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Purification and kinetics of Pectinase production from *Paenibacillus lactis* NRC1 locally isolated from Egyptian mangrove habitat

Manal, S. Selim*, Sahar, S. Mohamed, Manal, G. Mahmoud, Mohsen, M. Asker, and Osama, H. El Sayed

Microbial Biotechnology Department, National Research Centre, El-Buhouth Str., Cairo, Egypt

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ABSTRACT

Biotechnology is the use of microorganism to perform special industrial process, Therefore The aims of our study were to screen the bacteria that able to produce pectinase enzyme from locally marine by isolation technique. Pectinolytic activity, confirmed by the clear zones on the pectin medium plates. The highest pectinase was identified as Paenibacillus lactis NRC1 which produce 3.20 U/ml after 2 days of incubation at pH 7, 40°C, 5 g/L pectin and yeast extract 1 g/L. The purification fold of the enzyme was 98.718 with final recovery 3.844% using different chromatographic steps. The molecular weight of the purified pectinase determined by SDS-PAGE was 45 kD. The ideal pH was 7 and the enzyme safe up to 40 ºC for 60 min. The K_m and V_{max} was 0.772 and 7.936 respectively.

Keyword: screening, pectinase, purification, *Paenibacillus lactis* NRC1

INTRODUCTION

Pectinases are normally utilized as a part of numerous naturally agreeable and economical divisions on the grounds that there are expanding interest to supplant some traditional chemical processes which are enormous expansion in consciousness of the impacts of contamination, and media has affected both industry and government with biotechnological forms including microorganisms and enzymes such as pectinase [1,2]. Pectinolytic enzymes bring been expansive provisions in fruit juice commercial enterprises in place will upgrade fruit juice yield, furthermore clarity [3,4] and they have a share of over 25 % in the worldwide scale of the nourishment enzymes [5,6]. They would largely used on eradication also elucidation for apples and oranges juices, eradication about vegetable oil, preparing from claiming alcoholic beverages, maturation of espresso beans, furthermore tea sack leaves, retting and degumming from claiming fibers [7,8], and novel applications in the production of oligogalacturonides as functional food components. The enzymes hydrolyzing pectin include endo-polygalacturonase (EC 3.2.1.15), exopoly-galacturonase (EC 3.2.1.67), pectin lyase (EC 4.2.2.10) and pectin esterase (EC 3.1.1.11) [9,10]. Pectinases are generated by a significant numbers of organisms, counting bacteria, fungi, yeasts, insects, nematodes, protozoa and plants. However, determination of a specific strain remains a dull errand and the decision gets harder when financially enzyme outputs are to be accomplished. Bacterial strain yielding commercial catalysts are would dependably always favoring through contagious strains due to simplicity for maturation methodology, furthermore execution for strain change systems or whatever current strategy on increment those yield from claiming processing [11]. Pectinases from bacteria have functional warm reliability [10] likewise development through a wide degree for pH [9], also may be useful in various industrial applications that require usage regarding raised temperatures, extremes regarding pH additionally ionic concentrates. Therefore, it is significant with research interesting natural surroundings and studies the indigenous flora about microscopic organisms with exceptional enzymatic possibility. Sediment of mangrove tree gives suitableness corner for different bacteria because of plenitude of dampness, saltiness and natural flotsam and jetsam of plants and animals [12]. The current study focuses on screening of pectinolytic bacteria from mangrove trees, improved the production of pectinase through media optimization followed by its purification.

MATERIALS AND METHODS

Microorgainsms
All microorganisms used in the present study were taken from laboratory of Microbial Biotechnology. These strains were tested on citrus medium for pectinse production.

Screening for pectinase production
Purified colonies were spotted on to pectin enriched agar plates which contained (g/L) pectin 5, yeast extract 5, agar 16 (pH 7.0). After 2 days, clear zones were visualized using 1% cetrimide solution [13] and a potent producer of pectinase was selected.

Submerged fermentation of pectinase
Pectinase production from the selected bacterial strain was carried out in pectin broth containing (g/L): yeast extract, 1; pectin, 5; KH_2PO_4, 4; NaCl, 2; $MgSO_4.7H_2O$, 1; $MnSO_4$, 0.05; $FeSO_4.7H_2O$, 0.05; $CaCl_2.2H_2O$, 2; NH_4Cl, 2 and distilled water with 50% sea water at pH 7.3 [12]. Fermentation was carried out in 250-mL Erlenmeyer flask containing 50 mL of production medium with 1% (v/v) inoculum (10^6 cells/mL) and incubated at 40°C under shaking condition (150 rpm) for 2 days. The culture broth was centrifuged at 5,000 rpm for 20 min at 4°C. The supernatant was subjected to pectinase assay.

Assay of pectinase
Polygalacturonase activity was determined by quantifying the amount of reducing groups expressed as galacturonic acid units, liberated during the incubation of 1 mL of 1% (w/v) citrus pectin, prepared in 0.2 M phosphate buffer (pH 7) with 300 µL of the enzyme at 40°C for 20 min, by DNSA method [14]. One unit of polygalacturonase activity was defined as the amount of enzyme required to release 1 µmol of galacturonic acid per minute under standard assay conditions [15] and expressed as units per millimeter (U/mL).

Identification of bacteria
The bacterial strain which produces high amounts of pectinase, was identified in previous research about polysaccharide and it take accession number after submitted to Genebank [16].

Effect of physico-chemical parameters
The promising stain was subjected to different culture conditions to derive the optimum conditions for pectinase production. Pectinase production were estimated at regular time intervals (1, 2, 3, 4, and 5) days and selected temperatures (25, 30, 35, 40, 45, 50 and 55 °C), and pH (3, 4, 5, 6, 7, 8 and 9). All the experiments were carried out in 500-mL Erlenmeyer flask containing 100 mL of basal medium in triplicate.

Effect of nutritional parameters
The effect of substrate concentration on pectinase synthesis was investigated by incorporating various concentrations of pectin (1, 2, 3, 4, 5, 6 and 7 g/L) into the production medium. The effect of different organic (beef extract, yeast extract, peptone) and inorganic (NH_4Cl; $(NH_4)_2SO_4$; $NaNO_2$) nitrogen sources were investegated.

Protein estimation
Total soluble protein was determined by Bradford [17] using bovine serum albumin as the standard.

Purification of pectinase
The proteins were precipitated from the supernatant with ammonium sulfate (0–80% saturation. Pectinase active fraction was pooled, centrifuged (5,000 rpm, 25 min) and the precipitate was dissolved in minimal amount of 0.2 M sodium phosphate buffer, pH 7 and dialyzed overnight at 4 °C. The dialyzate was loaded on to a DEAE-cellulose anion exchange column (1.5×40 cm), which was equilibrated and washed with 0.2 M sodium phosphate buffer, pH 7. The enzyme was eluted with a linear gradient of NaCl (0-1.0 M) at the flow rate 1 mL/min and the eluate was monitored for absorbance at 280 nm, conductivity and pectinase activity. The most active fractions from DEAE-cellulose column were pooled, dialysed as described in previous step and loaded on a (1.5×60 cm) of Sephadex G-100 equilibrated with 0.2 M phosphate buffer pH 7 and eluted with one liter of the same buffer at flow rate 0.5 mL/min. Elute (5 mL fractions) was collected for measurement of absorbance at 280 nm and the enzyme activity was assayed [18]. The active fractions were pooled and dialysed against the same buffer. The protein content was measured and the pectinase activity was assayed as described before.

Characterization and properties of the purified pectinase
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
Molecular weight of the purified pectinase was determined by SDS-PAGE according to the method of Laemmli

[19]. The strength of the gel was 12% (w/v) and the protein bands were stained with Coomassie brilliant blue R 250. Molecular weight markers were used to determine the molecular mass of purified pectinase. The markers were: carbonicanhydrase (29 kDa), albumin (egg) (45 kDa), bovine serum albumin (66 kDa), phosphorylase (97 kDa), β-galactosidase (116 kDa) and myosin (205 kDa).

Optimum pH and thermal stability

To estimate the optimum pH of the pure pectinase , the activity was measured with 1 mL of citrus pectin 1% (w/v) in 0.2 M of citrate phosphate buffer pH (3.0-5.0), phosphate buffer pH (6.0-7.5) and Tris-HCl pH (8.0-10.0) [18]. To determine the thermal stability of purified enzyme, samples were pre-incubated with different range of temperature from 30ºC to 100ºC at different times (10, 20, 30, 40, 50 and 60) min. Assays were performed as described above.

Substrate concentrations

Different concentrations of citrus pectin (0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4 and 1.6 % w/v) have been used and the apparent K_m values of citrus pectin were determined through studies relating substrate concentrations to the velocities of the reaction. The apparent *Km* and V_{max} values for these substrates were determined from Lineweaver–Burke plots [20].

RESULTS AND DISCUSSION

Silt around the rhizosphere of the mangrove tree holds a considerable measure for debris from autolysis or microbial debasement of fallen leaves, blossoms, verdant sustenances [21]. These natural materials supply vitality and nutrition of a living structures found in the tree surroundings [22]. Bacteria, fungi , furthermore actinomycetes, which decay those plant based polysaccharides, abundantly flourish in the tree sediment [23]. In the introduce study, all the selected stains obtained from the rhizospheric sediment samples.

Screening of pectinolytic bacteria

Eight strains of bacteria were previously isolated from the mangrove sediments screened for pectinase production revealed that all the strains were pectinolytic. Strains number 1, 2, 3 and 4 exhibited great pectinolytic possibility with the strain number 7 Likewise the most noteworthy producer (data not shown). On the other hand, strains number 5 and 6 indicated moderate enzymatic potential, whereas, stain number 8 might have been discovered to process those most reduced zone from claiming pectin hydrolysis. Mangrove bacteria bring extraordinary pectinase, presumably due to those riches of natural residues in the debris. This perception may be bolstered by many discoveries whom noticed that 61.1% of the bacterial strains of the mangrove tree exhibited valuable pectinase activity [24] .

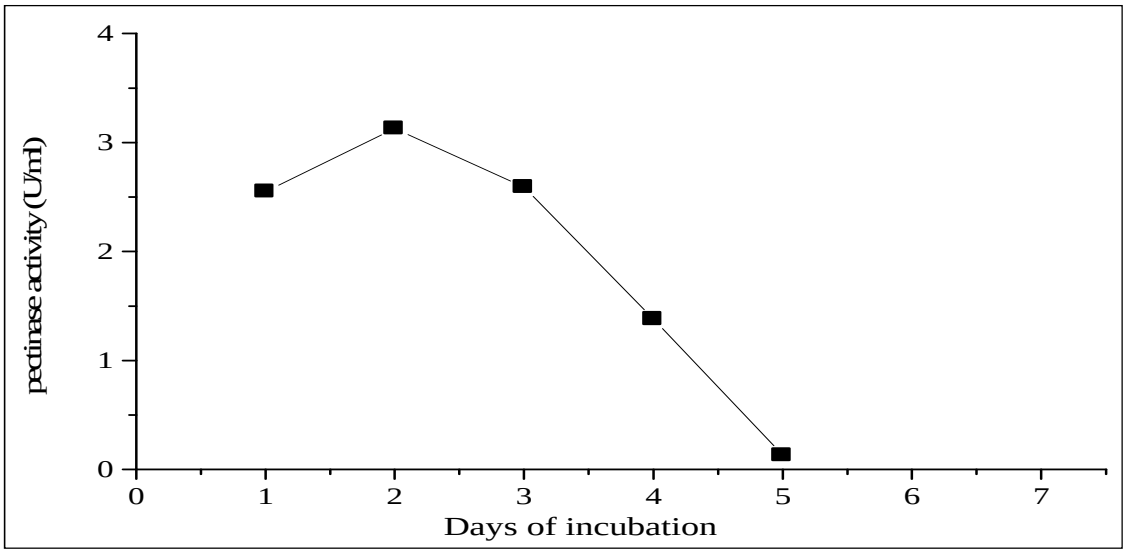

Fig. 1. Effect of incubation period on pectinase production

Characterization of the pectinolytic strain

The highest pectinase production strain number 7 have been identified as *Paenibacillus lactis* NRC1 in other study [16]. This strain produced several enzymes such as catalase, oxidase, arginine dihydrolase, and penicillinase **[**25].

Effect of physico-chemical parameters

In the current study, the yeild of enzyme production varies according to the time of icubation of the fermentation steps [26]. Pectinase processing might have been recognized following 1 days for brooding. A progressive augment in the pectinase level might have been distinguished till those second day of the fermentation process (3. 15 U/mL), whereas, there might have been a soak decrease in the pectinase action after the 4 th day for incubation, as shown in **Figure (1)**. Past this period the enzyme handling awfully declined, because of the exhaustion of crucial supplements in the production medium and/or collection of harmful auxiliary metabolites.

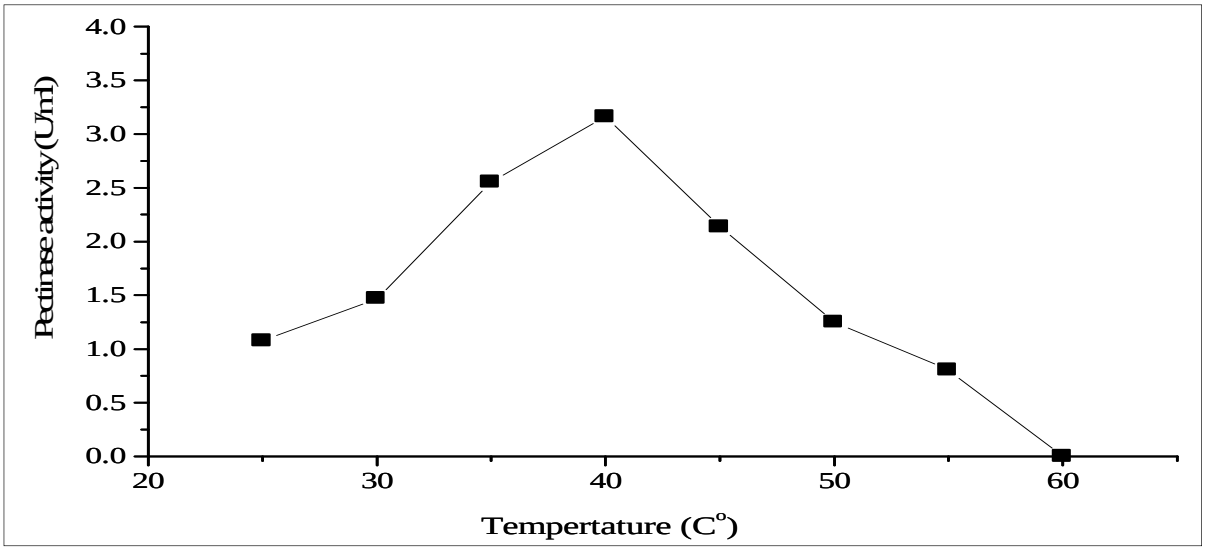

Fig.2. Effect of temperature on pectinase prouction

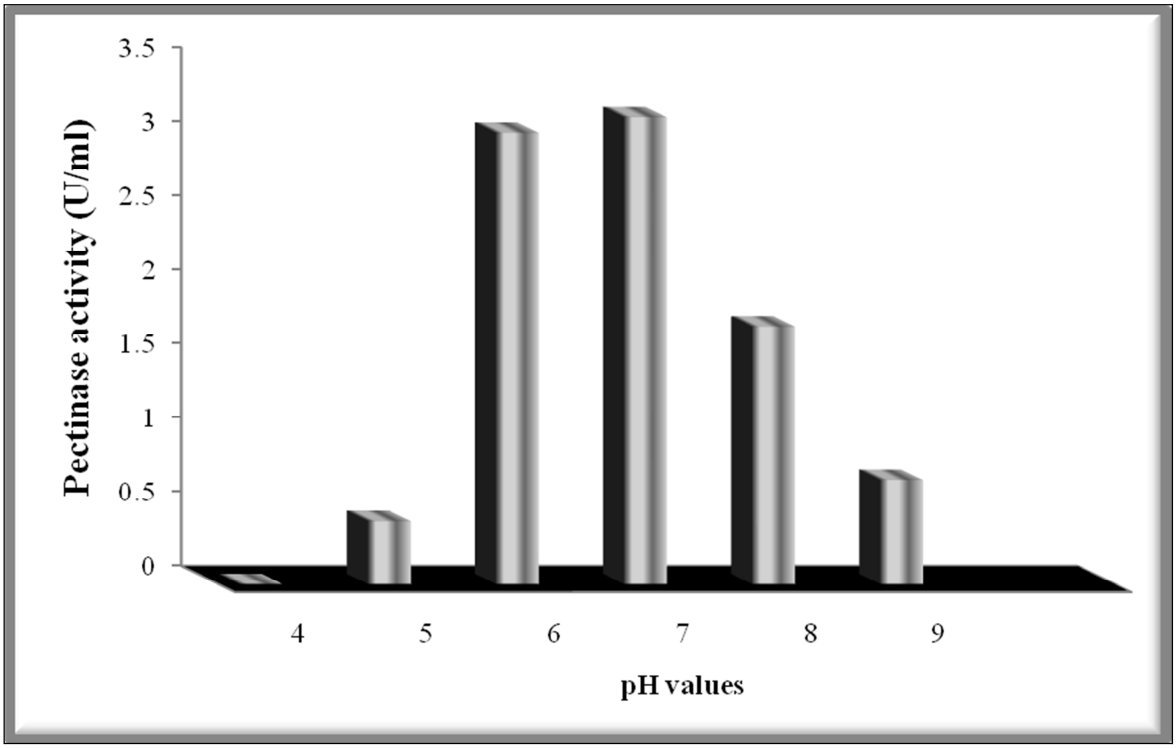

Fig.3. Effect of pH on pectinase prouction

The incubation temperature incredibly influences microbial development rate, catalyst secretion, catalyst restraint, also protein denaturation [27]. Forty celsius degree backed most extreme pectinase amalgamation (3.16 U/mL), concerning illustration exhibited on **Figure (2)**. Due to the mesophilic bacterial strain mood, this tempertaure might be support and enhance membrane permeability and in this manner most extreme pectinase emission under the

medium. A decline to pectinase production might have been watched when incubation temperature was over 40°C, this is owing to the impaired metabolic activity of the isolate. Banu *et al.* [18] expressed the same results.

pH of the fermentation medium accepts an essential a bit carried out figuring out the level for metabolite amalgamation. The balance of the microbial metabolite will be also subject to the hydrogen ion of the medium [27]. pH 7 enhanced the extreme pectinase production (3.18 U/mL) **Figure (3)**. The bacterial strains of the mangrove tree favour the neutral pH rather than alkaline pH for the growth and the productivity of the enzymes. These results are in a good accedence with Kashyap *et al*. [28]who stated that pectinase production of *Bacillus* sp.DT7 increased at pH 7.2.

Effect of nutritional parameters

The accomplishment of a fermentation procedure relies basically for notable system parameters that impact the microbial improvement furthermore metabolite generation [29]. Submerged fermentation is may be comprehensively used to the industerial processing for microbial enzymes. Approachability of useful measure about a utilizable substrate in the fermentation medium helps in high yeild of enzyme production.

Substrate concentration enhanced the highest pectinase activity of (3.20 U/ml) from 5 g/L pectin. Pectin percentages either lower or higher than 5 g/mL exhibited lesser production of the enzyme, as indicated in **Figure (4).** These outcomes were concurred with de Andrade *et al*.[30]

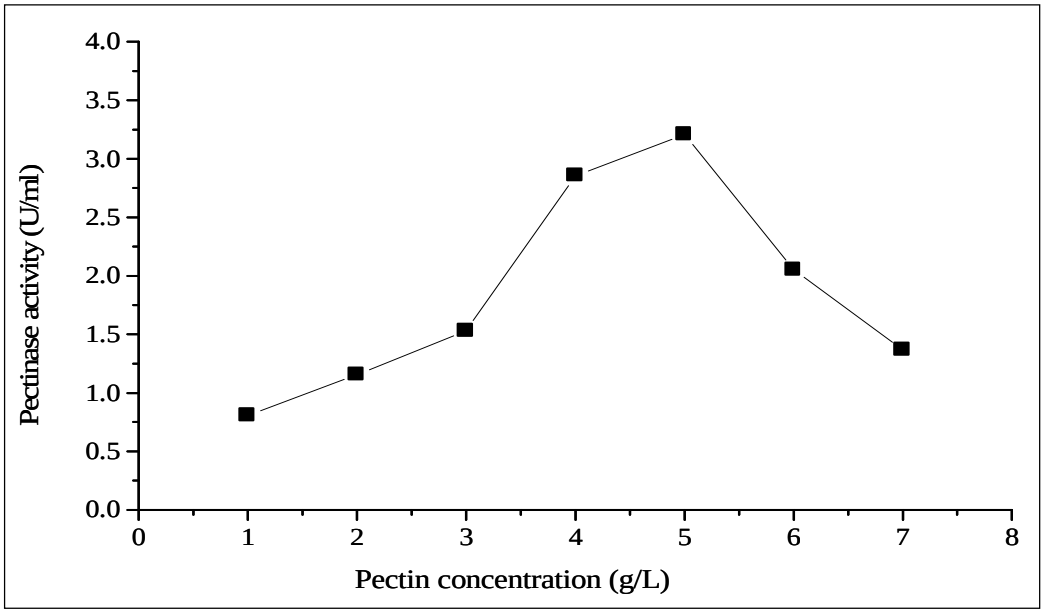

Fig. 4. Effect of pectin concentration on pectinase production

Nitrogen supplements, The point when combined under the production medium, sway better biomass preparation furthermore hence higher metabolite emission. Nitrogenous compounds would used by those microbial units to synthesis nucleotides, amino acids, proteins and other metabolites [26]. The effect of different organic and inorganic nitrogen supplements was investigated.

Organic nitrogen supplements like beef extract and peptone decreased the yield of pectinase yet, yeast extract enhanced the maximum productivity of the enzyme (3.21 U/mL) **Figure (5)**. This might make attributed of the vitamins, in addition accessory growth factors naturally present in these organic supplements. Among the inorganic nitrogen sources, both ammonium chloride and ammonium sulfate promoted greater pectinase production, whereas, sodium nitrite declined the activity as seen in **Figure (5)**. Many authers reported the same result [31,18] while others expressed that ammonium sulfate decrease the activity [12].

Purification of pectinase

Fractions from 60% ammonium sulfate saturation demonstrated high pectinase activity and specific activities in comparison with crude pectinase as noticed in **Table (1)**. The obtained pectinase produced after partially purified using 60% ammonium sulfate was subjected on column of DEAE-cellulose. The elution diagram of the enzyme is illustrated graphically in **Figure (6)**. The obtained result showed a single peak co-eluting with pectinase activity. The specific activity has elevated to 64.382 U/mg with a fold purification fold of 40.038. Subsequent purification by

gel filtration using Sephadex G-100 was applied. The elution diagram of the enzyme is illustrated graphically in **Figure (7)**. The obtained result showed a single peak co-eluting with laccase activity of active DEAE cellulose fractions. The purification results are summarized in **(Table 1)** with an overall recovery of 3.844%. The purification of pectinase is imperative from the viewpoint of developing a better understanding of the functioning of the enzyme. Precipitation is regularly utilized technique for the isolation and recovery of proteins from unrefined biological mixtures. Reporters found that pectinase is precipitated by 60% saturation of ammonium sulfate from *Hylocereus polyrhizus* and *Penicillium* sp. respectively [32,33,34]. It It might have been accounted the use of Sephadex G-100 for the purification of pectinase from *Penicillium chrysogenum* and *Penicillium chrysogenum* respectively [18,35], while additional investigations used Sephadex G-150 likewise G-200 for pectinase purification [28,12].

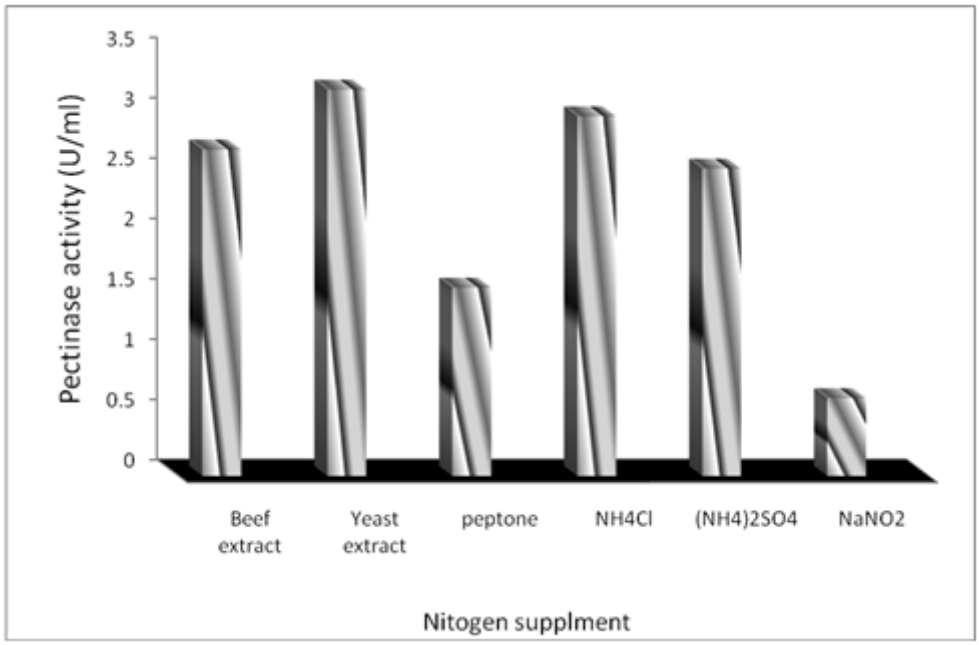

Fig. 5. Effect of nitrogen sources on pectinase production

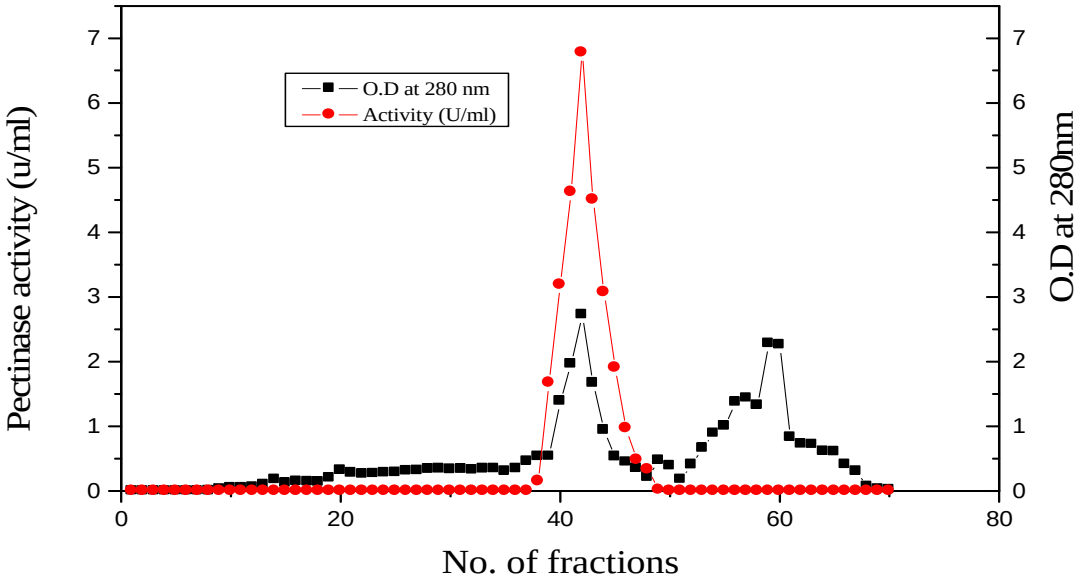

Fig.6. Elution profile of pectinase (60% saturation) on DEAE- cellulose

Table. 1. Summary of the purification of pectinase

Purification steps	Volume (ml)	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg)	Purification (fold)	Recovery (%)
Crude	1200	3840	2388	1.608	1	100
$(NH_4)_2SO_4$ (60%)	10	78.9	7.58	10.408	6.472	2.054
DEAE-cellulose	40	262.68	4.08	64.382	40.038	6.840
Sephadex G-100	30	147.63	0.93	158.741	98.719	3.844

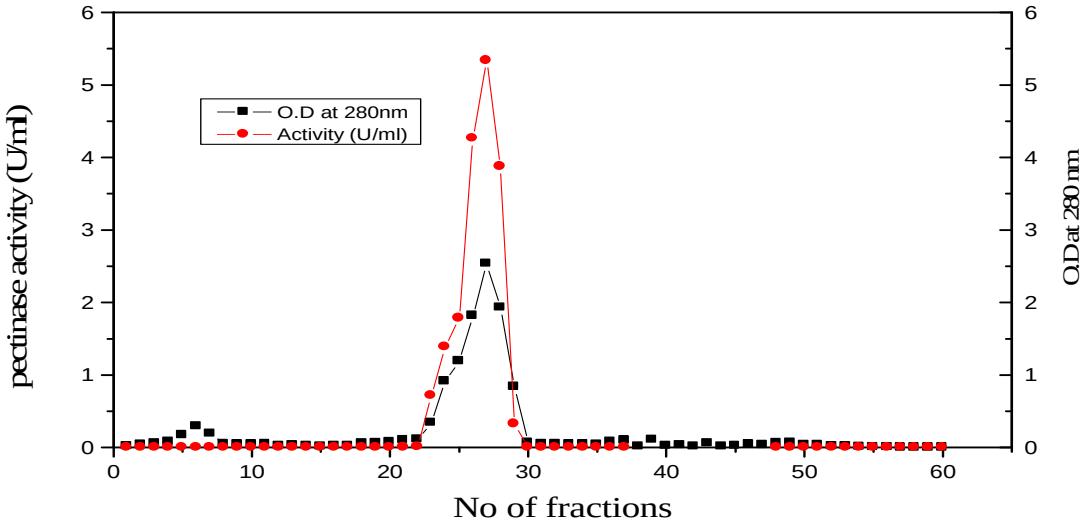

Fig. 7. Elution profile of pectinase (from activated fractions of DEAE-cellulose) on sephadex G-100

The apparent molecular weight of the purified pectinase was observed to be 45 kDa by SDSPAGE as seen in **Figure (8)**. This outcome is will be over end closeness with the consider that pectinase from *Streptomyces lydicus* was found to be 43 kDa [36]. In another reports, the molecular weight of purified exo-polygalacturonase from *Bacillus stearothermophillus* (24 kDa) [37], *Fusarium oxysporium f. sp. melonis* (58 kDa) [38], *Penicillium frequentans* (20 kDa) [39] and *Penicillium chrysogenum* was determined as 31 kDa [18].

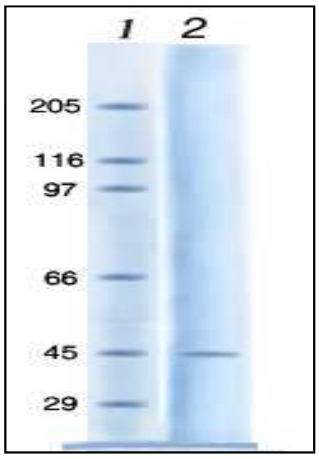

Fig. 8. SDS-PAGE of pectinase enzyme. Analysis performed on a polyacrylamide. From left to right; Lane 1, standard molecular weight markers, Lane 2, final purified pectinase.

Enzyme characterization

Perfect pH of the pectinase might have been determined on diverse buffer systems, as previously described. The pectinase demonstrated appropriate activity within range of pH values (6.0 to 8.0), and the most elevated enzyme activity (4.944 U/mL) was gotten at pH 7.0 **Figure (9)**.

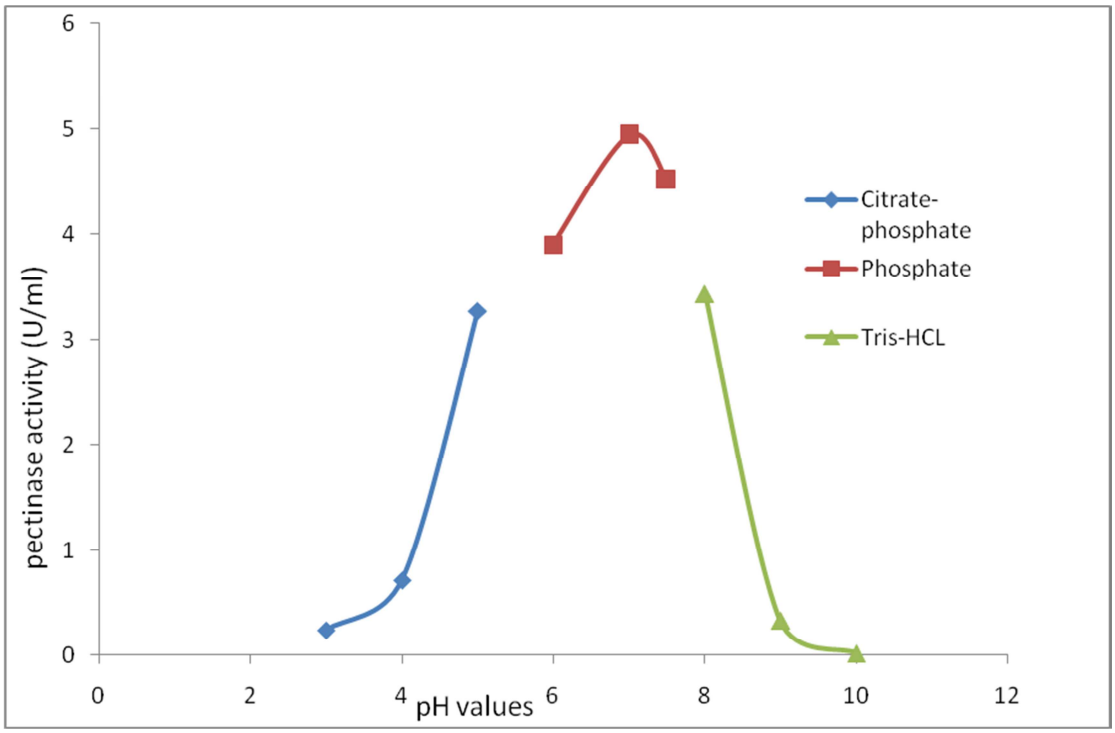

Fig.9. Effect of pH on purified pectinase

The ideal temperature of the pectinase was estimated by determining its activity at different temperatures. As shown in **Table 2** the enzyme was active over a wide extent of temperatures from 30 to 70 °C, and the optimal activity was gotten at 40 °C. The residual activities of pectinase at 30 and 70 °C were 98.2 and 22.87 %, respectively. The analysis of the thermal stability of pectinase indicated that the pectinase obtained more than 50 % of its activity in the temperature range of 30–60 °C, yet the enzyme activity was markedly diminished at temperatures higher over 60 °C. The relative activity of the enzyme at 70 °C was 22.87 %, but there was no notable activity at temperatures more over 70 °C. This might be owing to denaturation of the tertiary structure of the pectinase at the evaluated temperatures [40,41]. Moreover, the findings obtained in this study are consistent with those of several previous studies that analyzed the isolation of pectinase from other sources, such as fungi and actinomycetes; [42,12]; however, another previous study showed that a pectinase isolated from a fruit source was thermostable [34].

Table 2. Thermal stability of purified pectinase

Time (minutes)	Residual laccase activity (%)					
	Temperature ^{0}C					
	30	40	50	60	70	80
10	98.2	100	97.5	53.41	22.87	0
20	98	100	85.14	33.88	12.71	0
30	97.21	100	71.02	25.43	3.45	0
40	97.08	100	60.99	10.15	2.22	0
50	96.16	100	27.1	7.81	1.11	0
60	95	100	12.4	1.89	0	0

As seen in **Figure (10)** pectinase activity increased by increasing the concentration of citrus pectin till reached 1% (w/v), the activity was (4.944 U/mL) and after that the increase of the activity was very weak. The K_m and V_{max} was 0.772 and 7.936, respectively. A lower Km value indicates a higher binding of enzymes and substrates [43].

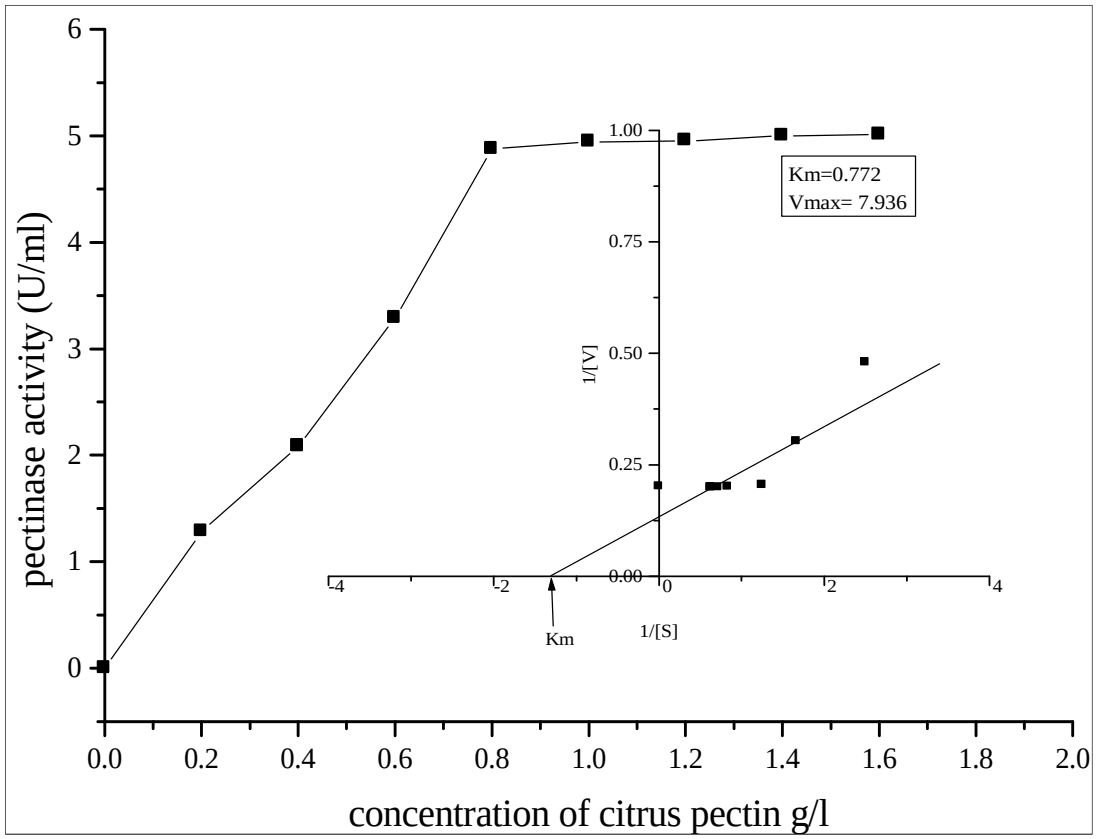

Fig. 10. Lineweaver-Burk plot of pectinase activity with pectin

CONCLUSION

Mangrove bacterial strains are a good source for pectinase production and the thermostable ability of the produced pectinase will be used in further studied in industrial and biotechnological application.

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