

Purification and Characterization of Contemporaneously Produced Alkaline Protease and α -amylase Enzymes from Locally Isolated *Bacillus methylotrophicus* SCJ4

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Abstract

The contemporaneously produced alkaline protease and α -amylase by locally isolated *Bacillus methylotrophicus* SCJ4 had been purified in two steps and some characteristics of the purified enzymes were studied. Crude enzymes were first salted out using ammonium sulfate, then concentrated proteins were applied to anion column chromatography Q-sepharose (HiPrep Q FF 16/10 column). The purified alkaline protease has an estimated molecular mass of 24 kDa with maximum enzyme activity (1185U/ml/min) at pH 9.0 and 55°C. This enzyme belongs to serine proteases family with remarkable stability up to 62% and 61.5% toward Sodium Dodecyl Sulfate (SDS) and Hg^{++} , respectively. On the other hand, the purified amylase is a calcium-independent α -amylase, showing a good stability against SDS up to 73%. The estimated molecular mass was 67.5 kDa with maximum enzyme activity (280U/ml/min) at pH 7.0 and 65°C.

Keywords: Purification, Characterization, Alkaline Protease, α -amylase, *Bacillus methylotrophicus*.

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INTRODUCTION

Protease and amylase are among the most important industrial enzymes regarding to their application and marketing (Merheb *et al.*, 2007; Kiran and Chandra, 2008). In application, both enzymes represent the main key in many industries including food, pharmaceuticals, and detergent industries (Hmidet *et al.*, 2009) so their marketing represented about 60% for proteases (Rao *et al.*, 1998) and about 25-30% for amylases (Rajagopalan and Krishnan, 2008; Azad *et al.*, 2009; Hmidet *et al.*, 2010) from the total microbial enzyme sales. Many detergent industries imply the combined use of protease and amylase together in the same formulation (Maurer, 2004; Joo and Chang, 2006). Amylase exerts a synergistic effect on the washing capacity of the protease containing detergent and removes starchy food stains from fabrics which are difficult to remove under normal washing conditions (Hmidet *et al.*, 2009). A major problem of using both enzymes together is proteolysis of amylase by protease. Therefore, using protease and amylase enzymes

from the same strain will enhance the applicability of the detergent formulation. The co-production of the protease and amylase was reported in few studies (Hmidet *et al.*, 2009; Correa *et al.*, 2011; Kumar *et al.*, 2013).

The growing protease and amylase marketing entails an extensive search for new enzymes with higher activity and better characteristics. Exploring microorganisms from niches with high contamination levels as, El-Max bay, Egypt may represent a very promising source for enzymes that could meet the industrial demands. Enzymes could be used industrially in crude or purified preparations depend on the needed applications (Sundaram and Murthy, 2014). Crude enzymes preparations are generally used for commercial applications however, purified enzymes are essential for better understanding of its specific properties and functions (Nadeem *et al.*, 2013). Many studies reported the increase in enzymes specific activities followed purification, which increase their industrial applications specificity (Kumar, 2002; Adinarayana *et al.*, 2003). The individual production and

purification of protease or amylase enzymes from different *Bacillus* sp. have been reported extensively (Annamalai et al., 2014; Kamran et al., 2015; Ghafoori et al., 2016; Wang et al., 2016). However, only one report is available for purification and characterization of amylase form *Bacillus methylotrophicus* (Xie et al., 2014), but no such report for purification of protease from the same strain. The scope of this study is directed toward purification and understanding some characteristics of purified protease and amylase enzymes produced by *Bacillus methylotrophicus* SCJ4.

MATERIALS AND METHODS

Microorganism

The bacterial strain used through this work is *Bacillus methylotrophicus* SCJ4 which was isolated from El-Max bay, Egypt. The used strain was identified on the molecular level using 16S rRNA by Amer and Abde-Fattah, (Amer and Abdel-Fattah, 2014). This strain was selected among group of marine bacterial isolates based on its high production potency for both protease and amylase enzymes (El-Gendi et al., 2016).

Enzymes production and preparation

After cultivation of *Bacillus methylotrophicus* SCJ4 (ac: KF217257) on protease and amylase optimized production media as reported by El-Gendi et al., 2016, the cell free supernatant containing alkaline protease and α -amylase enzymes was used as a source of both enzymes in the following purification steps.

Determination of enzymes activities

Estimation of alkaline protease activity was carried out according to Anson, 1938 using bovine casein as substrate and standard curve of tyrosine. Determination of α -amylase activity was carried out based on Iodine-Starch color reaction of Fuwa's colorimetric method (Fuwa, 1954).

Determination of protein concentration

Lowry method was applied to estimate the total protein contents using standard curve of bovine serum albumin (Lowry et al., 1951).

Ammonium sulfate precipitation

Crude protease and amylase in the cell free supernatant were slatted out using ammonium sulfate, 65% saturation, and stirred over night at 4°C. The precipitated proteins were recovered by centrifugation (12,000 rpm for 10 min. at 4°C). The precipitate proteins were dissolved in minimum amount of 50mM glycine-NaOH buffer, pH 10.0 in case of protease purification. While for amylase purification, the precipitated proteins were dissolved in 50mM Tris-HCl buffer, pH 8.0. Proteins in the two different buffers were dialyzed overnight against the same buffer with four buffer changes using dialysis bag membrane with cut off of 10 kDa. The enzymes activity and protein concentrations were measured and specific activities were calculated.

Separation of protease and amylase using anion-exchange chromatography

For protease purification the dialyzed proteins solution (5ml) was loaded into Q-sepharose column (HiPrep Q FF 16/10, Pharmacia, Sweden) equilibrated with 50mM glycine-NaOH pH 10.0 buffer using an automated ÄKTA Prime Plus system. To remove the unbound proteins, the column was washed with 5 column volumes of equilibration buffer. The column-bound proteins were eluted with a linear gradient (0-1M) NaCl in glycine-NaOH buffer, pH, 10.0 at a flow rate of 1 ml/min and fraction size of 3ml. For amylase purification the same procedures were followed, with 50mM Tris-HCl buffer, pH 8.0. Fractions contained alkaline protease or amylase activities were pooled, desalting and concentrated by Amicon-10 ultrafiltration concentrator (membrane cut off of 10 kDa).

SDS-Polyacrylamide gel electrophoresis and zymography

Different purification steps were evaluated through 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed as described by Laemmli, 1970. Blue-silver stain was applied to visualize the protein bands in SDS-PAGE according to Candiano et al., 2004. Spectra™ multicolor broad range protein ladder, (10-260 kDa) was used as molecular mass marker.

Protease activity zymogram was conducted on SDS-PAGE using a modified method of Garcia-Carreno et al., 1993. Enzyme sample were not boiled before electrophoresis and after electrophoresis, the SDS-PAGE was incubated with 1% (w/v) casein in 50mM glycine-NaOH buffer (pH 10.0) for 30min at 50°C. The appearance of clear zone after staining the gel with 0.25% Coomassie Brilliant Blue R250, indicated the protease activity. For amylase activity zymogram, after electrophoresis the gel was incubated in 1% soluble starch in 50mM phosphate buffer (pH 7.0) for 30 min at 60°C. the gel was stained with I_2 solution (0.01% I_2 in 0.1% KI), the appearance of clear zone indicated amylase activity.

Characterization of purified enzymes

Some characteristics of the purified protease and amylase were studied to evaluate optimum temperature and pH, thermal stability, pH stability, effect of some detergents, enzyme inhibitors, and some metal ions on both enzymes activity.

Effect of temperature on activity of purified enzymes

Effect of temperature on the activity of purified enzymes was assessed by incubating the reaction mixture at different temperature ranging from 30–80°C in 50mM glycine-NaOH, pH 10.0, for purified protease and 50mM phosphate buffer, pH 7.0 for purified amylase. The enzymes activities were expressed in percentage relative activity considering the maximum value as 100%.

Effect of temperature on stability of purified enzymes

Temperature stability was estimated by incubating the purified enzymes at different temperature ranges (40–60°C.) in case of protease and (50–70°C.) in case of amylase.

Purified enzymes were incubated at each temperature for different time intervals from 0, 10, 20, 30, 40, 50 to 60 min. The enzyme activities were measured as described before and expressed in percent residual activity.

Effect of pH on activity of purified enzymes

Activity of the purified protease was measured at different pH (7.0-12.0). The reaction mixture pH was adjusted by dissolving 1% casein in one of the following buffers 50mM sodium phosphate (pH 6.0-7.0), 50mM Tris-HCl (pH 8.0), 50mM glycine-NaOH (pH 9.0-10.0), 50mM sodium phosphate-NaOH (pH 11.0), and 50mM KCl-NaOH (pH 12.0). On the other hand, activity of the purified amylase was estimated at pH values of (4.0-10.0) by dissolving 1% soluble starch in one of the following buffers: 50mM citrate (pH 4.0- 5.0), 50mM sodium phosphate (pH 6.0-7.0), 50mM Tris-HCl (pH 8.0), 50mM glycine-NaOH (pH 9.0-10.0). The reaction mixture was incubated for 10 min at optimum temperature for each enzyme. The enzymes activity at different pH was determined as described earlier and expressed in percentage relative activity.

Effect of pH on stability of purified enzymes

Purified protease and amylase enzymes were incubated with one of the above-mentioned buffer at room temperature for 60 min. Samples were taken every 10 min interval where, the residual activity was determined at standard assay conditions.

Effect of some detergent and enzymes inhibitors on activity of purified enzymes

The effects of different surfactant as Triton X-100, Tween-80, Tween-20, SDS (0.1% and 0.5% final concentration), enzymes inhibitors including, phenylmethylsulphobnly fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA) with final

concentration of (1mM, 5mM) on enzymes activity were studied by pre-incubating the enzymes with each chemical for 15 min at room temperature. The residual activities were determined as mentioned before and compared to controls without any treating.

Effect of some metal ions on activity of purified enzymes

Activity of the purified enzymes was tested in presence of the following metal ions: Zinc sulfate, Iron sulfate, Magnesium sulfate, Copper sulfate, Cobalt chloride, Manganese chloride, Mercuric chloride and Calcium chloride. Purified enzymes were pre-incubated with each metal ion (5mM final concentration) for 15 min at room temperature. The residual activity was determined as mentioned before. The enzyme activity without any metal ion was taken as 100%.

RESULTS AND DISCUSSION

Protease and amylase purification

The crude enzymes in the cell free supernatant were first salted out with ammonium sulfate 65% saturation. The results indicated an increase in the specific activity about 9313.4 and 2046.12 (U/mg) for protease and amylase, respectively, compared to the cell free supernatant (Table 1). On one hand, the results indicated enhancement in both enzymes purification with ammonium sulfate to 5.53 and 7.36 purification fold for protease and amylase, respectively (Table 1). Concentration of protease and amylase enzymes with ammonium sulfate was reported in many studies (Raul et al., 2014; Bekler et al., 2015).

Table 1. Purification Scheme for Alkaline Protease and α -amylase produced by *Bacillus methylotrophicus* SCJ4.

Step		Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield %
Cell free supernatant	Protease	363600	177	2054.237	1	100
Ammonium sulfate	Amylase	60255.5	187.2	321.9	1	100
Anion – column	Protease	168240	14.8	11367.6	5.53	46.3
	Amylase	40730	17.2	2368.02	7.36	67.6
	Protease	2625	0.13	20192.31	9.83	0.72
	Amylase	1943	0.2	9715	30.1	3.22

* One unit of protease activity was defined as the amount of enzyme that yields the equivalent of 1 μ mol of tyrosine per minute under the assay conditions. Where, one unit of amylase activity was defined as the amount of enzyme decreased the absorbance of 660 nm by 0.1 in 10 min.

In the next step, the dialyzed concentrated enzymes were applied to anion-exchange (HiPrep Q FF 16/10 column), as an essential purification step. The alkaline protease was purified to 9.38 fold with increase in the specific activity 18138.1 U/mg, while in case of amylase, the purification fold and specific activity were increased by 30.18 fold and 9715 (U/mg), respectively. Proteins purification after each step was evaluated through 12% SDS-PAGE. The results revealed improvement in purification for both

enzymes indicated by reduction in proteins bands to a single protein band at molecular weight of 24 kDa for alkaline protease and 67.5 kDa for amylase (Figure 1). The usual molecular masses reported from genus *Bacillus* for protease between 15 and 38 kDa (Kumar and Takagi 1999; Doddapaneni et al., 2009), where in amylase were between 22.5 kDa to 68 kDa (Gupta et al., 2003; Liu, et al., 2008). Gessesse et al., 2003, reported an alkaline protease with analogues molecular weight (24 kDa) from *Bacillus*

pseudofirmus. The purified amylase in this study has a higher molecular mass than that reported by Xie et al., 2014 from *Bacillus methylotrophicus* P11-2 that estimated to be 44.0 kDa, indicating a different amylase (Figure 1), purified protease and amylase showed a single clear band (each) of zymogram indicating pure preparations.

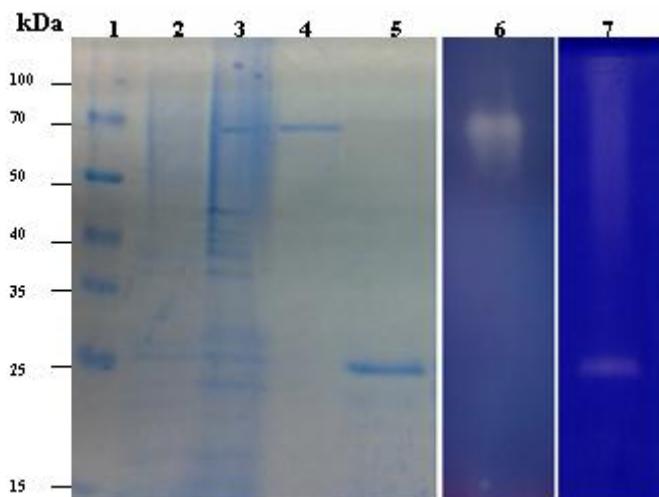


Fig. 1. SDS-PAGE (12%) and Zymography of Purified Protease and Amylase Enzymes. Standard molecular mass marker (lane 1), cell free supernatant (lane 2), ammonium sulfate precipitated proteins (lane 3), purified α -amylase (lane 4), purified protease (lane 5), zymogram activity of purified amylase (lane 6) and zymogram activity for purified protease (lane 7).

Characterization of the purified alkaline protease and amylase

The effect of temperature on enzymes activity was studied by measuring the activity at different temperature values from 30-80°C. The results (Figure 2) indicated an increase in protease activity with increase in temperature from 30-50°C with an optimum activity at 55°C. The activity was drastically affected with increase in temperature beyond 55°C, losing about 80% from the total activity at 80°C. Alkaline protease enzyme is generally known to has an optimum temperature range of 50-70°C (Ellaiah et al., 2002). On the other hand, the purified α -amylase showed a higher activity compared to protease, where the total amylase activity was more than 55% at 30°C and 80°C with an optimum activity at temperature range of 60-70°C. This result is in accordance with Xie et al., 2014, who reported that the purified amylase from *Bacillus methylotrophicus* P11-2 showed an optimum temperature at 70°C and pH 7.0.

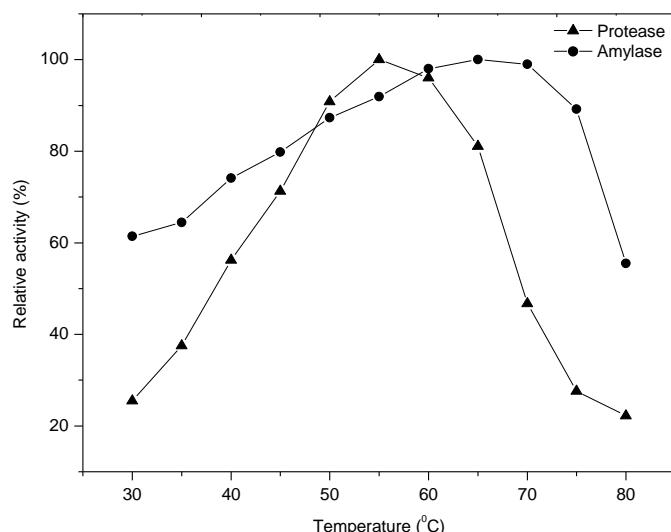


Fig. 2. Optimum Temperature for the Purified Alkaline Protease and α -amylase.

Effect of temperature on stability of purified enzymes

Thermal stability of protease and amylase enzymes was examined by incubating the purified enzymes at different temperature then the remaining activity was determined (Figure 3). The purified amylase showed higher thermal stability over the purified protease. Purified amylase was highly stable at temperature 50°C and retained more than 37% of its activity after 1h incubation at 60°C, while purified protease retained 84% from its activity after 1h at 40°C and 21% after 10 minutes at 60°C. purified amylase was completely inactivated at 70°C after 20 minutes, however, purified protease lost its activity at 60°C after 10 min.

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Effect of pH on activity of purified enzymes

Activity of the purified protease was measured at different pH values (7.0-12.0) (Figure 4). Protease enzyme was active over the entire tested pH range with a maximum activity at pH 9.0. Protease activity lost about 20% of total activity with increasing the pH values from 9.0 to 10.0. The same optimum pH had been reported by other investigators for alkaline protease from *Bacillus* sp (Annamalai et al., 2014; Badoei-Dalfard, 2015). The purified α -amylase showed a higher activity at acidic pH estimated to be 58% at pH 4.0

with optimum activity at pH 7.0. The results indicated a sharp decrease in the α -amylase activity in alkaline pH, about 40% loss in the total activity at pH 9.0. This finding is in accordance with many studies reported alpha amylase enzyme from *Bacillus* sp. with neutral pH optima, pH of 7.1 by Raul et al., 2014 and pH of 6.0–7.5 by Abdel-Fattah et al., 2013. Contrary to Annamalai et al., 2011 reported the optimum pH for amylase from *Bacillus cereus* in alkaline medium (pH 8.0) with 89% of its activity was sustained at pH 11.0.

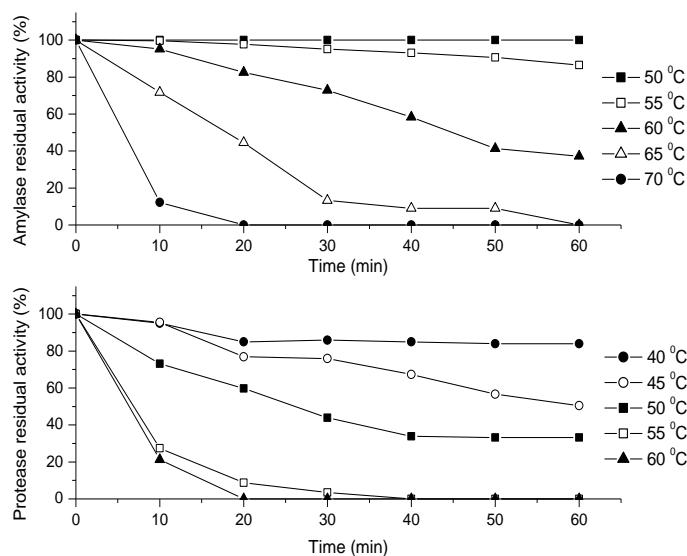


Fig. 3. Effect of Temperature on Stability of the Purified Alkaline Protease and α -amylase.

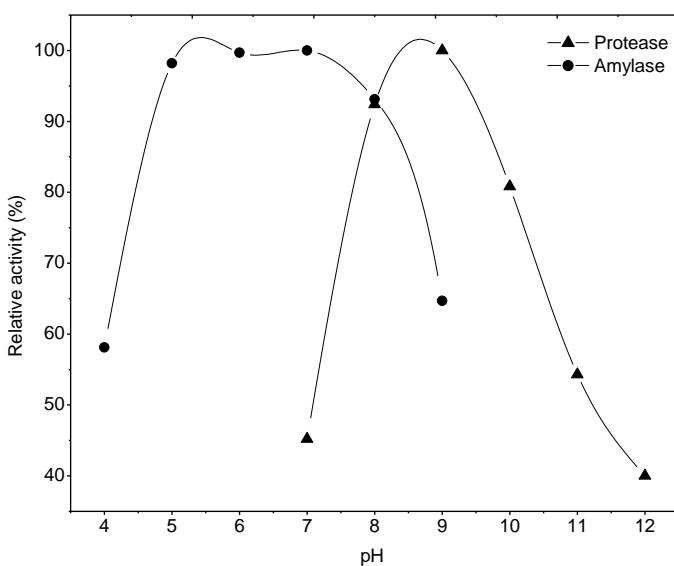


Fig. 4. Optimum pH for the Purified Alkaline Protease and α -amylase.

Effect of pH on stability of purified enzymes

Stability of purified protease was investigated at different pH values ranging from 7.0 to 11.0 (Figure 5). Purified protease showed a good stability in pH range of 7.0–10.0 with maximum stability at pH 8.0. Protease activity was gradually decreased at pH 11.0, where it completely inactivated after 50 min of incubation at the same pH. On the other hand, purified amylase showed high stability in acidic to neutral pH ranges with a little decrease in the enzyme activity in alkaline medium (pH 9.0). Stability of amylase in acidic pH is a privilege to starch industries as the usual pH of starch slurry is generally around 4.5 (Sivaramakrishnan et al., 2006).

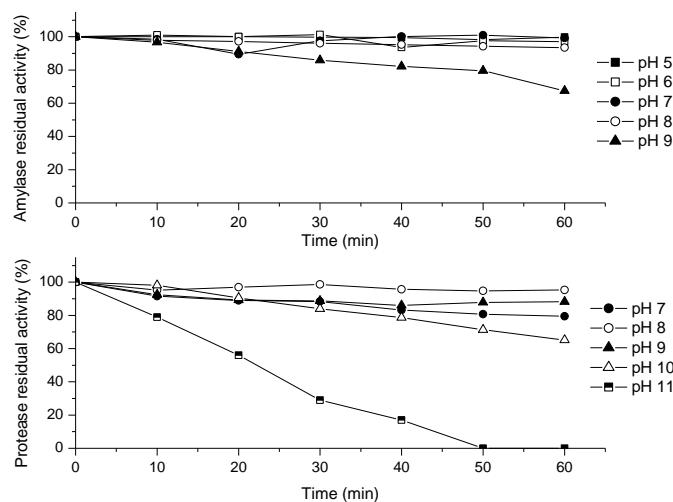


Fig. 5. Effect of pH on the Stability of Purified Alkaline Protease and α -amylase.

Effect of some detergents and inhibitors on activity of purified enzymes

The effects of detergent on purified enzymes are varied (Table 2). Non-anionic detergents like Triton-X-100 slightly increased the activity of both enzymes, contrary to Tween-80 that had a moderate negative effect on both enzymes. Tween-20 negatively affected the amylase activity, but showed no effect on protease. The strong ionic detergent SDS moderately affected both enzymes activity. At 0.5% SDS the estimated protease activity was 62%, where in amylase was 73%. Stability toward detergents is an important characteristic for industrial application of enzymes (Kumar and Takagi, 1999), particularly the SDS stable enzymes, which have been rarely reported (Hmidet et al., 2009). The results of enzymes inhibitors indicated a remarkable stability for both enzymes toward EDTA, The stability of amylase from *Bacillus methylotrophicus* SCJ4 to EDTA, indicating non-metalloenzyme which is contradictory to Xie et al., 2014 reported 78% reduction in the amylase activity from *Bacillus methylotrophicus* P11-2 in presence of EDTA. Alkaline protease was drastically inactivated in presence of PMSF indicating a serine protease. Beg and Gupta, 2003 stated that, the adverse effect of PMSF on

protease enzymes is due to sulfonating the essential serine amino acids in the active site.

Table 2. Effect of some Chemical Compounds on Activity of Purified Protease and Amylase.

Compounds	Final concentration	Residual activity (%)	
		Alkaline protease	Amylase
Control	-	100	100
Triton-X-100	0.1%	99	103.1
	0.5%	110	104.1
Tween-80	0.1%	98	90.5
	0.5%	88	86.8
Tween-20	0.1%	99	75.1
	0.5%	98	82.6
SDS	0.1%	97	73.3
	0.5%	62	73.8
EDTA	0.1%	100	100
	0.5%	98	99
PMSF	0.1%	20	100
	0.5%	5	100

Effect of some metal ions on activity of purified protease and amylase

The effect of some metal ions on the enzymes activity was examined by measuring the activity in presence of 5mM of each metal ion (Figure 6).

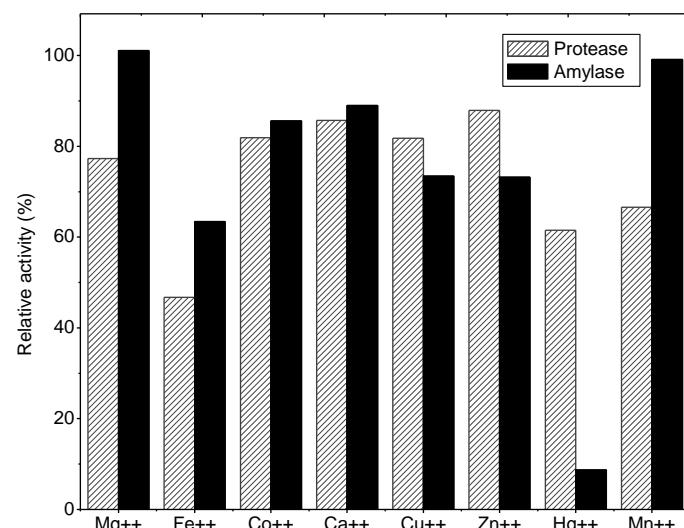


Fig. 6. Effect of some Metal ions on Activity of Purified Alkaline Protease and α -amylase.

The results indicated a good stability of both enzymes against most metals. Fe⁺⁺ and Hg⁺⁺ ions showed the most adverse effects on alkaline protease and amylase activity respectively. Inactivation of amylase with Hg⁺⁺ was reported by other authors (Annamalai et al., 2011; Lin et al., 1998). The inhibition of alkaline protease activity by Fe⁺⁺ is in

accordance with other studies Bhatiya and Jadeja, 2010 and Mokashe et al., 2015 and contrary to Badoei-Dalfard et al., 2015 reported 40% increase in alkaline protease activity in presence of Fe⁺⁺ion (5mM). The slight inactivation effect of metal ions on amylase activity, especially Ca⁺⁺ ion, indicated that this amylase is ions independent, required no metal for its activity. Our finding is in accordance with some other studies reported calcium independent amylase (Swain et al., 2006; Carvalho et al., 2008), contrary to most other studies confirming that α -amylases are metalloenzymes and Ca⁺⁺ ions are essential for α -amylases stability and activity (Gupta et al., 2003; Burhan et al., 2003).

CONCLUSION AND RECOMMENDATIONS

The present work focused on two main key enzymes in many industries namely: alkaline protease and α -amylase which were produced by a local isolate *Bacillus methylotrophicus* SCJ4. Both enzymes were purified till unity then fully characterized. The two enzymes showed an interesting feature concerning thermal and pH- stabilities which make them good candidates in many industrial applications.

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CONFLICT OF INTEREST

There is no conflict of interest.

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