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Purification and Characterization of Pectinase from Bacillus licheniformis obtained from a Cassava Waste Dump.

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ABSTRACT

This study investigated the properties of pectinase from Bacillus licheniformis. The pectinase secreted by the microorganism was purified using ion exchange and gel filtration chromatography on DEAE-Sephadex and Sephadex G-200 respectively. A purification fold of 10.5, yield of 23.9% and specific activity of 9.47 U/mg was obtained. The characteristics of the enzyme included a native molecular weight of 38 kDa, pH optimum 9.0, temperature stability between 35 and 45ºC; temperature optimum 50°C. Inhibition of the enzyme was observed with metal and chemicals, such as $Ba^{2+,}$ $Al^{3+,}$ $Mg^{2+,}$ EDTA, benzoic acid, tannic acid, citric acid and sodium oxalate, however the enzyme activity was enhanced in the presence of K^+ and Na^+. Other enzymatic properties was K_m values of 1.37, 1.62, and 9.09 mg/ml for polygalacturonic acid, 7.8% methylated pectin and 67% methylated pectin respectively The pectinase produced have some desirable characteristics that could be utilised in many industrial processes.

Keywords: pectinase; pectin; Bacillus licheniformis; purification

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1.0 INTRODUCTION

Pectinases are enzymes with enormous potential to offer in the many industries. The wide spread applications of pectinases have placed it among the most important enzymes of great significance for biotechnology (Bhat, 2000). Due to the increasing demand for this enzyme in various industries such as the fruit, wine, paper, textile, coffee and tea fermentation industries, the search for pectinases which fit these industrial uses is on the rise (Sharma et al. 2013).

Pectinases comprise a heterogeneous group of enzymes that catalyze the breakdown of pectin containing substrates (Gummadi and Panda, 2003). The major types of pectinase include polygalacturonase (EC 3.2.1.15 and EC 3.2.1.67), pectin lyase (EC 4.2.2.10), pectate lyase (EC 4.2.2.2), and pectin esterase (EC 3.1.1.11) (Celestino et al. 2006).

Several microbes, including bacterial and fungal strains have been used in the production of different types of pectinolytic enzymes. Pectinases from fungal sources are known to work best under acidic pH and low temperature which makes them more applicable to industrial processes which operates optimally at acidic to neutral pH, bacteria pectinases however, are known to perform optimally at alkaline pH and therefore are best applied to industrial processes operating in the alkaline pH. They are also known to withstand high temperatures (Hoondal et al. 2002; Torimiro and Okonji 2013).

Although, a large number of microorganisms are capable of producing pectinase, the rising demand for this enzyme with desirable characteristics has made it necessary to study these enzymes produced by different microorganisms for applications in industries. This present study aims to characterise the purified pectinase produced by B. licheniformis isolated from agro waste dumpsites in order to ascertain its pectinolytic enzyme potentials under various physicochemical conditions.

2.0 MATERIALS AND METHODS

2.1 Production of pectinase using submerged fermentation

Bacillus licheniformis isolated from an agro waste dumpsite was cultured and identified as described (Torimiro and Okonji, 2013). The inoculums were standardised to absorbance of 0.2 at 650 nm (Demirkan, 2011) and were inoculated at 1% (v/v) into the defined enzyme production medium. The enzyme production medium contained 1 g of citrus pectin, 0.14 g of $(NH_4)_2SO_4$, 0.6 g of K_2HPO_4, 0.2 g of KH_2PO_4 and 0.01 of $MgSO_4.7H_2O$, dissolved in 100 ml of distilled water with the initial pH adjusted to 8.0. Sterilisation was done under pressure at 121°C for 15 min. The media were inoculated in duplicates and were incubated at in a rotary shaker incubator of 150 rpm at 35ºC for 48 h. Thereafter, the culture was centrifuged at 12000 rpm for 20 min and the cell free supernatants were used to evaluate the crude pectinase activity (Kumar and Sharma, 2012).

2.2 Protein determination

The total protein contents of the samples were determined according to the method Lowry et al., 1951, using bovine serum albumin (BSA) as standard.

2.3 Pectinase assay

The pectinase activity was assayed according to the modified method of Miller (1959) as reported by Wang et al. (1997). The reaction mixture consisted of 0.80 ml of 1.0% w/v polygalacturonic acid in 100 mM sodium carbonate buffer (pH 9.0) and 0.20 ml of supernatant (enzyme solution). Control tubes (Enzyme blank) contained the same amount of substrate and 0.2 ml of enzyme solution but were boiled for 10 min. Both the experimental and control tubes were incubated at 40 °C for 30 min. The reactions were terminated by the addition of 1.5 ml of 3, 5-Dintrosalicyclic acid (DNSA) reagent and the absorbance was taken at 540 nm. One unit of Pectinase activity was defined as the amount of enzyme that liberated reducing sugar equivalent to 1 µmol galacturonic acid per minute under the specified assay conditions. A standard calibration curve of galacturonic acid was constructed and used for the estimation of the polygalacturonic acid equivalent in µmol/ml and then converted in units (U).

2.4 Purification of the enzyme

2.4.1 DEAE-Sephadex ion-exchange chromatography and gel filtration

The cell free supernatant (enzyme solution), was precipitated using 60% (v/v) of chilled acetone. The enzyme solution obtained was dialyzed and further purified by Ion-exchange chromatography, using DEAE-Sephadex resin. The DEAE-Sephadex was carefully packed in column (1.5 x 25.0 cm); the column was pre-equilibrated with 0.1 M phosphate buffer pH 7.5. The dialyzed sample was then applied on the equilibrated DEAE-Sephadex column. The column was first washed with 0.1 M phosphate buffer, pH 7.5, to remove unbound proteins followed by stepwise elution with 1.0 M NaCl in phosphate buffer 100 mM, pH 7.5 at a flow rate of 12 ml/ hour. Fractions of 2 ml were collected; the protein content and pectinase activity of each fraction was estimated. The active fractions from the column were pooled, concentrated and assayed for pectinase activity and protein content. The yield and fold of purifications were calculated. Thereafter, the enzyme from the preceding step was applied to 1.0 x 45 cm Sephadex G-200 column. Fractions of 1.5 ml were collected at a flow rate of 10 ml/h. Each fraction was assayed for pectinase activity and protein content. The yield and fold of purifications from the active fractions from the column were calculated.

2.5 Determination of molecular weight

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The native molecular weight of the enzyme was determined on Biogel P-100 while the subunit molecular weight of the enzyme was determined by SDS-PAGE using the method of Weber and Osborn (1975). Standard proteins were as contained in Molecular Weight Markers Calibration Kit for SDS polyacrylamide gel electrophoresis.

2.6 Determination of kinetic parameters of the pectinase produced

The kinetic parameters (V_{max} and K_m) of the enzyme were determined by varying the concentration of polygalacturonic acid or esterified pectin 7.8% apple methyl pectin and 67% citrus pectin (0.1-10mg/ml) and measuring the initial reaction velocities. V_{max} and K_m were then determined from the double reciprocal plot (Lineweaver and Burk 1934).

2.7 Effects of temperature on the pectinase produced

Optimum temperature for pectinase activity was determined according to the method of Tari et al. (2008). The pectinase activity was measured using polygalacturonic acid substrate with a temperature range of 25 to 65ºC.

2.8 Thermal stability of the pectinase produced

Pectinase thermo stability was determined using the method described by Tari et al. (2008) at temperature range from 40 to 60ºC. At intervals of 15, 30, 45, 60, 75 and 90 min, aliquots of samples were taken and the residual pectinase activity was assayed using polygalacturonic acid.

2.9 Effects of pH on Bacillus licheniformis pectinase

The effect of pH on the enzyme was studied using the following buffer systems: citrate (4.0-5.5), sodium phosphate (6.0-8.0), Tris (8.5) and sodium carbonate (9.0-10.5) to determine the optimum pH of pectinase produced. Polygalacturonic acid was used as substrate.

2.10 Effects of Salts on Bacillus licheniformis Pectinase

The assay was performed in the presence of various salts at final concentrations of 1 mM, 5 mM and 10 mM using the following salts: $SnCl_2$, $CaCl_2$, NaCl, KCl, $MnCl_2$, $MgCl_2$ and $AlCl_3$. The salts were solubilised in distilled water. The enzyme with the various salts were pre-incubated for 10 min at 40ºC, before initiating the reaction with polygalacturonic acid, the residual pectinase activity was subsequently evaluated as previously described in the assay protocol. The enzyme samples without the salts were taken as control with 100% activity.

2.11 Inhibition by preservatives and other chemicals on pectinase activity

The inhibitory effects of preservatives and other chemicals: benzoic acid, oxalic acid, citric acid, gallic acid, EDTA and tannic acid on pectinase activity were studied. The assay was performed in the presence of the acids at final concentrations of 1 mM and 5 mM in 100 mM sodium carbonate buffer, pH 9.0, by pre-incubating the enzyme for 10 minutes at 40ºC in presence of these acids before starting the reaction with polygalacturonic acid. All chemicals were solubilized in 100 mM sodium carbonate buffer (pH 9.0). The enzyme samples without the chemicals were taken as control with 100% activity.

3.0 RESULTS

A 10.52 fold increase in specific activity of the enzyme with a 23.92% recovery of the purified enzyme was observed **Table 1**. The ion-exchange profile of pectinase produced is shown in **Figure 1** while purification profile on Sephadex G-200 gel filtration chromatography is as shown in **Figure 2.**

The molecular weight of the enzyme was estimated to be approximately 38 kDa by gel filtration and SDS-PAGE. As shown in **Figures 3 and 4**.

Figures 5a, b and c shows the Lineweaver-Burk plot for the determination of the kinetic parameters of the purified enzyme using 7.8% methylated pectinase, 67% methylated pectin and polygalacturonic acid respectively. The summary of the kinetic parameters and relative activities of the enzyme is as shown in Table **2**.

The activity of enzyme increased as the temperature increased and reached its maximum activity at 50 ºC, it then decline above 50 ºC (**Figure 6**). The thermal stability of the enzyme is shown in **Figure 7**, the enzyme was stable for 1 h 30 min as it retain most of its initial activity at temperature of 35-45 ºC, it however lost 30% of its initial activity when pre-incubated at 50 ºC (its optimum temperature) and about 50% at 55 ºC.

Studies on the effect of pH on the enzyme indicated that the enzyme was active over a broad pH range with optimum activity at pH 9.0 (**Figure 8**)

Table 3 shows the effects of salts on the enzyme activity. Apart from potassium and sodium, all the salts investigated exhibited inhibitory effect on the enzyme activity at high concentration (10 mM) compared to lower concentration of the ions. The inhibitory effects of tannic acid, gallic acid, citric acid, benzoic acid and EDTA showed that the enzyme was able to retain its activity in the presence of these chemicals with only a moderate loss of activity (**Table 4**).

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Table 1 Purification of Bacillus Licheniformis Pectinase

Purification Steps	Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification fold
Crude Enzyme	198.70	45.15	40.64	0.90	100.00	1.00
60% (v/v) Acetone Precipitation	21.60	14.17	30.39	2.15	74.78	2.39
DEAE-Sephadex Chromatography	18.50	1.85	15.91	8.61	39.15	9.57
Sephadex G-200 Gel Filtration	14.30	1.03	9.72	9.47	23.92	10.52

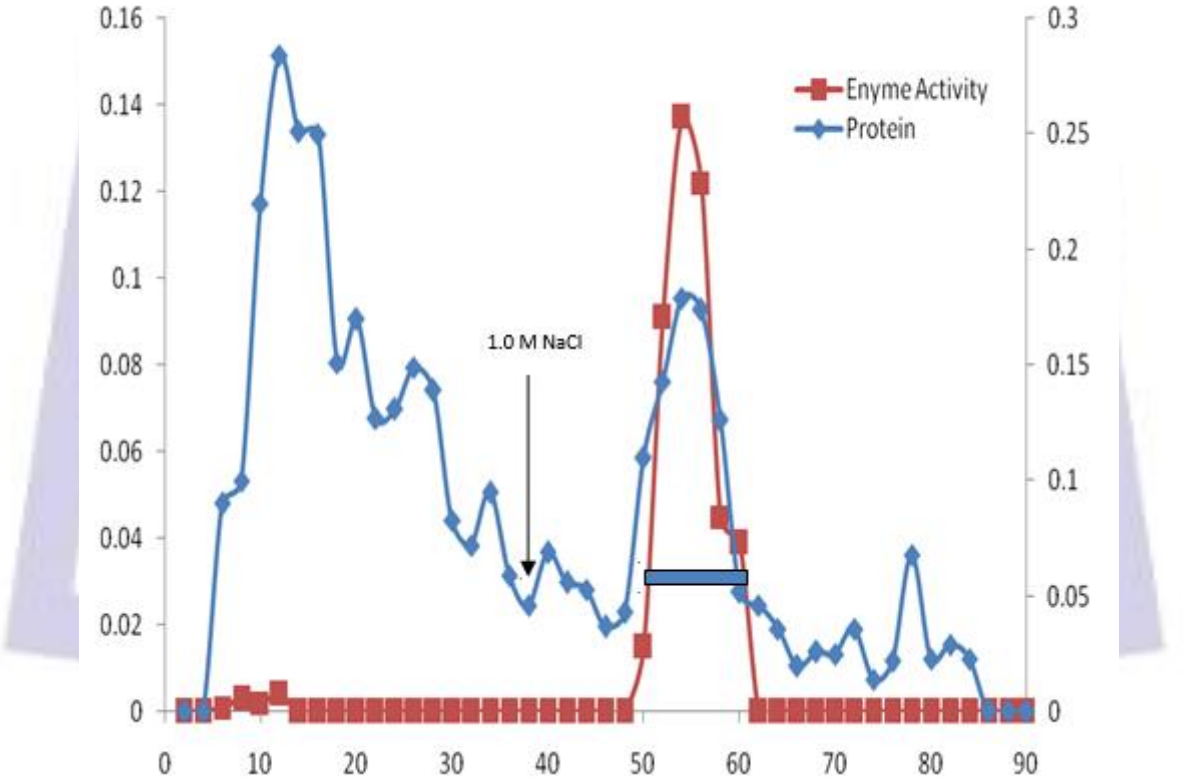

Figure 1 DEAE-Sephadex Ion-Exchange Chromatography (column 1.5 x 25 cm) of Bacillus licheniformis pectinase. The column was washed with 0.1 M sodium phosphate buffer to remove unbound protein. Fraction of 2 ml were collected at a flow rate of 12 ml/h. The column was further eluted with 1.0 M NaCl in 0.1 M sodium phosphate buffer to remove bound protein. Pooled fractions, ▬ stepwise elution →

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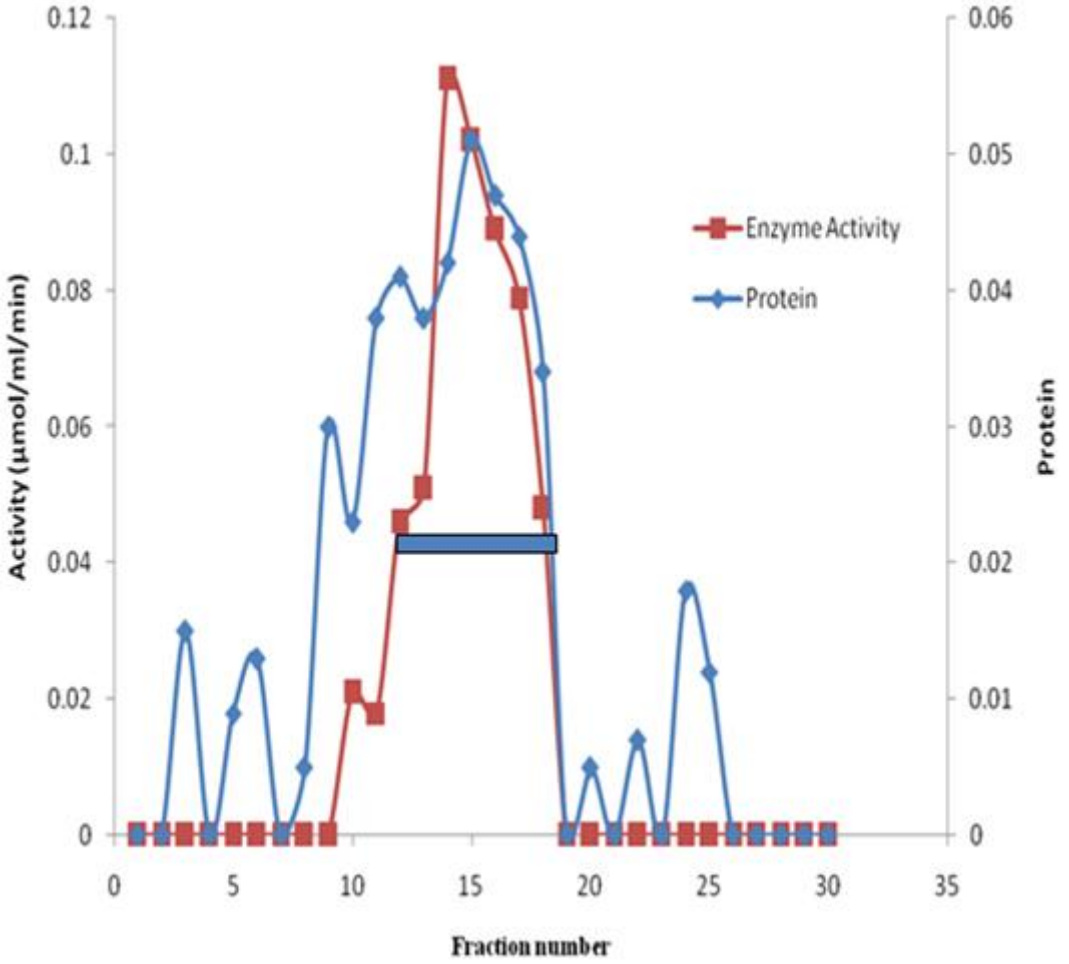

Figure 2 Sephadex G-200 Gel Filtration Chromatography (column 1.0 x 45 cm) of Bacillus licheniformisPectinase. The eluant was 10 mM Sodium phosphate buffer pH 7.5 Fractions of 1.5 ml were collected from the column at a flow rate of 10 ml per hour. ▭ Pooled fractions.

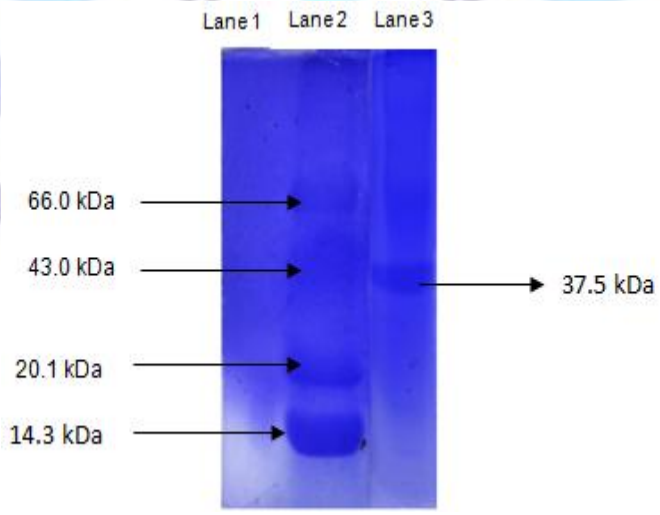

Figure 3 SDS-PAGE for the Determination of Subunit Molecular Weight.

SDS PAGE of purified pectinase from Bacillus licheniformis. Lane 1 control without sample. Lane 2 is the Molecular weights marker: Bovine Serum Albumin—66 kDa, Ovalbumin—43.0 kDa, Trypsin Inhibitor—20.1 kDa, Lysozyme—14.3 kDa and Lane 3 is the purified enzyme.

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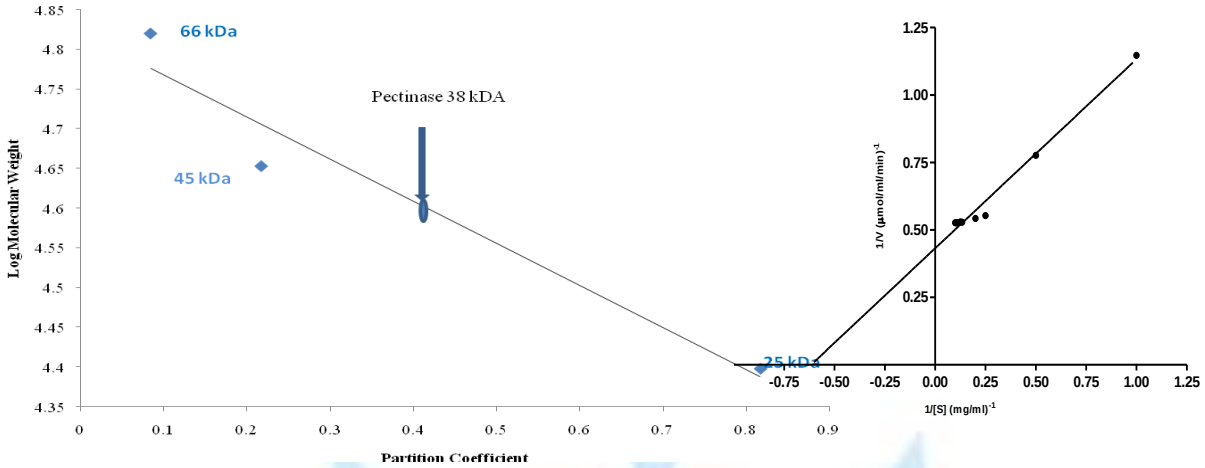

Figure 4 Calibration Curve for the Determination of Native Molecular Weight.

Figure 5b Lineweaver-Burk Plot for varying concentration of 7.8% Methylated pectin on the initial reaction velocity at pH 9.0 and 40ºC.

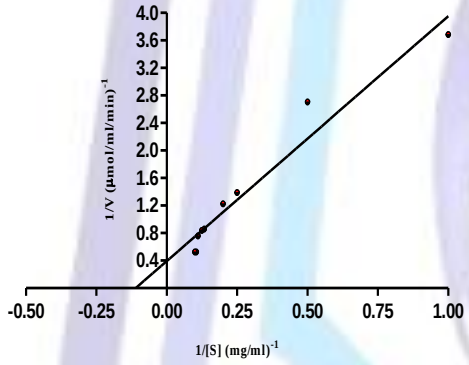

Figure 5a Lineweaver-Burk Plot for varying concentration of 67% Methylated pectin on the initial reaction velocity at pH 9.0 and 40ºC.

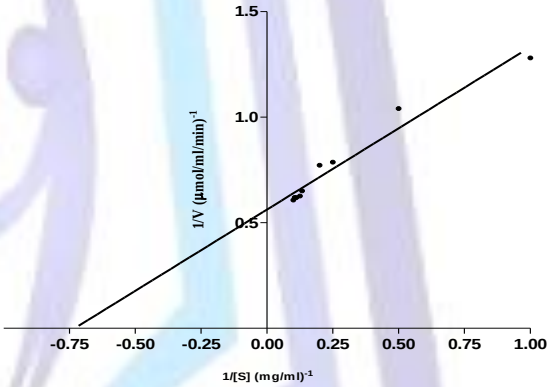

Figure 5c Lineweaver-Burk Plot showing the effect of varying concentration of polygalacturonic acid on the initial reaction velocity at pH 9.0 and 40ºC.

Table 2 Summary of Kinetic Parameters and Substrate Specificity of the Enzyme

Substrates	K_m (mg/ml)	V_{max} (µmol/ml/min)	% Residual Activity
Polygalacturonic acid	1.37	1.78	100
7.8% methylated pectin	1.62	2.56	102.02
67% methylated pectin	9.09	2.32	33.59

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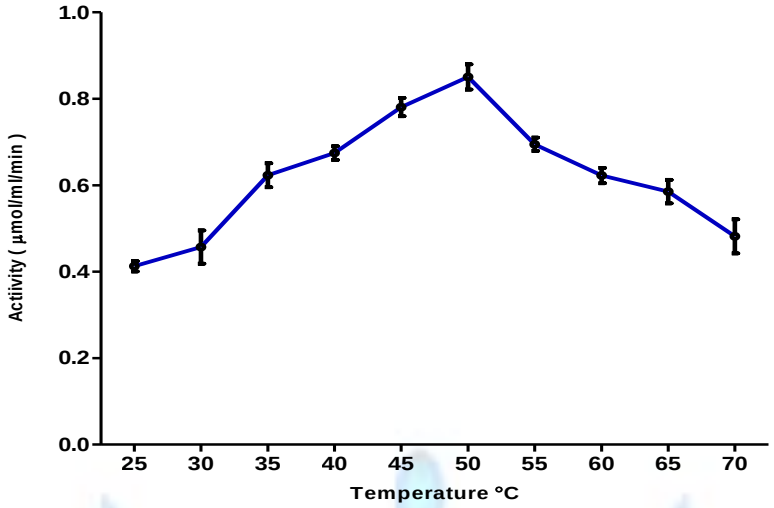

Figure 8 Effect of Temperature on the Activity of B. licheniformis Pectinase

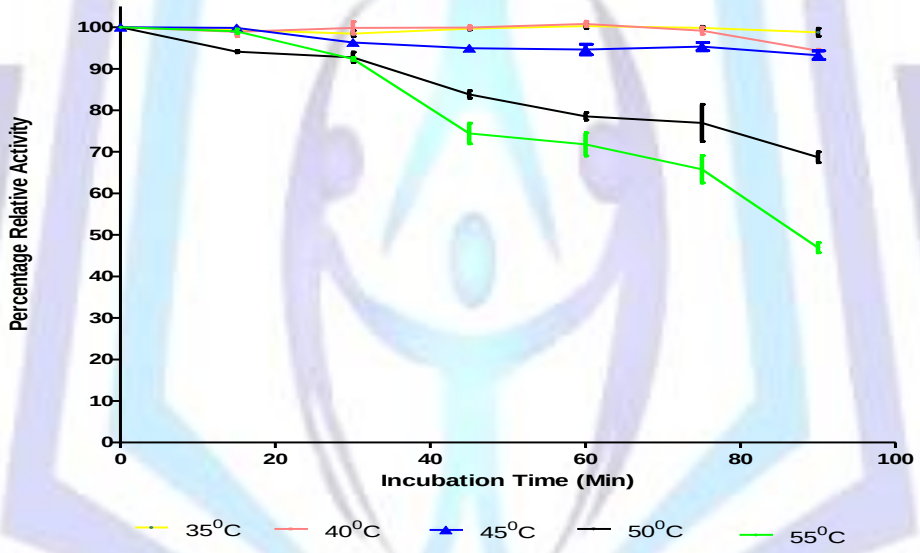

Figure 9 Thermal Stability of B. licheniformis Pectinase at pH 9.0

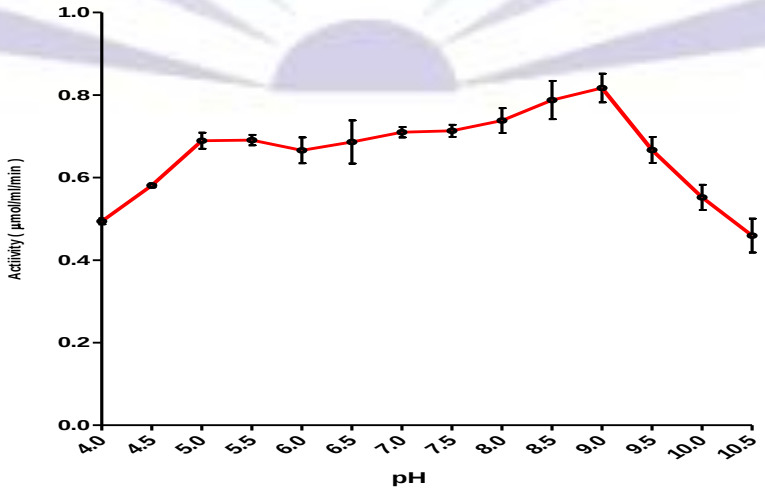

Figure 10 Effect of pH on B. licheniformis Pectinase

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The enzyme showed activity over a broad pH range with maximum activity at pH 9.0.

Table 3 Effect of Salts on Bacillus licheniformis Pectinase

	% Residual Activity		
Salts	**1 mM**	**5 mM**	**10 mM**
Control	100.00	100.00	100.00
KCl	174.10±6.00	166.67±2.18	166.98±6.32
NaCl	127.10±5.15	117.41±1.12	98.75±1.12
$CaCl_2$	107.48±4.07	111.21±2.47	86.73±3.24
$SnCl_2$	113.99±7.86	72.02±3.00	71.04±3.39
$MnCl_2$	102.38±8.15	81.30±7.25	18.38±3.59
$BaCl_2$	81.93±6.065	70.72±4.19	60.12±4.65
$AlCl_3$	95.95±2.714	79.13±3.30	73.21±2.43
$MgCl_2$	92.83±8.40	81.30±7.25	53.27±2.16

Table 4: Effect of Preservatives and Other Chemicals on B. licheniformis Pectinase

	% Relative Activity	
Chemicals	**1 mM**	**5 mM**
Control	100.00	100.00
Tannnic Acid	40.16±1.97	28.97±4.29
Gallic Acid	59.55±4.41	15.69±0.71
Citric Acid	83.98±4.41	71.85±2.57
Tri-Sodium Citrate	83.50±1.22	61.98±4.70
Sodium Oxalate	79.62±3.89	68.93±4.40
Benzoic Acid	94.33±2.80	71.68±2.10
EDTA	77.83±3.02	59.81±2.25
Sodium Benzoate	85.28±4.64	67.80±2.81

DISCUSSION

Pectinases are mostly used for industrial processes in the crude form; however, purification of these enzyme enhance the understanding of the biochemical properties and mode of action of the enzymes. Acetone precipitation of the crude enzyme had 2.4 purification fold and percentage yield of 74.8%. The precipitate was further purified on DEAE-Sephadex and Sephadex G-200. The purification steps of pectinase produced from Bacillus licheniformis had 10.5 fold increase in specific activity and 23.9 percentage yield. Similar yield within the range 6.1-17.2 has been reported on fold increase in specific activity of purified enzyme (Mohsen et al. 2009; Banu et al. 2010). The molecular weight of the pectinase determined was 38 kDa. Pectinase of the same molecular weight has been isolated from Bacillus gibsonii (Li et al. 2008) and Aspergillus niger U-86 (Mohsen et al. 2009). Generally the molecular weight of pectinase from microorganisms has been reported to fall within 30-70 kDa range (Klug-Santner et al. 2006; Tari et al. 2008; Banu et al. 2010). However there are reports on pectinases from microbial sources having a molecular weight greater than 70 kDa (Kashyap et al. 2000; Kobayashi et al. 2001; Arotupin et al. 2012) have been reported. The result of the subunit molecular weight indicates that the enzyme had a molecular weight of 37.5 kDa, in comparison to the native molecular weight it can be inferred that the enzyme is a monomer. Most pectinase of microbial origin have been found to be monomeric in structure (Polizeli et al. 1991; Sang et al. 2001; Schols et al. 2009; Asif Siddiqui et al. 2012).

The kinetic parameters [K_m and V_{max}] (**Table 2**) of B. licheniformis pectinase showed that the pectinase from this microorganism, shows preference for polygalacturonic acid as substrate, these values are similar to the results of previous workers. Polizeli et al. 1997; Saad et al. 2007; Asif-Siddiqui et al. (2012), working on from Rhizomucor pusillus reported a K_m and V_{max} of 0.22 mg/ml and 4.34 U/ml respectively using polygalacturonic acid as substrate. Saad et al. (2007)

reported a K_m of 1.88 mg/mL and V_{max} of 0.045 mole/ml/min for Mucor rouxii using polygalacturonic acid as substrate. Polizeli et al. (1991), obtained a K_m of 5 mg/ml for an endo-polygalacturonase from Neurospora crassa. These studies establish the fact that the kinetic properties of pectinases vary with the source of the enzyme as well as substrate used for the assay.

In this study, optimal activity of the pectinase produced was observed at 50^{o}C. A similar optimum temperature for action of pectinase has been reported for pectinase from other sources (Silva et al. 2002; Phutela et al. 2005; Yadav et al. 2009) as well as from Bacillus subtilis (Al-Ajlani et al. 2012). Pectinase from most bacteria have their optimum temperature between 45-60 ºC (Silva et al. 2002; Phutela et al. 2005; Yadav et al. 2009; Al-Ajlani et al. 2012; Torimiro and Okonji, 2013). However, exceptionally elevated optimum temperature of 70ºC and 80^{o}C for alkaline pectinase has been reported for Bacilus pumilus BK2 and Thermotoga maritima respectively (Klug-Santner et al., 2006; Kluskens et al., 2005).

The stability and catalytic activity of pectinases is of prime importance to food processors since desirable and/or deleterious reactions can be tailored (accelerated or inhibited) to meet specific quality targets (Duvetter et al. 2009). The thermo stability of the enzyme was examined at various temperatures for different periods. The purified enzyme exhibited high thermal stability between 35 and 45 ºC. Above these temperatures reduction in the activity of the purified pectinase was observed. Similar trend have been reported for pectinases from other sources (Tari et al. 2008) and among Bacillus spp (Jayani et al. 2005). Even the pectinase (Pectinex 3XL) used in many commercial applications gets inactivated on extended exposures to heat at temperature of 50 ºC or more (Ortega et al. 2004). The denaturation of the enzyme by heat, followed by inactivation due to the prolonged exposure at high temperatures may be responsible for this drop in pectinase activity (Bhatti et al. 2006).

The optimum pH of pectinases has been a determining factor for the industrial process to which it can be applied. The pectinase from Bacillus licheniformis showed high activity at a broad pH range with maximum activity at pH 9.0. Pectinases from different species of bacteria were reported to have different optimum pH ranging from neutral to alkaline pH (Kobayashi et al. 1999; Rehman et al. 2012; Yadav et al. 2013). Other bacteria were found to produce pectinases with a similar pH optimum of 8.5 (Kaur et al. 2010). A pH optimum of 10.5 has been recorded for the pectinase from Bacillus gibsonni (Li et al. 2008). The pH profile of the pectinase of Bacillus licheniformis meets the criteria for its possible industrial processes that are carried out at warm alkaline pH range.

The effects of salts showed that KCl salt activated the enzyme activity at all the tested concentration. Similar observations have been reported by Hamdy (2005). However, inhibitory effect at all concentration for potassium salt was observed on pectinase from Bacillus firmus (Roosdiana et al. 2013). In this study NaCl, $CaCl_2$, $SnCl_2$, $MnCl_2$ were able to activate B. licheniformis pectinase at 1 mM, while NaCl and $CaCl_2$ maintained the activation of the enzyme at 5 mM. On the other hand, Sn, Mn, Ba and Al salts had inhibitory effect on the enzyme activity. Similar results of activation of pectinolytic enzyme by different metals have been reported for other pectinases (Al-Najada et al. 2012). Likewise, Hamdy (2005) reported stimulatory effects for K, Na, Ca and Mg salts.

Al-Najada et al. (2012) working on pectinolytic fruit spoilage by Fusarium oxysporum and Aspergillus tubingensis used benzoic and citric acids as anti-fungal compounds against the growth of these organisms. They found these chemicals to be effective against fungal growth caused by pectinolytic organisms. Therefore, in the present study, the effect of these chemicals (Benzoic acid, citric acid, EDTA, gallic acid, tannic acid, sodium benzoate and sodium oxalate) on the activity of B. licheniformis pectinase was investigated. The enzyme was was inhibited up to 39% by EDTA similar result have been recorded by Banu et al. (2010) and Al-Najada et al. (2012). However, Yadav and Shastri, (2005) reported that EDTA had no inhibitory effect on Penicillium oxalicum polygalacturonase while stimulatory effect by EDTA was recorded by Chen et al. (1998). Gallic acid and tannic acid had profound inhibitory effect on the activity of B. licheniformis pectinase; other chemical substances evaluated also inhibited the activity of the enzyme. The inhibition of food spoilage enzymes produced by microorganisms could serve as control strategy in microbial food spoilage (Doyle, 2007).

CONCLUSION

In conclusion the pectinase from Bacillus licheniformis exhibited desirable thermal stability, broad pH, substrate specificity and activity at moderate temperatures in the presence metal ions and which could be utilized in some industrial processes.

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Conflicts of Interest Statement

No competing interests.

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