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Production, purification and characterization of pectin lyase from *Bacillus subtilis* isolated from moong beans leaves (*Vigna radiata*)

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ABSTRACT

Pectin lyase (E.C. 4.2.2.10) is the enzyme known to act on highly esterified pectin substances via β-elimination mechanism without producing methanol. It is potentially applied in fruit juice extraction and clarification in food and beverages industry. In pulp and paper industry, its application is explored for bio-bleaching of kraft paper and recycling of wastepaper. It is also in high demand in textile industry for retting of natural fibers and to eliminate pectin present in it. In the present study pectin lyase production was optimized for parameters as concentration of substrate and K_2HPO_4, pH of medium, nitrogen source and time course of pectin lyase production from *Bacillus subtilis*. The enzyme was purified and found to be of 38 kDa in molecular weight. EDTA, Ba^{+2} and Hg^{+2}showed stimulatory effects on the enzyme activity while Zn^{+2}, Mn^{+2} and Fe^{+2} inhibits the enzyme activity. To the best of our knowledge the study describes first time alkalophilic pectin lyase form a bacterium isolated from diseased plants.

1. Introduction

Pectinases are group of enzymes involved in pectin degradation. These are classified according to their mode of action and substrate specificity into two categories: (1) de-esterifying enzymes (Pectin esterase) and (2) De-polymerizing enzymes. Later are divided into two groups: (i) Hydrolyases (Polygalacturonase; 3.2.1.15) and (ii) Lyases (Pectate lyase; 4.2.2.2 and pectin lyase; 4.2.2.10) (Satyanarayana et al., 2013). Amongst these, Pectin lyases are of particular interest as they degrade highly esterified pectin without the prior action of any other pectinase by the β elimination mechanism. Pectin lyase is produced by many fungi (Yadav et al., 2017a; Carrasco et al., 2019), but rarely reported in bacteria (Demir et al., 2014) and Yeast (Alimardani-Theuil et al., 2011).

Pectin caused turbidity and viscosity interferes in fruit juice extraction. Its hydrophobicity causes problem in dyeing process in textile industry. Conventionally pectin removal requires harsh chemicals and use of high temperature in most of the industrial processes. Bioscouring is an ecofriendly approach for removal of non-cellulosic impurities from fibers with pectinolytic enzymes to make fiber more hydrophilic (Garg et al., 2016). Several reports of pectinases from microbial sources besides pectin lyase from bacterial sources are available in literature (Yadav et al., 2009a). Among fungus pectin lyase have been exploited from *Aspergillus flavus* (Yadav et al., 2008), *Aspergillus terricola* (Yadav et al., 2009b), *Penicillium citrinum* (Yadav et al., 2009c), *Aspergillus flavus* MTCC 10938 (Yadav et al., 2013), *Aspergillus niger* (Xu et al., 2015), *Penicillium griseooseum* (Lima et al., 2017), *Aspergillus brasiliensis* (Pili et al., 2018) and *Fusarium oxyspoum* (Yadav et al., 2017b) etc. The purified alkaline pectin lyase of *Fusarium lateritum* MTCC 8794, showed optimum temperature of enzyme was 40 °C and stability up to 50 °C for 30 mins (Yadav et al., 2017a). *Bacillus* sp. DT7 (Kashyap et al., 2000), *Paenibacillus amylolyticus* (Sakiyama et al., 2001) and *Geobacillus stearothermophilus* (Demir et al., 2011) are characterized for pectin lyase production. Kashyap et al. (2000) purified the enzyme from *Bacillus* sp. DT7 and found its molecular weight 106 kDa, optimum temperature and pH of enzyme was 60 °C and 8.0 respectively. In another study an acid stable and thermostable pectin lyase having molecular weight 36 kDa was purified. The enzyme activity was affected by Ca^{+2}, Cu^{+2}, Hg^{+2}, Zn^{+2}, Fe^{+2}, Mg^{+2}, Mn^{+2}, EDTA, L-cysteine and ascorbic acid (Demir et al., 2011). Extracellular pectin lyase of *Bacillus pumilus* (P9) was purified with 36.36-fold purification and 1.736 U/mg specific activity (Nadaroğlu et al., 2010).

Pectin is naturally present in plants as structural element. The possibility of finding potential isolate for pectin degradation is thus likely to be infected plant sources. In view of pectinolytic enzymes applications at high pH the study in present manuscript is aimed to purify and

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Table 1
Nutrient conditions range for optimization of pectin lyase production.

Selected parameters	Concentration range (w/v)
Pectin	0.5%, 1%, 1.5%, 2% and 2.5%
Inorganic nitrogen sources	Ammonium nitrate, Sodium nitrate, Potassium nitrate, Ammonium sulphate, Ammonium persulfate, Ammonium oxalate and Ammonium chloride) Each at 0.2% concentration
K_2HPO_4	0.1–0.5%

characterize the enzyme from an earlier isolated strain *Bacillus subtilis*.

2. Material and methods

2.1. Chemicals and microorganisms

Unless otherwise specified all chemicals were analytical grade and obtained from Hi Media, Merck and Sigma-Aldrich. Pectin was obtained from CDH (P) Ltd. India. The bacterium was isolated from infected moong bean leaves (*Vigna radiata*) and identified (data not shown here).

2.2. Optimization of enzyme production

2.2.1. Growth curve and time course of enzyme production

The methodology suggested by Sunnotel and Nigam (2002) was adopted with minor modifications. A loop full of the bacterial culture was inoculated into 25 ml pectin broth (pectin, 10 g/l; $(NH_4)_2HPO_4$, 2 g/l; $MgSO_4.7\ H_2O$, 0.5 g/l; K_2HPO_4, 2 g/l; pH 7.2). The inoculated medium was incubated overnight at 37 °C with an rpm of 120 along with a control. Absorbance values were noted down at 660 nm, followed by pectin lyase activity estimation till 30 hrs with an interval of every 3 hrs to determine growth and enzyme production respectively.

2.2.2. Enzyme assay

Pectin lyase was assayed by following the method of Poturcu et al. (2017) with some modification. The activity was determined by monitoring increase in optical density at 235 nm due to the formation of unsaturated uronide product using UV/Vis Spectrophotometer. Enzyme solution (0.1 ml) was added to a reaction mixture containing 2 ml pectin solution (0.05% w/v) in 50 mM Glycine NaOH buffer of pH 9.0 incubated at 40 °C. Optical density was measured at zero time and after 30 mins, and the steady-state velocity was calculated by absorbance change per minute. Enzyme activity was defined in terms of micromole of unsaturated product (Satyanarayana et al., 2013) released by 1 ml of enzyme in a minute, by using molar extinction coefficient value of 5550 M^{-1} cm^{-1}.

2.2.3. Optimization of nutrient and growth conditions

The nutrient and growth conditions of the isolate were optimized with below parameters (Table 1).

2.2.4. Effect of pH

A loopful of culture was inoculated aseptically into each flask of pectin broth (25 ml) of pH 5, 6,7, 8, 9, 10 and 11. All the flasks were incubated at 37 °C with an rpm of 120 along with a control. The pectin lyase activity was determined in the supernatant with above optimized conditions as described earlier.

2.3. Enzyme purification

Enzyme production was carried out under optimized of growth and nutrients. A bacterial growth of exponential phase at a concentration of 5% (v/v) was used as an inoculum into four flasks of 125 ml each (1.5% pectin, 0.2% NH_4Cl, 0.05% $MgSO_4.7\ H_20$ and 0.4% K_2HPO_4; pH 9.0). The inoculated medium was incubated at 37 °C with 120 rpm for optimized time period. The culture was centrifuged (7000 g, 10 mins, 4 °C) and pectin lyase activity was determined in cell-free supernatant. Protein concentration was also determined by the Lowry method (Lowry et al., 1951).

2.3.1. Ammonium sulphate precipitation

The pectin lyase produced by the isolate was precipitated using ammonium sulphate. To the 500 ml of cell free supernatant, ammonium sulphate was added slowly with constant stirring to achieve 0–30%, followed by up to 30–60% concentration of it. Each mixture was then kept overnight at 4 °C followed by centrifugation at 13,500*g* for 20 mins. The precipitate obtained was dissolved in 10 ml of 50 mM Glycine-NaOH buffer (pH 9.0). The precipitated enzyme was dialyzed through 10 kDa cut off membrane overnight with 50 mM Glycine-NaOH buffer (pH 9.0) in the ratio 1:1000 ml with 4 consequent changes. Enzyme activity and protein content were determined in each dialyzed fraction as described earlier.

2.3.2. Ion exchange chromatography

A column of DEAE-Cellulose (2.5 cm × 10 cm) was packed and equilibrated with 100 ml Glycine-NaOH buffer (50 mM, pH 9.0). 10.0 ml of dialyzed sample was loaded on to it. Unbound proteins were eluted with 50 ml Glycine-NaOH buffer (50 mM, pH 9.0) followed by elution of desired protein. It was performed with same buffer containing NaCl concentrations gradient (0.1M, 0.2M, 0.3M, 0.4M, 0.5M, 0.75M and 1M). Fractions of 3 ml volumes were collected. The presence of protein in fractions was determined spectrophotometrically at 280 nm. Pectin lyase activity and protein concentration were determined as described earlier. The fractions showing pectin lyase activity were pooled and loaded on gel filtration chromatography.

2.3.3. Gel filtration chromatography

A glass column of Sephadex G 200 (1.5 cm × 50 cm) was prepared to purify the protein. 3 ml of the pooled sample obtained above was loaded on to this column. The proteins were eluted using Glycine-NaOH buffer (50 mM, pH 9.0). Fractions of 3 ml volumes were collected and checked for protein presence. The enzyme activity and protein concentration were determined as described earlier.

2.3.4. Polyacrylamide gel electrophoresis (PAGE)

Gel filtration eluted fraction showing enzyme activity was subjected to SDS-PAGE (10%) to know the purity of enzyme preparation by following the method of Lämmli (1970). The protein bands were visualized by silver staining method (Merril et al., 1981).

2.3.5. Activity staining for detecting pectin lyase

Zymography of purified pectin lyase was done by following the method of Hadj-Taieb et al. (2011) with some modifications. PAGE under non denaturating conditions was performed. The pectin lyase gel (electrophoretic gel containing pectin lyase) was then applied against an overlay of 1% agarose containing 0.1% of pectin dissolved in 50 mM Glycine NaOH (pH 9.0) buffer solution. The sandwiched gels were incubated at 37 °C for 4 hrs followed by staining with 1% CTAB for 30 mins to observe the presence of clear zone.

2.4. Characterization of purified pectin lyase

The purified enzyme was characterized to determine the effects of metal ion on enzyme activity. Ba^{+2}, Ca^{+2}, Co^{+2}, Cu^{+2}, Hg^{+2}, Zn^{+2}, Na^{+}, Mg^{+2}, Mn^{+2}, Fe^{+2} and EDTA were added in 50 mM Glycine NaOH buffer (pH 9.0) at 1 mM concentration and activity was determined as described earlier.

3. Results and discussion

The enzyme production was optimized for isolate *Bacillus subtilis*. In

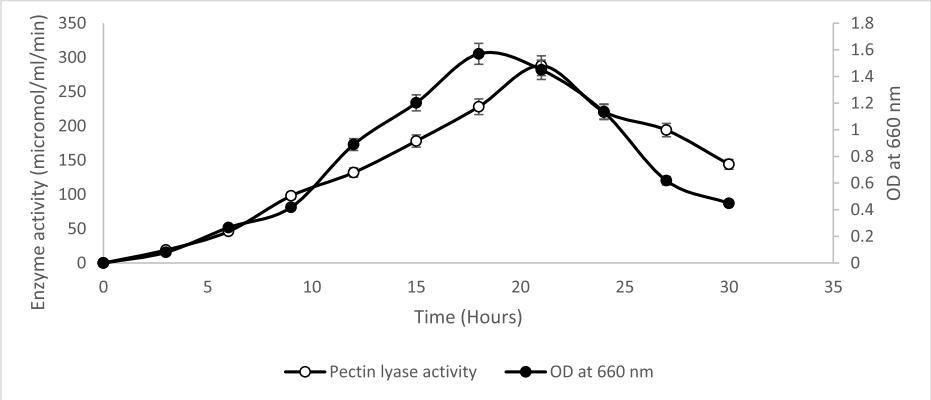

Fig. 1. Growth curve and pectin lyase production in pectin broth (Data points represents the means of three replicates with a SD ± 5%).

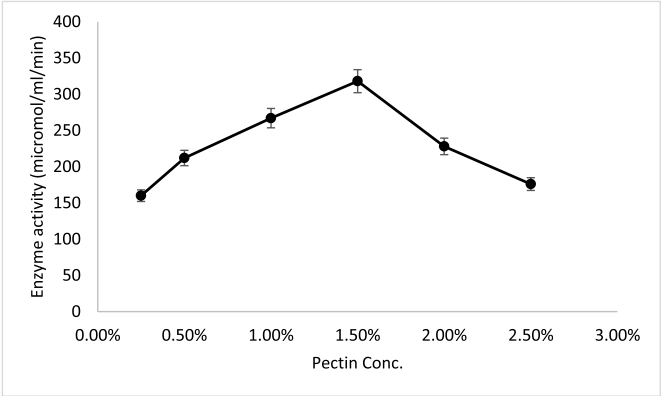

Fig. 2. Effect of pectin concentration (g %) on pectin lyase production (Data points represents the means of three replicates with a SD ± 5%).

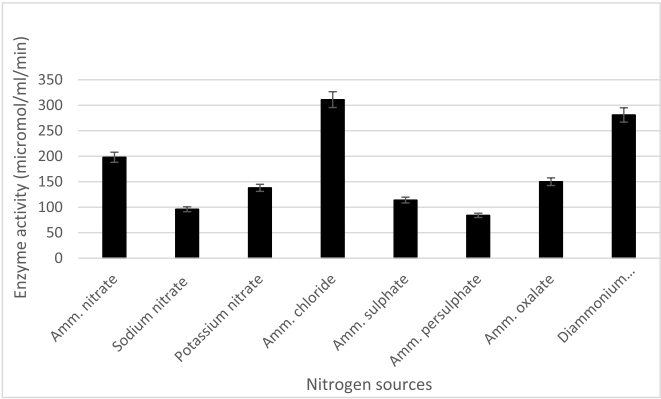

Fig. 3. Effect of various nitrogen sources on pectin lyase production (Data points represents the means of three replicates with a SD ± 5%).

this study, pectin lyase was produced by *Bacillus subtilis* using submerged fermentation. A minimal basal media with some modification was used for the production of pectin lyase. The growth curve suggests that bacteria stays 9–18 hrs into exponential phase after inoculation. The microorganism produces enzyme maximally between 18 to 21 hrs of incubation. A low level of pectin lyase activity was observed during earlier stages of incubation but gradually increased to maximum at 21 hrs of incubation (Fig. 1). Decrease in enzyme production after 21 hrs may be due to accumulation of end product which inhibits its own production by feed-back mechanism. Further it may be attributed to the depletion of nutrients due to which stationary phase comes and bacterial

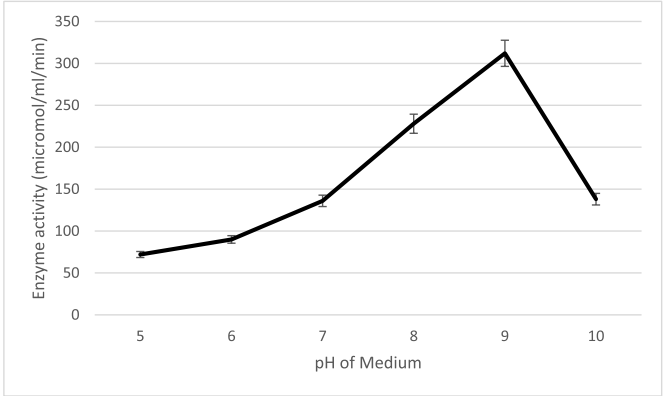

Fig. 4. Effect of pH on pectin lyase production. (Data points represents the means of three replicates with a SD ± 5%).

growth as well as enzyme production ceases (Ahlawat et al., 2007). Similar incubation period for pectin lyase production has been also observed from *Bacillus* sp. DT7 (Kashyap et al., 2000).

The isolate was optimized for nutrient and growth conditions with respect to various parameters as given in Table 1. At first, pectin was used as a sole source of carbon at its various concentrations. The optimum concentration obtained was 1.5% w/v pectin in production medium (Fig. 2). Pectin lyase is an inducible enzyme, its production is induced by presence of its substrate i.e. pectin (Lima et al., 2017). The effect of various nitrogen sources on pectin lyase production was determined at a concentration of 0.2% each. Among the various nitrogen source chosen, NH_4Cl gave maximum pectin lyase activity (Fig. 3). The

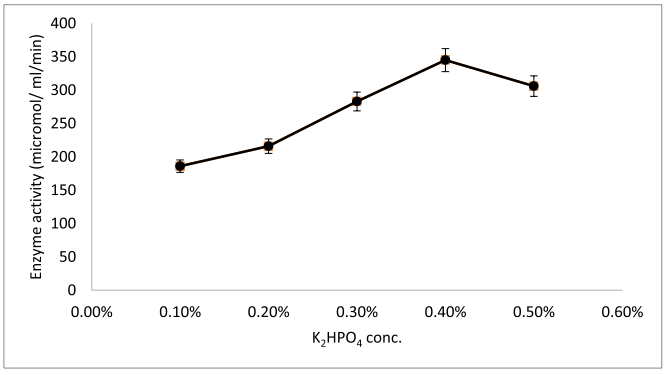

Fig. 5. Effect of K_2HPO_4 (g %) concentration on pectin lyase production (Data points represents the means of three replicates with a SD ± 5%).

Table 2
Purification of pectin lyase.

Purification step	Volume (ml)	Activity (micromol/ml/min)	Protein conc (mg/ml)	Total Activity (Units)	Total Protein (mg)	Specific Activity (Units/g)	Purification Fold	Yield (%)
Crude	500	2850	2	1425000	1000	1.42	1	100
Precipitated	13	1500	1.2	19500	15.6	1.25	0.88028169	1.368421053
DEAE Cellulose	10	2664	0.0204	26640	0.204	130.58	91.90140845	1.869473684
G-200 Sephadex	3	2040	0.007	6120	0.021	291.4	205.2112676	0.429473684

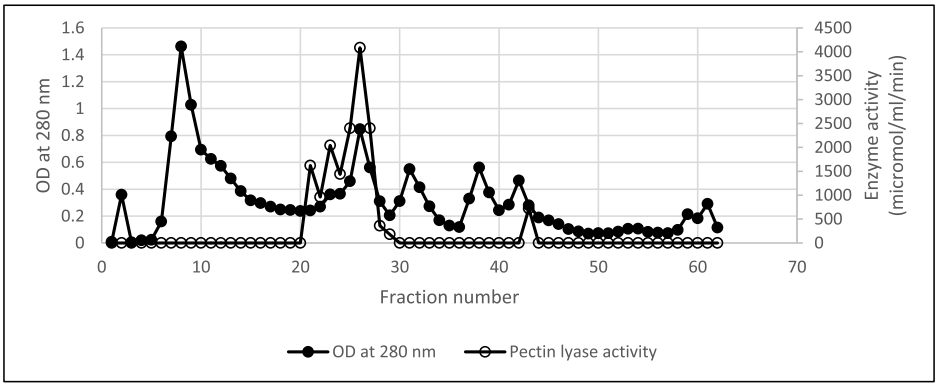

Fig. 6. Purification of pectin lyase on Ion exchange chromatography.

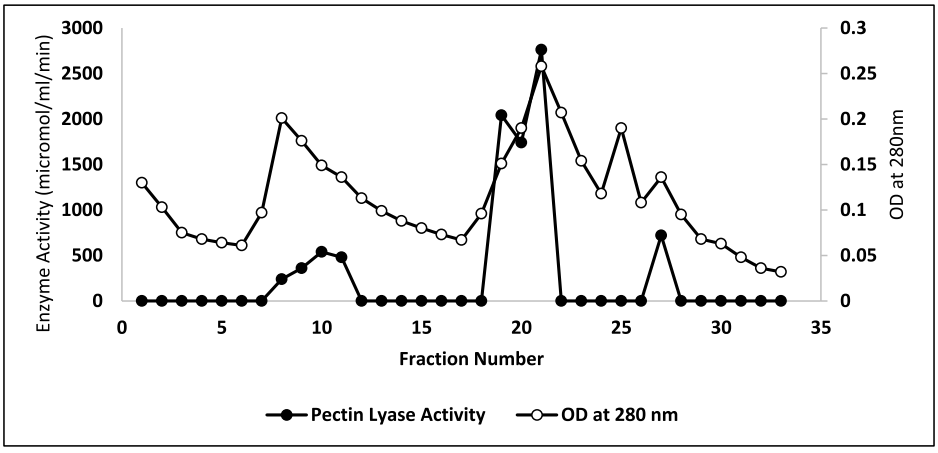

Fig. 7. Elution profile of pectin lyase activity on Gel filtration chromatography.

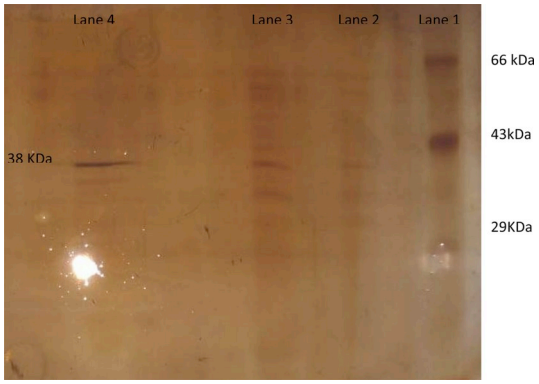

Fig. 8. 10% SDS PAGE of Purified Pectin Lyase. Lane 1: Molecular weight marker protein, Lane 2 and 3 Pectin lyase positive fractions of Ion exchange chromatography fractions, Lane 4: Gel filtration eluted fraction.

results are also in accordance to Hamdy (2006) and Ghazala et al. (2015) study in which the use of ammonium chloride was suggested to produce pectin lyase.

The pectin lyase production was determined at various pH of medium (Fig. 4). The isolate was able to grow in acidic as well as in alkaline pH. Maximum activity was observed at pH 9.0. Pectin lyase production decreases upon changing the pH of medium to either side. It is due to the fact that a change in pH changes enzyme structure and that's why enzyme activity decreases. Pectin lyase from other sources was also reported to work best at pH 9 (Kohli and Gupta, 2015). 0.4% of K_2HPO_4 was found to be optimum for pectin lyase production (Fig. 5). Similar results were also suggested by Sharma and Satyanarayana, (2006) and Guo et al. (2019) to increase pectinase production.

The study revealed that the isolate produces pectin lyase maximally in a medium containing 1.5% pectin, 0.2% NH_4Cl and 0.4% K_2HPO_4 (w/v) at pH 9.0 after 21 hrs under submerged condition. A 1.4-fold increase in enzyme production was observed under optimized conditions. The enzyme was purified up to homogeneity using three steps of

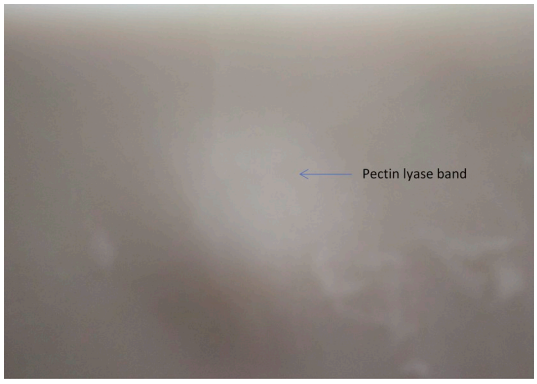

Fig. 9. Zymography analysis of Purified Pectin Lyase.

Table 3
Effect of metal ion on purified pectin lyase.

S.No.	Metal ion (1 mM)	Relative activity (%)
1	Control	100
2	Fe^{+2}	0
3	Ba^{+2}	113
4	Mg^{+2}	84
5	Cu^{+2}	36
6	Ca^{+2}	25
7	Zn^{+2}	0
8	Mn^{+2}	0
9	Hg^{+2}	124
10	Na^{+}	36
11	Co^{+}	33
12	EDTA	108

purification. The purification data are summarized in Table 2. Enzyme activity was obtained at 0–30% ammonium sulphate precipitation. The 30–60% ammonium sulphate precipitation did not reveal the presence of enzyme. Pectin lyase was eluted using NaCl gradient at a concentration between 0.1M and 0.2M. The elution profile of DEAE-cellulose yields a single protein peak in multiple fractions having pectin lyase activity (Fig. 6).

The enzyme was recovered with a total activity 6120 Units with a specific activity of 291.4 (Units/g) through Gel filtration chromatography (Fig. 7). The enzyme was purified up to 205.2 folds with a yield of 0.4294 (Table 2). Demir et al. (2011) obtained 40.77-fold purification of pectin lyase from *Geobacillus stearothermophilus* Ah22. Purified pectin lyase of *Bacillus subtilis* was found to be composed of a single unit of 38 kDa molecular weight (Fig. 8). The literature reported most of the purified pectin lyases have molecular weight in the range of 30–50 kDa (Jayani et al., 2005); (Li et al., 2012); (Yadav et al., 2012); (Xu et al., 2015); (Hadj Sassi et al., 2017); (Yadav et al., 2017a).

Zymography of purified pectin lyase revealed a zone of clearance around the enzyme band on polyacrylamide gel. It suggests the presence of active from of enzyme. Hadj-Taieb et al. (2011) attributed above phenomena as CTAB binds with intact pectin molecule and precipitate it to form the medium opaque, while a zone of clearance was obtained where pectin is degraded by enzymatic action (Fig. 9). Ba^{+2}, Hg^{+2} and EDTA enhances the enzyme activity, whereas presence of Zn^{+2}, Mn^{+2} and Fe^{+2} completely inhibits the enzyme activity (Table 3). A slight decrease in enzyme activity was observed in the presence of Co^{+2}, Cu^{+2} and Na^{+}. Afifi et al. (2015) reported similar behavior of pectin lyase towards above ions. The calcium ion decreases pectin lyase activity which is also earlier reported by Yadav et al., 2017a and Rashad et al. (2011). Although, this behavior deviates to the report of Kashyap et al. (2000) and Demir et al. (2011) where they observed an increase in enzyme activity. Sharma et al. (2013) suggested that Pectin lyase did not require calcium ions for their reaction mechanism. Perhaps the presence of it interferes in the binding of other activator ions to the active site. The effect of EDTA on enzyme activity is usually explained in terms of its metal complexing capacity as it does not allow the participation of metal ions in the reaction mechanism. In present study EDTA is acting as activator for the purified pectin lyase activity. It might be due the fact that it chelates inhibitor ions present in the environment and thus does not allow a decrease in enzyme activity (Kirkeby, 1976).

4. Conclusions

The present investigation purifies pectin lyase with 205.2-fold purification and reports an alkalophilic pectin lyase with a 38 kDa with purification. To the best of our knowledge it is first study where an alklophilic pectin lyase was purified and characterized from *Bacillus subtilis*, isolated from diseased *Vigna radiata*. The study may be useful for cotton industry waste effluent treatment. In addition, to above pectin lyase inhibitors can be designed to restrict pathogen entry. Thus, the present outcome is also useful for plant pathologist to characterize pectin lyase in phyto-pathogens as a virulence factor under alkali conditions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101306.

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