J. Appl. Environ. Biol. Sci., 7(12)9-19, 2017

ISSN: 2090-4274
Journal of Applied Environmental and Biological Sciences
www.textroad.com

Paenibacillus sp. strain NBR–10, A Thermophilic Soil-Isolated Bacterium with Thermo-Alkali Stable Pectinase Activity

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Received: August 10, 2017
Accepted: November 1, 2017

ABSTRACT

Bacterial pectinases are one of the most important industrial enzymes where their sale constitutes more than 25% of all food enzymes and their marketing is increasing daily. The use of thermo- and alkali-stable bacterial pectinases is considered to be important biological alternative to chemical processing as well as it can cover the industrial demands. Stability of pectinase enzyme athigh values of temperature and pH gives the producing organism great advantages. Therefore, isolation of bacterial strain with novel pectinolytic activities is the target of the present study. To achieve this goal, twenty-one bacterial cultures were isolated from samples of different pectin rich sources collected from different locations of Rafha governorate at the Northern Border region in Kingdom of Saudi Arabia. The obtained bacterial isolates were screened qualitatively for pectinase activity using pectin agar medium under thermal and alkaline conditions. Out of 21 isolates, 4(19.0%) isolates showed pectinase activity with varied degrees, among them isolate NBR–10was found to be the highest pectinase producer thus it was selected for production of pectinase in fermentation conditions. The selected isolate was identified by partial sequencing of16S rRNA gene as *Paenibacillus sp.* strain NBR-10(the sequence had been deposited in Gen Bankunder accession number KT957624.1). The obtained pectinase enzyme showed high activity at temperatures ranged from 20 to 65°C and pH values ranged from5.0to10.5 with optimum activity at 60 °C and 9.5pH. At these conditions,90 % of the enzyme still active for 1 h. The high activity of pectinase enzyme produced by *Paenibacillus sp.* strain NBR–10 and its stability for a long time athigh values of temperature and pH, candidates this enzyme for application in many industrial processes.
KEY WORDS: Soil, *Paenibacillus sp.* strain NBR-10, Thermo-alkali stable, Pectinase, Production.

INTRODUCTION

Pectin is a complex high-molecular weight polysaccharide. It is the major constituent of many plants such as vegetables, fruits, fibers and cereals. Degradation of this polymer is carried out by extracellular enzymatic pectinases which produced by many of pectinolytic microorganisms [1].
The enzymes of pectin hydrolysis are known as pectinases or pectinolytic enzymes [2]. Generally pectinases is a common name for a family of enzymes which can be classified into two main types: methyl esterases, which convert pectin to pectate via removing of the methoxyl group and depolymerases, which hydrolysis the pectin and pectate by hydrolytic cleavage [3]. According to the mechanism of pectinases action, they are classified into three types; pectin lyase (PL), pectinesterase (PE) and polygalcturonase (PG)[1].
Recently, pectinases have received more attention in the industrial sector and their market is in gradual increase. Among the commercially produced industrial enzymes, pectinases hold a leading position due to its ecofriendly nature which allows them to be applied in various industries[4].
Pectinases have diverse applications in various industries such as fruit juices extraction and clarification [5,6],paper pulp bleaching [7],fibers degumming[8],extraction of oil [9]in addition to fermentation coffee and tea [10,11]. Also pectinases were found to be use in extraction of plants DNA [12]and production of some prebiotic food components such as pectic oligosaccharides [13].
Pectinases are classified into acidic and alkaline enzymes, based on the optimal pH for the enzyme activity. Acidic pectinases can be used in wine making, animal feed and fruit juice industries [10]. While, alkaline pectinases attracted more attention due to their extensive uses and industrial applications in bioscouring of cotton fiber [14], plant fibers degumming [15], production of papers; treatment of pectic waste water [16,17], fermentation of coffee/tea [18], in addition to purification of plant viruses and extraction of vegetable oils[19].

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Most of the microorganism's produces pectinolytic enzymes are grown at acidic and neutral pH. Although pectinolysis at the thermal and alkaline conditions are rare, it is great useful for extensive applications in enzymatic polishing and biopreparation of cotton fabrics [20–22], also to remedy the retention problems in mechanical bleaching of the pulp[7]. Any way using of traditional methods in treatment and processing of fibers are inefficient to remove the gummy material as well as it causes environmental pollution. Thus treatment with the pectinase in alkaline solution is the overcoming of these problems [23].
There are many researches focused on bacterial alkaline pectinases, but most of the enzymes reported in these researches were found to be limited in the short half-lives as well as their thermostability. Thermo-alki stable pectinases are produced by many bacterial genera such as *Bacillus* [24,25], *Xanthomonas* [26], *Aspergillus*[27], *Alkaliflexus* [28], *Natronoflexus* [29], *Natranaerovirga* [30] and *Paenibacillus* [31,32]. Production of pectinases with high thermo-stability and long half-life are promising factors for the practical applications of such enzymes. In the present study, a thermophilic bacterial strain isolated from soil and identified as *Paenibacillus sp.* strain NBR-10, was found to produce alkaline thermo-stable pectinase. This study investigated the physicochemical properties of pectinase produced from the locally isolated strain.

MATERIALS AND METHODS

Sampling

Seven samples included; four fruit peel wastes collected from pectin rich sources (spoiled fruits and rotten vegetables) at Rafha local market in addition to three sandy soils collected from the upper 5-15 cm depth around wild plants at desert of Rafha governorate at the Northern Border region in Kingdom of Saudi Arabia. The collected samples were taken in clean plastic bags, till further proceedings, samples were stored in the refrigerator at 4 °C.

Selective isolation of pectinase-producing bacteria

Samples (1.0 g) were added in 100 ml of 0.9% saline as a diluent and made 10 fold serial dilutions [33]. 100 µl from each dilution were plated onto pectin agar plates [34] contained (% w/v): pectin (Sigma) 1.0, yeast extract 0.5, peptone 0.5, K_2HPO_4 0.1, $MgSO_4.7H_2O$ 0.02, $NaCO_3$ 0.6, the initial pH was adjusted to 10.0. The inoculated plates were incubated for 2–3 days at 55 °C, the grown colonies were selected and purified. The obtained pure bacterial cultures were kept on nutrient slants at 4 °C, and stored in glycerol at −20 °C until use.

Qualitative screening for thermo-alkali pectinase producing bacteria

The obtained pure bacterial isolates were allowed to grow on sterilized pectin agar medium [35]. Medium was composed of (% w/v): 1% of citrus pectin (Sigma), $(NH_4)_2.SO_4$0.14, $KH_2PO_4$0.2, K_2HPO_4 0.6, $MgSO_4.7H_2O$ 0.01 and agar 2.0, pH was adjusted at 10.0. The inoculated plates were incubated at 55 °C for 72 h. Appearance of clear zone around the grown colony was visualized using solution of potassium–iodide [36]. The isolates with thermo-alkaline pectinase activity were selected based on the formed clear zones.

Identification of the most potent pectinase-producing isolate

The selected bacterial isolate NBR – 10 was identified by partial sequencing of 16S rRNA gene.

Pectinase production in submerged fermentation conditions

Inoculum preparation

A loopful of the bacterial strain NBR – 10, showing the maximum clear zone of pectin hydrolysis was inoculated on liquid nutrient medium contained (% w/v): 0.3 yeast extract, 1.5 peptone, 0.2 glucose, 0.2 NaCl, 0.12 K_2HPO_4, 0.3 Na_2CO_3, pH 10. 250-ml Erlenmeyer flasks contained 60 ml of this medium were inoculated and incubated at 50 °C on a rotary shaker at 150 rpm for 24 h.

Production of pectinase enzyme

Production of pectinase enzyme was carried out under submerged fermentation (SmF) condition in 250-ml Erlenmeyer flasks, each flask contains 90 ml of the fermentation medium (the same medium used in qualitative screening without agar-agar) then inoculated with 10 ml of a 24h-old of the prepared inoculum. Fermentation was carried out under a shaking condition at 150 rpm, 55 °C for 48 h. The cell free supernatant containing the crud pectinase was obtained by filtering using Whatman No. 1 paper. Extraction of the pectinase was achieved by adding 100 ml of $Na_2CO_3/NaHCO_3$ buffer (pH 10.5) for 30 min, to remove the cells and debris, the solution was centrifuged for 20 min at 10,000 rpm [34]. The collected supernatant was designated as the crude enzyme which then purified and used for enzyme assay and characterization.

Purification of pectinase

For obtaining the pure pectinase, the filtrate (cell-free supernatant) was mixed with three volumes of ice cold acetone then allowed to stand in ice cold condition for15 min [37]. The entire solution contents were centrifuged for 20 minat 4000 rpm, the supernatant was discarded and the obtained precipitate was dissolved in small volume of sodium acetate buffer (pH 4.2) then applied to Sephadex G-75 column equilibrated with phosphate buffer (pH 7) as an elution solvent in one liter volume with flow rate 0.5 mL/min. Elute of volume 5 mL/fraction were collected and the enzyme activity was assayed by measurement of absorbance at 280 nm [38]. The active fractions were collected and dialysed using the same buffer.

Enzyme assay

The obtained pure enzyme was subjected for assay of its activity according to the method of Miller[39]. Briefly, 1 ml of the pure enzyme mixed with equal volume of 2% pectin (Sigma) in aqueous solution. The mixture was incubated for 15 min at 55 °C, 2 ml of Dinitrosalicylic acid reagent were added to the mixture and boiled for 5 min, after cooling the absorbance was read at 540 nm against a blank (sample with pure water instead of the substrate). By using galacturonic acid as standard, one unit of pectinase activity is equal to 1 µmol of galacturonic acid released per min under the assay conditions.

Protein estimation

Estimation of the total soluble protein was carried out by the method of Bradford [40] using the standard of bovine serum albumin. The specific pectinase activity was recorded as units per mg protein.

Physicochemical characterization of the purified pectinase

Effect of different temperature degrees on enzyme activity

The influence of temperature on pectinase activity was investigated by incubating the enzyme and the substrate at different temperatures ranged from 20 to 70 °C, at pH value 10.0. Assay of the activity was carried out for 10 min according to the procedure described above.

Effect of different pH values on enzyme activity

The pectinase enzyme mixed with 1% of citrus pectin (Sigma) was subjected for different pH values ranged from 4 to 10.5 and incubated at 60 °C for 10 min[41]. The buffers used for adjusting pH were acetate (pH 3.0–5.0), citrate–phosphate (pH 5.0–7.0), Tris–HCl (pH 7.0–8.5) and glycine–NaOH (pH 8.5–11.0).

Thermostability

To investigate the thermal stability of pectinase, the enzymes solution was incubated for 1 h, in the absence of the substrate at wide range of temperatures from 30 to 70 °C. The remaining enzyme activity was measured at the optimum conditions of pH and temperature.

Alkaline stability

The alkaline stability of the produced pectinase enzyme was tested by mixing the crude enzyme with 0.1 M of different pH buffers; sodium acetate of pH (3.0 – 5.0), citrate–phosphate of pH (5.0 – 7.0), Tris–HCl of pH (7.0 – 8.5) and glycine–NaOH of pH(8.5 – 11.0), with final proportion of the mixture 1:1 v/v. The mixtures were incubated at 25°C for 24 h in the absence of the substrate, then the remaining enzyme activity was measured at the optimum conditions of both temperature and pH.

Pectin concentration and pectinase activity

To detect the best pectin concentration for pectinase activity, different concentrations 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 & 4.0 % pectin were tested individually at the optimum temperature and pH values.

Incubation time and pectinase activity

To study the influence of incubation time on pectinase activity, 1 ml enzyme fixed at 60 °C was added to 1 ml of pectin substrate (3.0 %) dissolved in sodium acetate buffer of pH 4.2 for 10 min. Assay of the enzyme activity was performed for 1.5 h at the interval of 10 min.

RESULTS AND DISCUSSION

Microbial pectinase are one of the extremely valuable enzymes, which have captivated much attention. This can be seen from the increased demand for these enzymes by many industrial sectors[42]. Exploring of microbial strains with improved pectinolytic activities is important to covering such demands [43].

Screening for thermo-alkali pectinolytic bacteria

Isolation of bacterial strains capable of producing pectinase with a novel properties such as stability for a long time at high range of temperature and pH, have been the focus of recent research. In this study, an attempt has been made to characterize alkali-thermo stable pectinase enzyme produced by the soil-isolated bacterial strain. Twenty-one bacterial cultures were isolated from different samples of pectin rich sources collected from different localities from governorate of Rafha at the Northern Border region in Kingdom of Saudi Arabia. The obtained bacterial isolates were screened qualitatively for pectinase production under alkaline (pH 10) and thermal (55°C) conditions using pectin agar medium activity. The qualitative assay of pectinase activity has been used by researchers via spotting the isolates grown on pectin plates and then analyzing the zone of pectin digestion [44].

Out of 21 isolates, 4(19.0%) isolates were found to be pectinase positive through appearance of clear zone on the pectin agar medium. Among them, strain NBR – 10 which isolated from soil sample from rhizosphere of wild plants, being the highest pectinase producer (Fig. 1). This isolate was selected for production of the enzyme in the fermentation medium. Presence of the hydrolytic enzymes in the soil rhizosphere is included in organic residues decomposition, recycling of nutrients, maintaining soil fertility as well as productivity of plants. Extracellular enzymes released by rhizospheric microrganisms have initial role in degradation of high molecular weight polymers such as pectin, cellulose, chitin and lignin [45].

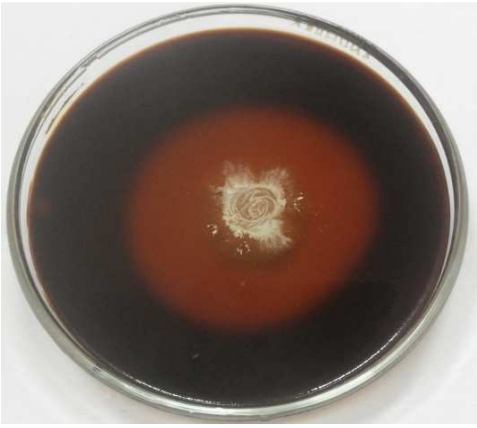

Figure 1. Qualitative screening of the most potent, *Paenibacillussp* strain NBR – 10for pectinolytic activity on pectin agar medium.

Characterization of selected pectinolytic strain

The highest pectinase producing strain NBR – 10was identified by the phylogentic analysis (data not shown) of 16S rRNA sequencing (450 bp). It was found that, the isolate under study have 99% similarity to *Paenibacillus sp.* and designated as *Paenibacillus sp.* strain NBR – 10. The sequence was submitted to the Gen Bankunder accession number KT957624.1. Many authors reported isolation of bacterial strains from soils with pectinolytic activity and identified as a *Paenibacillus* [31,32,46-48].

Pectinase production and purification

For the purpose of pectinase production, *Paenibacillus sp.* strain NBR – 10 was allowed to grow in submerged fermentation conditions using pectin liquid medium. After incubation for 48 h the crude enzyme was harvested and extracted. Results of purification of the crude enzyme (Table 1) showed that, purification using cold acetone resulted in obtaining 6.2-fold with12.42 U/mg specific activity. While purification using Sephadex resulted in obtaining 97.65-folds with 187.66 U/mg specific activity. The activity of the pure pectinase enzyme obtained in our study, to some extent similar to activity of pectinase obtained from *Paenibacillusamylolyticus*[49] and *Paenibacilluslactis* NRC1 [32], but it differs from pectinase produced by *Paenibacilluspolymyxa* Z6 [48].

Table 1. Overall scheme of purification and activity of pectinase from *Paenibacillussp* strain NBR – 10

Phase	Volume (ml)	Total activity (U)	Protein Total (mg)	Specific activity (U/mg)	Yield (%)	Purification fold (x)
Crude extract	1000	3730	2556	1.721	100	1
Acetone ppt.	8	76.53	8.399	12.42	2.240	6.25
Sephadex G-75	25	139.17	1.1	187.66	3.730	97.65

Physicochemical properties of pectinase enzyme

Effect of different temperatures on pectinase activity

The incubation temperature incredibly influences microbial development rate, catalyst secretion, catalyst restraint, also protein denaturation [50]. The obtained pectinase showed high activity at a broad range of temperature 20 – 65 °C with optimum activity (3.2 U/ml)at 60 °C. A strong decrease in the pectinase activity (2.6 U/ml) was recorded with increasing of temperature above 60 °C. These results ensure the thermo-stability nature of the pectinase enzyme by *Paenibacillus sp.* strain NBR – 10 (Fig. 2). Similar activity was recorded at temperature 60 – 65 °C for pectinase produced by *Paenibacillus sp.* BP-23 [31].

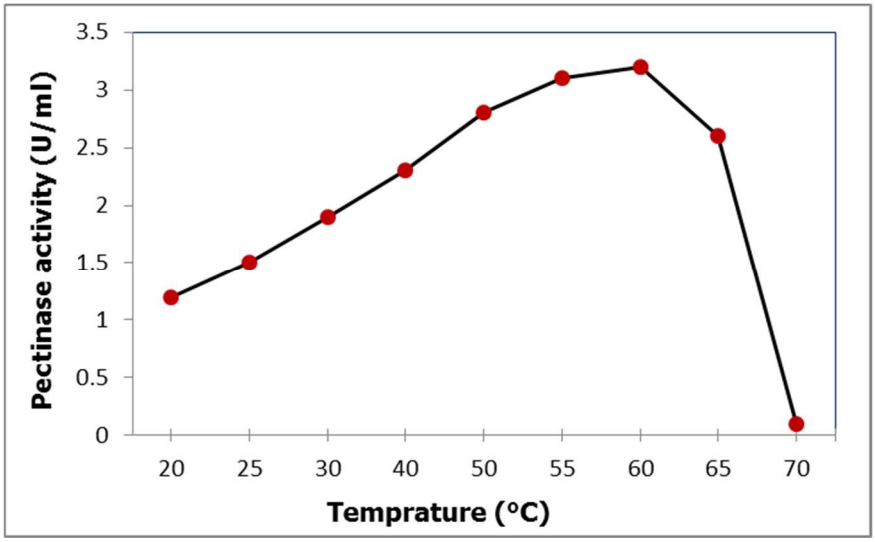

Figure 2. Effect of temperature on pectinase production

Effect of different pH values on pectinase activity

pH is one of the most restrictive factors that highly influence the enzyme activity. 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 [50]. In this study, the obtained pectinase enzyme showed high activity at pH range 5–10.5 with maximum activity (3.1 U/ml) at pH 9.5. Thus we can indicate that, the pectinase enzyme from *Paenibacillus sp.* strain NBR – 10havingalkaline nature (Fig. 3). The alkaline nature of our pectinase is the same of pectinase produced by *Paenibacillusxylanolyticus*, *Paenibacillus sp.* 0602 and *Paenibacillus sp.* BP-23which showed optimum activity at pH values 9.0, 9.8 & 10.0 respectively [31,46,51].

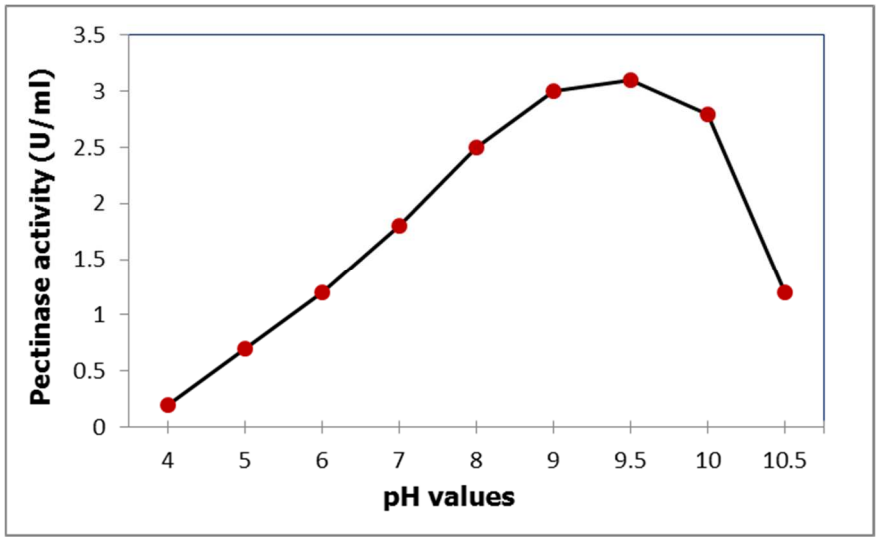

Figure 3. Effect of pH on pectinase activity

Effect of pectin concentration on activity of pectinase

The optimum pectinase enzyme (3.3 U/ml) was obtained at 3.0 % pectin. No significant effect on pectinase activity was observed with increasing in the substrate concentration (Fig. 4). The obtained pectinase activity are in agreement with activity of *Paenibacilluspolymyxa* pectinase which showed that the maximum activity in presence of 3.0 % pectin [52].

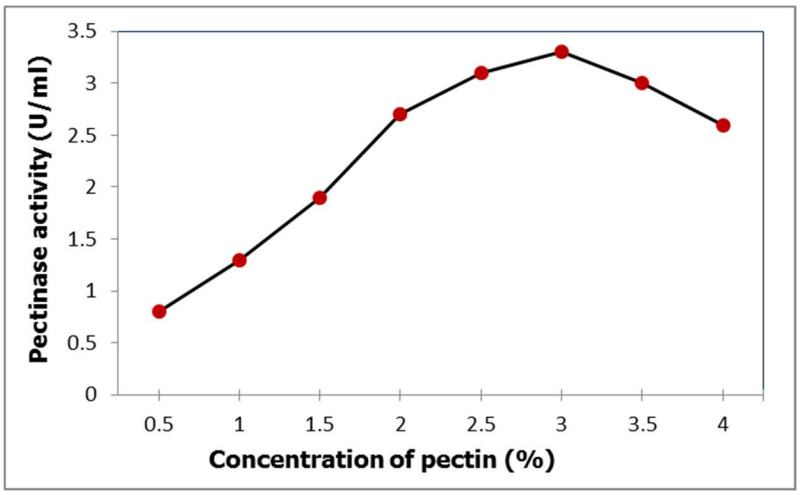

Figure 4. Effect of pectin concentration on pectinase production

Thermo-stability of pectinase

The produced pectinase enzyme was found to be stable in the temperature range 30 – 60 °C with 92% of its activity when incubated in the absence of the substrate. But it showed slight decrease in its activity with the increasing in temperature at 65 °C and retains with 80 % of its activity (Fig. 5) which ensured the thermostablility nature of the obtained enzymes. The reached results are similar to *Paenibacillusxylanolyticus* pectinase which showed thermal stability at temperature between 60 and 70 °C[51].

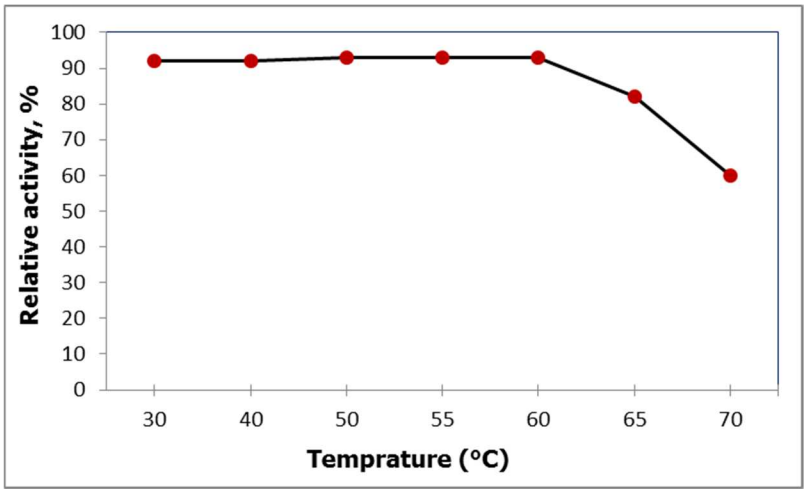

Figure 5. Thermo stability of pectinase

pH stability of pectinase

It was found that, the pectinase enzyme retains93% of its activity when incubated in absence of substrate at pH range 5 – 10. A slight decrease in its activity with the increasing pH at 11 and about 80% of pectinase activity was still retained. These results ensured that, the obtained pectinase is alkali-stable enzyme (Fig. 6). Our results are in agreement with the results of *Paenibacillusxylanolyticus* pectinase which showed stability of pectinase at the optimum pH 9.0 [51]. Also the obtained pH value for optimum pectinase activity is more than pH of pectinase produced by *Paenibacilluspolymyxa N10* which showed optimum pH activity at 8.0 [53].

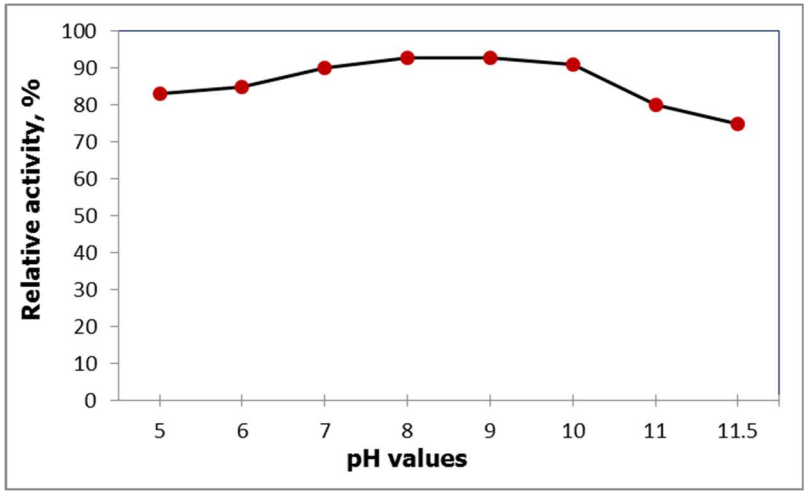

Figure 6. pH stability of pectinase

Effect of incubation time on stability of pectinase activity

No change in pectinase activity was observed when the enzyme incubated for period of time ranged from 15 to 60 mininpH9.5 and 60 °C. Although the enzyme activity decreased when incubation time increased for more than 60 min, 98 % of original pectinase activity was retained.

The obtained results ensured stability of pectinase enzyme produced by *Paenibacillus sp.* strain NBR – 10in thermo-alkali conditions for long time (Fig. 7). Our results were in agreement with the results of *Paenibacilluspolymyxa* pectinase which showed no changes in the enzyme activity after incubation for 60 min at 65°C with original activity reached to 93 % of [52].

The results obtained from this study indicate that, the pectinase enzyme produced by the locally isolated *Paenibacillus sp.* strain NBR – 10 showed high activity at temperatures (20 to 65°C) and pH values (5.0 to 10.5)

with optimum activity at 60 °C and 9.5pHwhich makes it an enzyme with thermostable and alkaline nature. Thermal and alkaline stability are very important characteristics of the enzymes for their use in industrial processes. Thus, this thermo-alkali stable nature of the pectinase enzyme obtained in this study makes it an alternative to chemical pectinases and may find its use in various industries. Many results reviewed the microbial sources of either alkaline pectinase [34,54,55] or thermostable stable pectinase [17,56,57]. However, work on thermo-alkali stable pectinases remains under discovering and few reports are available about such enzyme from the microbial origin specifically from bacteria[58]. Comparing with the results published in these papers, *Paenibacillus sp.* strain NBR – 10 can be considered as a potential thermo-alkali stable pectinase producing strain.

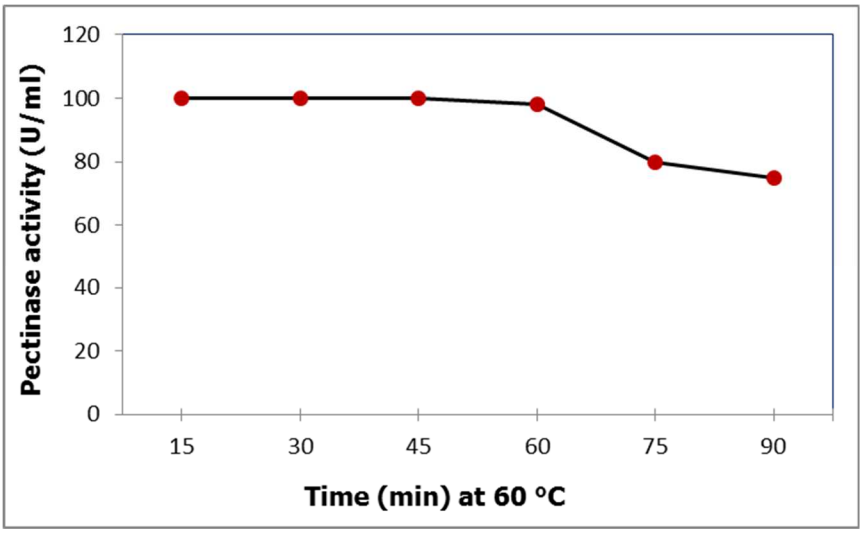

Figure 7. Effect of incubation time on pectinase activity

CONCLUSION

The locally isolated *Paenibacillus sp.* strain NBR – 10 isolated from soil sample collected from governorate of Rafha at Northern Border region in Kingdom of Saudi Arabia was found to produce extracellular thermo-alkali stable pectinase with a promising stability at high temperature and pH values for a long time; candidates this enzyme to be used extensively in various applications.

ACKNOWLEDGEMENT

Mohamed H. El-Sayed and Islam A. Elsehemy gratefully acknowledge the approval and support of this research study by the grant No. 6940-SAR-2017-1-7-F from Deanship of Scientific Research, Northern Border University, Arar, KSA.

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