Influence of Culture Conditions on Pectinase Production by *Streptomyces* sp. (Strain J9)

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***Abstract*- The purpose of this study was to determine the influence of growth conditions and medium composition on the pectinase enzyme production by *Streptomyces sp.* Production of pectinase by a *Streptomyces* strain (J9) was detected on pectin agar medium after 4 days of incubation at 28 ℃ which exhibited a clear zone of 12 mm around the colony. Pectinase production was assayed by measuring the amount of reducing groups (D–galacturonic acid) liberated from the substrate in µmol/ml/min using the dinitrosalicylic acid assay method. The highest crude enzyme relative activity (>80%) was observed after 2 days of incubation at pH 6 and 35 ℃. Strain J9 produced higher amount of crude pectinase when pectin and peptone were used as carbon and nitrogen sources, respectively. Crude pectinase was maximally active at day 4 (1122 U/L) and day 5 (923 U/L) when apple and orange pomaces were used as a sole carbon source, respectively. The findings of this investigation form an avenue for production of pectinase enzyme from *Streptomyces* strain (J9) to be used for commercial fruit clarification and for production of transgenic plants with extended shelf life as pectinase genes cloned from this active strain.**

Keywords- Conditions; Culture; Fruit Pomace; Pectinase; Streptomyces sp.

I. INTRODUCTION

Pectic substances are widely distributed in fruits and vegetables (10-30%). They are found in pulps of tomato, pineapple, orange, apple and lemon. In addition, they are found in turnips and in peels of oranges and other citrus fruits. Hence, such substances form important natural substrates for the pectinase [1, 2, 3]. Therefore, pectinases are mainly used for enhancing filtration efficiency and clarification of certain fruit juices. Furthermore, they are widely used in maceration, liquefaction and extraction of vegetable tissues and have some uses in wood preservation [3, 4].

Pectinase is a generic name for a family of enzymes that catalyze the hydrolysis of glycosidic bonds in pectic polymers. Pectinases are classified based on their mode of action into: polygalacturonase (EC 3.2.1.15), pectin esterase (EC 3.1.1.11), pectin lyase (EC 4.2.2.10) and pectate lyase (EC 4.2.2.2) [5, 6, 2]. The above pectinolytic enzymes act on O-α-(1, 4) polygalacturonopyanoside structures with activities and specificities that depend partly upon the degree of methyl esterefication.

Pectinolytic microorganisms are widely spread in soil. Yeast and filamentous fungi are known to produce pectinases [7, 8]. A wide variety of bacteria such as *Pseudomonas*, *Xanthomonas*, *Erwinia*, and *Bacillus* [9, 10] are known as pectinase enzyme producers. Actinomycetes and particularly streptomycetes are the best known enzyme producers [11]. Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-ll-3 was isolated by Beg et al. [12].

Few studies have been conducted on *Streptomyces* isolated from Jordan soil for their potential to produce enzymes of industrial importance. Rawashdeh et al. [13] isolated several *Streptomyces* isolates that were able to grow on tomato pomace. Upon further characterization, these isolates were able to produce cellulase, pectinase and relatively large amount of xylanase. Tahtamouni et al. [14] isolated indigenous *Streptomyces* isolates that were capable of producing chitinase.

Streptomycetes isolated from Jordanian habitats are poorly studied especially by pectinolytic enzyme producers. Therefore, the present investigation was conducted to isolate soil streptomycetes from different habitats in Jordan and screen them for their ability to utilize pectin as a sole source of carbon. The influence of different culture conditions on production of crude pectinase by the most active *Sterptomyces* isolated in submerged cultures was also studied. In addition, the most active pectinase *Streptomyces* (strain J9) producer was investigated for its potential to degrade apple and orange pomaces.

II. MATERIALS AND METHODS

A. Location, Sampling, Sample Processing and Treatment

Twenty soil samples were collected from 20 different regions in Jordan during fall 2004. Samples were collected by scraping off an approximately 3 cm of surface material with a spatula and taking an approximately 500 g sample at 10 cm

DOI: 10.5963/LSMR0304002

below the surface. Samples were placed in plastic bags, transferred to the laboratory and stored in a refrigerator at 4 ℃. Each soil sample was crushed, thoroughly mixed and sieved through a 2 mm pore size mesh (Retsch, Germany) to get rid of large debris [15]. One g of sieved soil samples were placed in a crucible dish then heated in an oven (Supertek, India) at 45 ℃ for 12 h [16]. After drying, soil samples were mixed with 0.1 g of $CaCO_3$ then incubated at 26 ℃ for 7 days in a water bath (GFL, Germany) [17].

B. Isolation Technique

One gram of pretreated soil samples were suspended in 100 ml sterile distilled water then incubated in an orbital shaker incubator (TEQ, Portugal) at 28 ℃ with shaking at 140 rpm for 30 min. Mixtures were allowed to settle then serial ten-fold dilutions were prepared. 0.1 ml was taken from each dilution and spread evenly over the surface of starch casein nitrate agar (SCNA) [17, 18] plates (in triplicate) with sterile L-shaped glass rod, and incubated at 28 ℃ for 10 days. Cyclohexamide (50 μg/1) was added to the media after autoclaving in order to prevent fungal growth. Plates were incubated for 10 days at 28 ℃ under aerobic conditions in an ordinary incubator (WTB Binder, Germany) [19]. Selected colonies were purified by repeated streaking. *Streptomyces*-like colonies were selected and screened for their ability to produce pectinase enzyme on a specified medium [20].

C. Screening for Pectinase-producing Streptomyces

A seven days old culture of each isolate was suspended in sterile vial containing 3 ml distilled water, to give a spore suspension of 10^7 spores/ml. The pre-inoculum was prepared by inoculating a loopful of each isolate growing on starch casein nitrate broth (25 ml/flask), and incubated at 28 ℃ with shaking at 100 rpm for 7 days. Serial ten-fold dilutions were prepared. 0.1 ml was taken from each dilution and spread evenly over the surface of starch casein nitrate agar (SCNA) [17, 18] plates (in triplicate) with sterile L-shaped glass rod, and incubated at 28 ℃ for 48 hours. Plates were counted and the spore suspension was calculated accordingly by referring to the corresponding flask containing the spore suspension. A drop (0.1 ml) from the suspension was cultured on the center of a pectin agar plate [21]. Pure *Streptomyces* isolates were cultured onto pectin agar plates and incubated for 4 days at 28 ℃. Plates were then flooded with a solution of 0.1% polysaccharide precipitant cetavlon (acetyl trimethyl ammonium bromide) dissolved in a 15% alcohol. After 20-30 minutes of incubation, colonies exhibiting clear zones against an opaque color of the non-hydrolyzed medium were considered pectinase producers and were tested again for confirmation [21, 22].

D. Characterization of the Most Active Streptomyces Isolates

Streptomyces colonies that showed the largest clear zone (>1cm, the distance from the edge of the colony to the rim of the clear zone) were characterized morphologically and physiologically according to the International *Streptomyces* Project (ISP) [23] and as described by Saadoun et al. [15].

E. Pectinolytic Activity Assay

This study focused on the assay of pectinase enzyme for the most active *Streptomyces* isolate. Pectinase assay was performed following the procedure of Miller [24] with some modification. Briefly, a reaction mixture composed of 0.2 ml of crude enzyme solution, plus 1.8 ml of 1.0% (w/v) citrus pectin in 50 mm sodium phosphate buffer (pH 7.0) was incubated at 37 ℃ in a shaker water bath (GFL, Germany) for 30 min. The reaction was terminated by adding 3 ml of DNS reagent. The color was then developed by boiling the mixture for 5 min. Optical densities of samples were measured at 575 nm against a blank containing the reaction mixture minus the crude enzyme. Results were then compared to controls inoculated with an inactive pectinolytic streptomycete isolate. Results were interpreted in terms of enzyme activity in which one unit of enzyme activity (U) was defined as the mount of enzyme releasing one μmol reducing groups (D – galacturonic acid) per min under these assay conditions [24].

F. Optimization of Growth Conditions and Pectinase Production

Erlenmeyer flasks of 250 ml containing 50 ml of pectin broth medium were inoculated with 1 ml of spore suspension of a 7 days old culture. Cultures were incubated in an orbital shaker incubator (TEQ, Portugal) for 7 days. Pectinase activity was then assayed daily by the DNS method as mentioned above.

G. Effects of PH and Temperature on Pectinase Production

The influence of pH on pectinase production was performed after growing the bacteria in pectin broth at different pH values. To adjust the pH of the medium broth, different buffers were used. These include sodium acetate (pH 4), citrate buffer (pH 5 and 6), phosphate buffer (pH 7), tris buffer (pH 8 and 9) at a final concentration of 50 mm. Samples from bacterial cultures growing in these broths were assayed daily for pectinase activity using the standard DNS method [24]. In addition, the effect of temperature on pectinase production was performed after growing the bacteria in pectin broth at different temperatures (15, 25, 28, 30, 35, 37, 40 and 45 ℃) in an orbital incubator shaker. The pH value was adjusted to 7 at the time of inoculation. Enzyme production was assayed on a daily basis following the standard calorimetric DNS assay [24].

DOI: 10.5963/LSMR0304002

H. Effect of Various Carbon and Nitrogen Sources on the Enzyme Production by Streptomyces

Streptomyces were grown in 100 ml of mineral salts medium [$CuSO_4.5H_2O$ (64 mg), $FeSO_4.7H_2O$ (110 mg), $MnCl_2.4H_2O$ (790 mg) and $ZnSO_4.7H_2O$ (150 mg)] supplemented with 0.2% (W/V) of one of the following carbon sources: glucose, arabinose, starch, glycerol, citrus pectin, and CMC (Carboxymethyl cellulose). Different inorganic (KNO_3, NH_4Cl) and organic nitrogen (peptone, asparagines and yeast extract) sources were tested at 0.2% (W/V) concentration. Cultures were incubated at 37℃ for 30 min, and the enzyme production was determined by the standard DNS method [24].

I. Effects of pH and Temperature on Enzyme Activity

This experiment was designed to test the effect of the combination of both temperature and pH on pectinase activity. Buffers (pH 4-9) at 50 mm were used to prepare 1.0% (W/V) citrus pectin. Culture filtrates were used as crude enzyme preparation. Enzyme activities were assayed following standard DNS method [24] at a wide range of temperatures from 4 to 100℃.

J. Pectinase Production in Apple and Orange Pomaces Media

The ability of the active *Streptomyces* sp. (strain J9) to grow in apple and orange broth media was tested. The media was prepared as follows: 1 g of dried fruit pomaces (apple or orange) was suspended in 100 ml distilled water, then supplemented with 0.1 ml trace salt solution composed of 1 ml/L of $FeSO_4.7H_2O$, 0.1 g of $MnCL_2.4\ H_2O$ and 0.1 g of $ZnSO_4.7H_2O$ (20). The pH of the media was adjusted to 7.5 prior to autoclaving. Flasks containing the media were inoculated with 1 ml spore suspension. Cultures were incubated at 28℃ for 6 days. Enzyme activity was performed using the standard DNS method as mentioned before.

III. RESULTS AND DISCUSSION

By employing the enrichment methods, a total of 340 different *Streptomyces* isolates were recovered from 20 soil samples that were collected from different habitats in Jordan. All of these isolates matched the genus description as reported by Shirling and Gottlieb [25], Nonomura [25] and Williams et al. [26].

Data showed that only 102 isolates (30%) were able to produce pectinase enzyme. Pectinase producing isolates were categorized into 3 groups: strong (group 1), moderate (group 2), and weak (group 3) with a clear zone diameter of 10-15 mm, 5-10 mm and 0-5 mm, respectively. Group 1, 2 and 3 represented 2, 13 and 85% of the pectinase active-producing isolates, respectively.

The isolate J9 was chosen as the most active pectin-degrading isolated *Streptomyces*, which exhibited a 12 mm diameter of clear zone on pectin agar (PA) medium (Fig. 1). Morphological and physiological characterization of the most active strain revealed that it belonged to the white colour series with a distinctive reverse side colour (grey), did not produce diffusable and melanin pigments and had a rectiflexible (RF) sporophore arrangement. The isolate was unable to utilize D-xylose and D-fructose. However, it utilizes D-glucose, L-arabinose, sucrose, D-mannitol and raffinose, I-inositol and rhaminose.

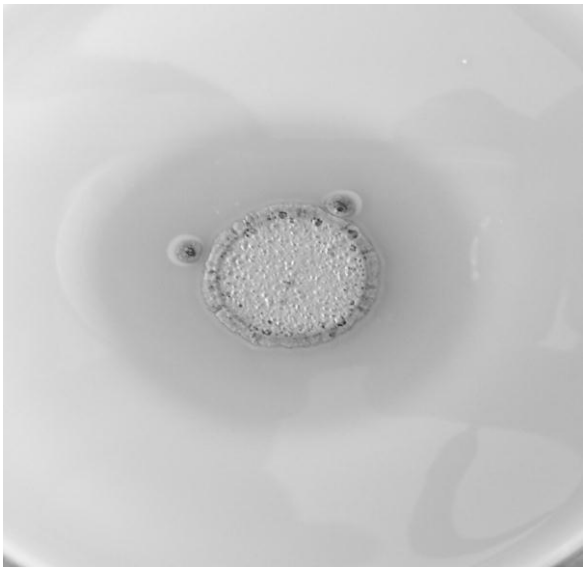

Fig. 1 Pectinolytic activity of *Streptomyces* sp. (strain J9) on pectin agar (PA) indicated by clearing zone surrounding the colony

The optimum pH for enzymes production was determined by using four types of buffers in the range of (4-9) as mentioned above. Fig. 2 shows that the maximal enzyme yield was observed after 2 days at pH 6 and 7 with relative activities of 100%

DOI: 10.5963/LSMR0304002

and 94%, respectively. However, pectinase production was suppressed at pH 4 and 5 with minimal relative activities of 20% and 23%, respectively.

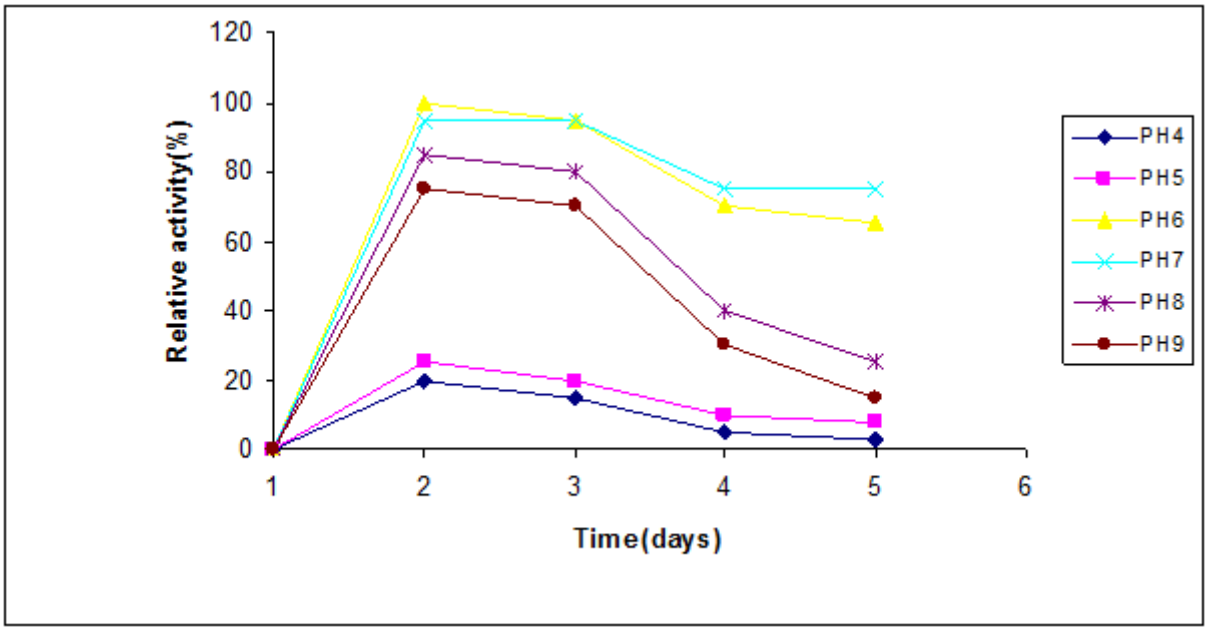

Fig. 2 Determination of optimum pH for pectinase production by the J9 isolate. Enzyme activities at the different conditions are compared to the highest value, considered as 100%

The optimum temperature was relatively similar between 28-37 ℃ (Fig. 3), whereas higher enzyme production was achieved at 35 ℃. However, the relative enzyme activity increased with increasing temperature and minimized at 37 ℃.

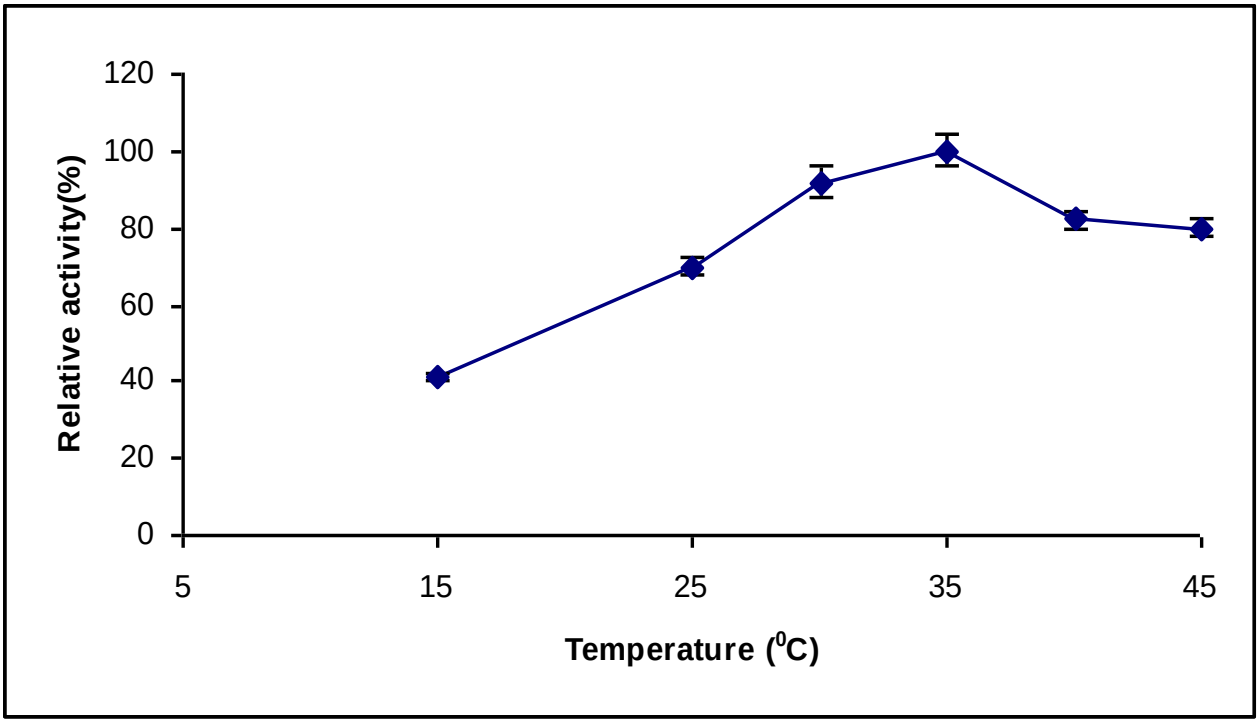

Fig. 3 Determination of the optimum temperature for pectinase production by the J9 isolate

Enzyme activities at the different conditions are compared to the highest value, considered as 100%. Points represent the standard errors for means at α=0.05.

As indicated in Fig. 4, the maximal pectinase yield was obtained when pectin and arabinose were used as a sole carbon source, but when CMC was used as a carbon source the production of enzyme was suppressed with relative activity reached to 60%. This reduction could be attributed to the production of CMCase enzyme, which in turn hydrolyzed CMC to produce cellobiose that has a repression effect on pectinase production [27].

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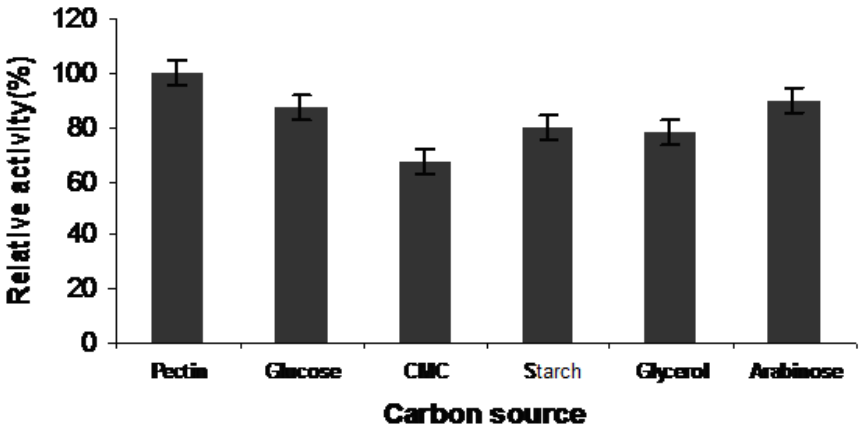


Fig. 4 Effect of different carbon sources on pectinase enzyme production by the J9 isolate.

Enzyme activities at the different carbon sources are compared to the highest value, considered as 100%. Pars represent the standard errors for means at α=0.05.

Synthesis of extracellular enzymes may be inducible (partially or totally) or constitutive, depending on the microorganism and the enzyme. In all cases, it has been found essential to keep the required nutrients at low level to insure maximum accumulation of fermentation products [27]. Overall study indicated that pectinase production from J9 isolate was constitutive in nature.

Al-Rajabi (3) reported a new strain of the genus *Bacillus* produced pectin lyase and pectate lyase constitutively in a medium containing glucose, fructose and pectin as a carbon source. However, maximum production of enzymes was observed when pectin was used as the sole carbon source in the medium. Constitutive polygalacturonate layse production was not repressed by glucose and this is similar to our results as shown in Fig. 4. Similarly, Kuhad et al. [28] reported that a *Streptomyces* isolate (RCK-SC) produced optimum amount of pectinase when pectin plus glucose were used with media (76,000 U/L). The highest level of pectinase production was achieved when peptone, NH_4CL, and yeast extract were used as nitrogen sources with relative activities of 100%, 94% and 91%, respectively, at day 2, which suggests that there is no significant difference between them in the induction of pectinase production. Various amino acids and their analogues like DL-norleucine, L-leucine, DL-isoleucine, L-lysine monohydrochloride and DL-β-phenylalanin were found to stimulate production of xylanase and pectinase from *Streptomyces* sp. QG-11-3 by up to 3.72- and 2.78-fold, respectively, whereas DL-aspartic acid showed no significant stimulatory effect on enzyme production [6]. This study also showed that the lowest level of enzyme production was observed when asparagine was used as a nitrogen source (Fig. 5).

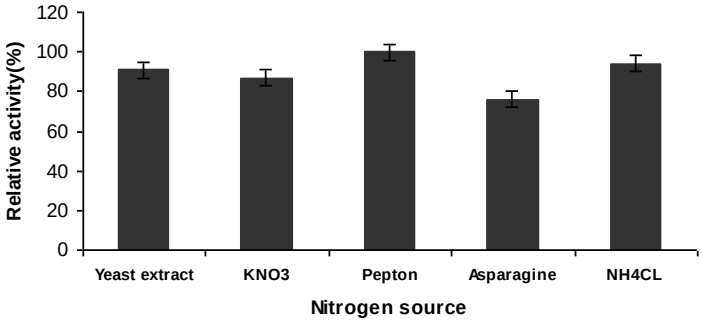
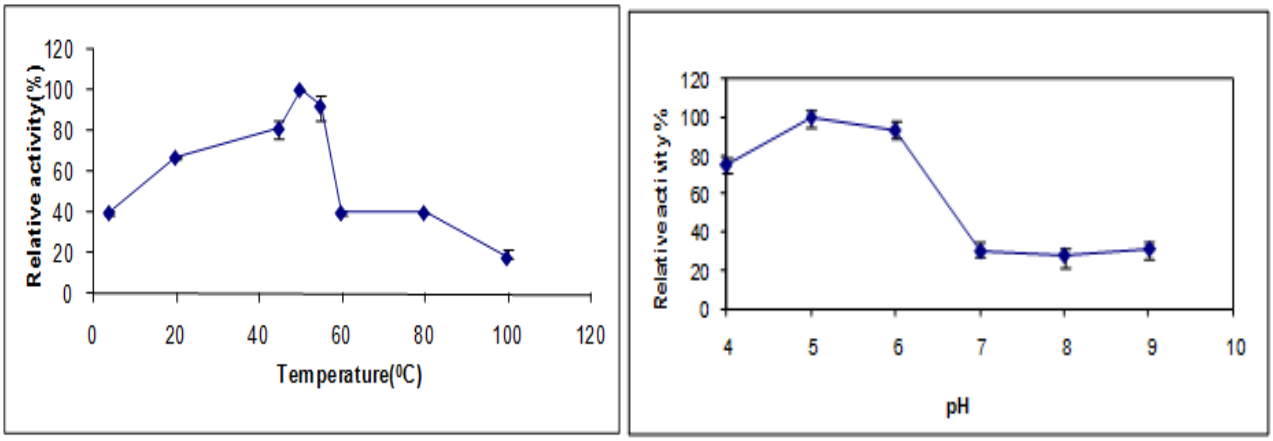


Fig. 5 Effect of different nitrogen sources on pectinase enzyme production by the J9 isolate

Enzyme activities at the different nitrogen sources are compared to the highest value, considered as 100%. Pars represent the standard errors for means at α=0.05.

The temperature and pH profiles were presented in Fig. 6. Results showed that pectinase was active by more than 80% at a temperature that ranged between 35 and 55 ℃, with maximal activity at 50 ℃ (Fig. 6). Nevertheless about 40% of pectinase activity was lost at 60 ℃. The crude enzyme was active over a pH range of 4-7 with maximum activity at pH 5. This result agrees with the finding of Beg et al. [12], who reported a pectinase from *Streptomyces* sp.QG-11-3 with pH optimum at 5. This appears to contrast findings of Kuhad et al. [28], who reported an alkalophilic *Streptomyces* sp. RCK-SC producing a thermostable alkaline pectinase. Variability of pH depends on the type of pectinase enzyme. The two pectolytic enzymes pectin lyase and pectate lase have high optimum pH values at 8.5, while the polygalacturonase is acidic pectolytic enzyme [29]. *Bacillus* sp. DT7 has been found to produce an extracellular pectinase subsequently characterized as pectin lyase (EC4.2.2.10), by optimizing growth conditions, *Bacillus* sp. DT7 produced higher amount of pectin lyase (53,000 U/L) at pH 8 [30].

DOI: 10.5963/LSMR0304002



(a) (b)

Fig. 6 Effect of (a) temperature and (b) pH on pectinase enzyme activities

Enzyme activities at the different conditions are compared to the highest value, considered as 100%.

To evaluate the production of pectinase in media containing fruit pomaces, two different types of fruit pomaces were used as carbon sources. When apple pomace was used, data indicated that the highest pectinase activity (1122 U/L) in the culture filtrate is recorded at day 4 (Fig. 7), while when orange pomace was used, the highest pectinase activity was 923U/L at day 5 (Fig. 7). Level of pectinase produced by the *Streptomyces* (J9) in orange pomace medium was lower than the value obtained in apple pomace medium. However, these values were also lower than the values obtained in synthetic media (pectinase with mineral salts). This could be attributed to the fact that the fruit pomaces contain simple sugars and could result in the reduction or repression of enzyme production. Zheng and Shetty [31] reported the production of polygalacturonase (PG) by *Lentinus edodes* using fruit processing wastes including: apple pomace, cranberry pomace and strawberry pomace. The later pomace was the best substrate for highest PG yield, followed by apple pomace, while cranberry pomace was not a suitable substrate for PG production. PG activity was increased by the addition of polygalacturonic acid in the apple pomace and cranberry pomace media. The highest PG activity was obtained after 40 days of culture and the yields from strawberry pomace, apple pomace and cranberry pomace were 29.4 U, 20.1 U and 14.0 U per gram of pomace, respectively. *Lentinus edodes*, also known as Chinese mushroom, was successfully grown on apple pomace and other lignocellulosic wastes [30]. Furthermore, Hours et al. [4] reported the production of pectinase by *Aspergillus foetidus* from apple pomace fermentation, the highest rate of pectinase production was after 12 h.

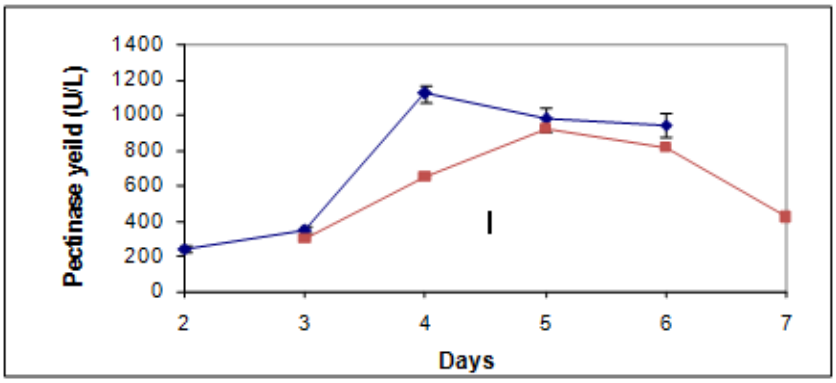


Fig. 7 Production of pectinase enzyme by the J9 isolate in apple (♦) and orange (▪) pomace medium

Points represent the standard errors for means at α=0.05

IV. CONCLUSION

The results of the findings of present study form an avenue for production of pectinase enzyme from *Streptomyces* strain (J9) to be used for commercial fruit clarification and for production of transgenic plants with extended shelf life as pectinase genes cloned from this active strain.

ACKNOWLEDGMENTS

Deanship of Scientific Research at Jordan University of Science and Technology funded this research (Grant No. 167/04).

DOI: 10.5963/LSMR0304002

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