme has the capacity to withstand the severity of industrial and medical applications.

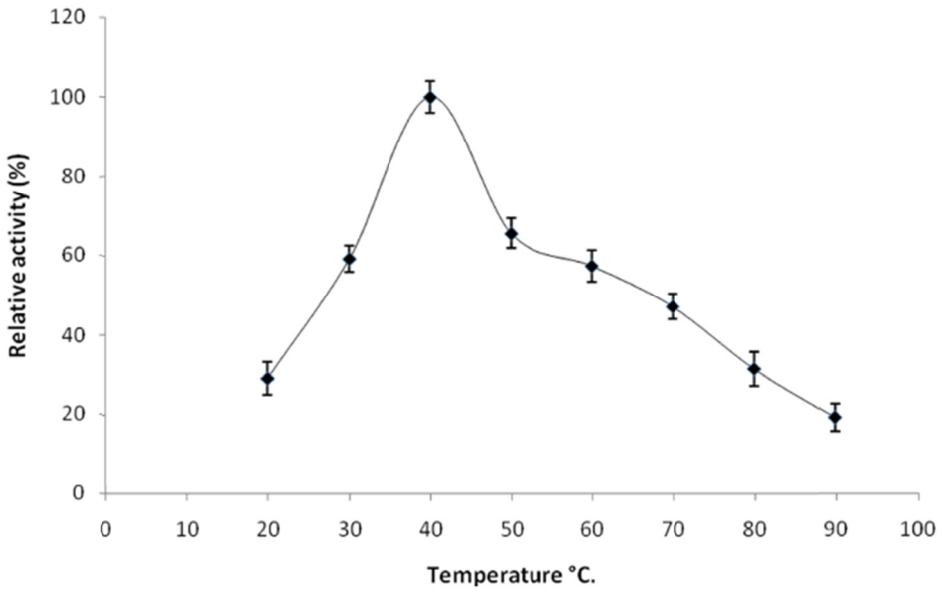

Figure 2. Relative activity (%) of β-galactosidase in the crude extracts of *Pleurotus ostreatus* at different temperature values using ONPG as a substrate. Mean±SD (n=3).

3.3 Kinetic Parameters

The kinetic parameters of β-galactosidase from *Pleurotus ostreatus* for hydrolysis towards ONPG at pH 3.0 and 40ºC were determined by a typical double reciprocal Line-weaver Burk plot. The K_m and V_{max} values for hydrolyzing ONPG were found to be 0.307 mM and 0.571μmol/min, respectively (Figure 3).

V_{max} is the rate of reaction when the enzyme is saturated with substrate. Increasing the substrate concentration indefinitely does not increase the rate of an enzyme-catalyzed reaction beyond a certain point (Bar-Even et al., 2011). Besides, the relationship between rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate. This is usually expressed as the K_m (Michaelis constant) of the enzyme, and reflects the affinity of the enzyme for its substrate. K_m is the concentration of substrate which permits the enzyme to achieve half V_{max}. The lower the K_m, the greater the affinity (so the lower the concentration of substrate needed to achieve a given rate) (Lu, 2004; Reuveni et al., 2014).

However, K_m value was less than that of other organisms, 11.3 mM for *Thermomyces lanuginosus* (Fischer et al., 1995), 0.785 for *Rhizomucor* sp. (Shaikh et al., 1999), 1.78 mM for *Aspergillus fonsecaeus* (Gonzalez & Monsan, 1991), 1.81 mM for *Penicillium chrysogenum* (Nagy et al., 2001) and 1,61 mM for *Aspergillus Oryzae* (Gargova et al., 1995).

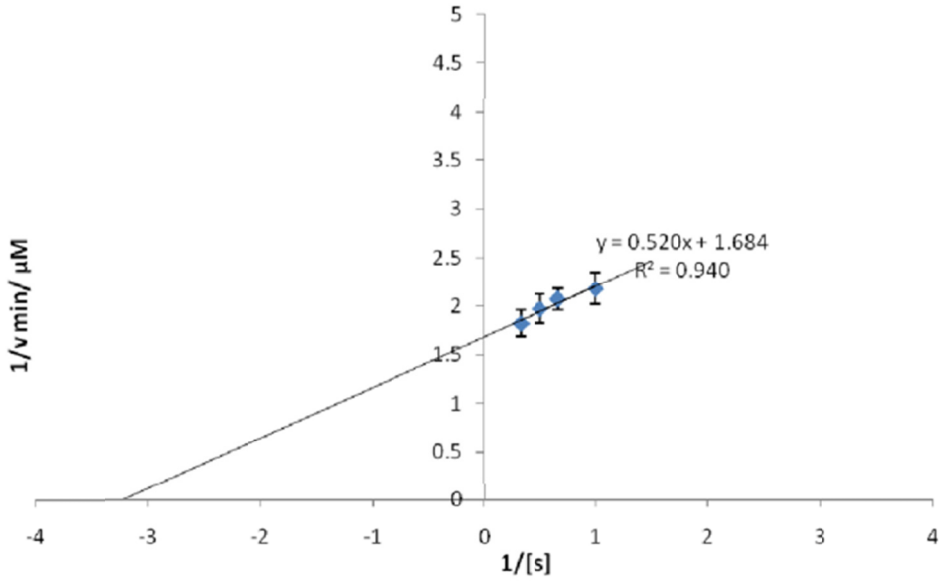

Figure 3. Determination of V_{max} and K_m values for β-galactosidase in the crude extracts of *Pleurotus ostreatus* using ONPG as a substrate. Mean±SD (n=3).

3.4 Effect of Different Heavy Metals on β-galactosidase Activity

Heavy metals at higher concentrations are toxic to living organisms primarily because of their protein binding capacity and hence to their ability to inhibit enzymes (Pritsch et al., 2006). All results are analyzed according to their effects: uncompetitive inhibition, mixed inhibition

and noncompetitive inhibition.

The effect of an uncompetitive inhibitor is to decrease K_m and to decrease V_{max} (Voet et al., 2013; Gonze & Kaufman, 2015). Uncompetitive inhibition cannot be reversed by increasing the substrate towards a saturating concentration, leading to decrease both K_m and V_{max} (Sharma, 2012). As shown in table (1), Hg^{2+} and Mo^{2+} have an uncompetitive inhibition on the β-galactosidase extract activity in *Pleurotus ostreatus* by decreasing both K_m value from 0.307 mM to 0.285 mM and 0.253 mM, and V_{max} from 0.571µmol/min to 0.189 and 0.377 µmol/min, respectively. Figure (4) showed that the relative activity (%) of the enzyme in the presence of Hg^{2+} and Mo^{2+} was 33.1% and 66.1%, respectively.

However, the effect of a mixed inhibitor is to increase K_m and to decrease V_{max} (Voet et al., 2013). If the ability of the inhibitor to bind the enzyme is exactly the same whether or not the enzyme has already bound the substrate, it is known as a non-competitive inhibitor. Non-competitive inhibition is sometimes thought of as a special case of mixed inhibition (Storey, 2005). In the present findings, Al^{3+}, Cu^{2+}, Cr^{3+}, Zn^{2+} and Ni^{2+} have mixed inhibition on the β-galactosidase extract activity in *Pleurotus ostreatus* by decreasing V_{max} from 0.571 µmol/min to 0.476, 0.43, 0.363, 0.425 and 0.39 µmol/min, and by increasing K_m from 0.307 mM to 1.162, 0.91,1.28, 0.66 and 0.892 mM, respectively, while Pb^{2+} showed non-competitive inhibition by decreasing V_{max} from 0.571 µmol/min to 0.408, and showed no change on K_m value (Table 1). In addition, the relative activity of β-galactosidase in the presence of Al^{3+}, Cu^{2+}, Cr^{3+}, Zn^{2+}, Ni^{2+} and Pb^{2+} was 83.3%, 75%, 63.5%, 74.4%, 68.3% and 71.4%, respectively (Figure 4).

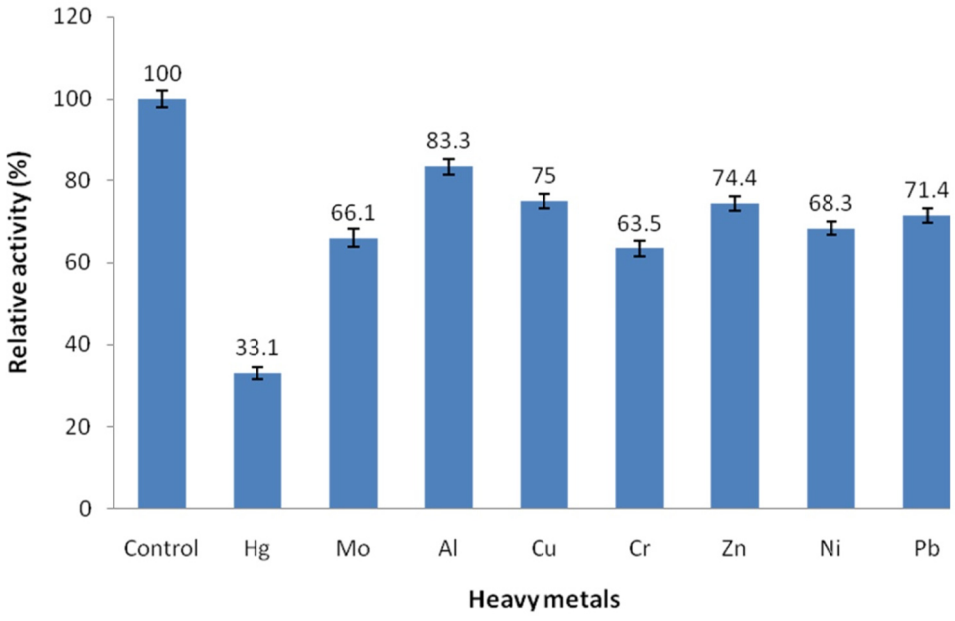

Figure 4. Relative activity (%) for β-galactosidase in the crude extracts of *Pleurotus ostreatus* in control and in presence of different heavy metals (600 µM) using ONPG as a substrate. Mean±SD (n=3).

Table 1. Kinetic values of β-galactosidase in the crude extracts of *Pleurotus ostreatus* in control and in presence of different heavy metals (600 µM). Mean±SD (n=3).

Heavy metals	K_m(mM)	V_{max}(µmol/min)	Effects
Control	0.307	0.571	Normal
Hg^2	0.285	0.189	Uncompetitive inhibition
Mo^{2+}	0.253	0.377	Uncompetitive inhibition
Al^{3+}	1.162	0.476	Mixed inhibition
Cu^{2+}	0.91	0.430	Mixed inhibition
Cr^{3+}	1.28	0.363	Mixed inhibition
Zn^{2+}	0.66	0.425	Mixed inhibition
Ni^{2+}	0.892	0.390	Mixed inhibition
Pb^{2+}	0.307	0.408	Non-competitive

5. Conclusion

In the present study, characterization and kinetic parameters of the β- galactosidase in the crude extracts of *Pleurotus ostreatus* in the presence and absence of various heavy metals were investigated. The present findings showed that the crude enzyme β-galactosidase extracts of *Pleurotus ostreatus* has a potential activity according to its ability to hydrolyze the substrate ONPG evidenced by the K_m and V_{max} values. The optimum reaction conditions for crude β-galactosidase in the crude extracts of *Pleurotus ostreatus* was found to be at pH 3.0 and at temperature 40°C. The results of this study indicated that the values of K_m and V_{max} of β-galactosidase in the crude extracts of *Pleurotus ostreatus* for hydrolyzing ONPG were found to be 0.307 mM and 0.571µmol/min, respectively. These results suggested that the enzyme β-galactosidase in the crude extract of *Pleurotus ostreatus* could be applied to industrial, medical and other production processes. The results indicated that heavy metals such as, Hg^{2+} and Mo^{2+} have an uncompetitive inhibition, while Al^{3+}, Cu^{2+}, Cr^{3+}, Zn^{2+} and Ni^{2+} have a mixed inhibition, and Pb^{2+} has a non-competitive inhibition.

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