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Polygalacturonase Production by *Sarocladium strictum* T4 Isolate using Apricot Pulp as Substrate in Non-Sterile Culture Conditions

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ABSTRACT

In this study, 34 molds showing pectin degradation activity were isolated from the soil of orchards, by several tests. By using these isolates, pectinase group enzymes; studies on pectin lyase and polygalacturonase production were performed in the batch system and under non-sterile culture conditions. 5 isolates denoting polygalacturonase activity were coded as: T1, T2, T3, T4, T5 and the best polygalacturonase activity among these isolates was determined as 354.4 U/L in T4 isolate. Thus, optimization analyses continued by the use of this isolate. Initial apricot pulp concentration, temperature, pH and incubation period were tested as optimization parameters. The effects on enzyme activities were investigated by changing the initial apricot pulp concentration in the range of 5-100 (g/L), and in this regard, polygalacturonase activity was determined as 397.4 U/L at 50 g/L. In addition, the pH parameter was analyzed in each unit between pH = 3 – 8, and the temperature was tested by increasing 5 units in the range of 5-25°C. Consequently, the maximum polygalacturonase activity was determined as 405.7 U/L at pH 5 and 406.3 U/L at 15°C. Besides, the effect of the incubation period was studied within 1-5 days and the maximum polygalacturonase activity was determined as 429.0 U/L on the 4th day (after 96 hours). As a result, the above-mentioned T4 isolate, with which the optimization studies were conducted, was identified as *Sarocladium strictum* (Top ekinküfü) T4 by molecular methods.

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Introduction

Pectin is a carbohydrate compound from the pectic substance group with colloidal properties and is an additive providing benefits with its gel-forming ability. It is partially soluble in water. It is mostly found in foods, in the structure of vegetables and fruits, among the cells and in cell walls of all plantlets (Wang et al. 2014). It is critical to protect pectic compounds during the drying of fruits and vegetables. The pectin content of apricots is at the level of 1%. There are reports of losses in invert sugars such as galactose and arabinose in the pectin chain and a decrease in cell wall stiffness (Femenia 1998). Having great industrial importance, the enzymes that break down pectic substances are referred to as pectinolytic enzymes or pectinases (Fogarty and Kelly 1983; Saad et al. 2007). Research demonstrated a positive correlation between the polygalacturonase and pectin methyl esterase activity and the onset of softening in fruits such as apricot, papaya and mango (Roe and Bruemmer 1981; Femenia 1998). Pectinases are one of the enzymes that are mostly common in bacteria, fungi and plants. Pectinase production by filamentous fungi varies according to strain, composition of the growth medium and culture conditions (pH, temperature, aeration, shaking and incubation period (Souza et al. 2003). Having a large share in the world enzyme market, pectinase enzymes are often used in breeding flax and vegetable fibers, extraction of essential oils from the peels of vegetables and citrus fruits, pre-treatment of pectic wastewater, fermentation of coffee and tea, producing poultry feed, distilling plant viruses, extracting fruit juice and the decantation process (Hoondal et al. 2002; Saito et al. 2004; Uzuner and Çekmecelioğlu 2016). The pectinase enzyme has a 25% share in the global sales of food enzymes, and this rate is expected to increase over time with the discovery of new areas to use the enzyme (Jayani et al. 2005). This enzyme is most commonly obtained commercially from microbial pathways and especially from molds. Microorganisms are the most suitable organisms for enzyme production and currently represent 90% of the total market (Sanchez and Demain 2011).

The last few years have witnessed prominence in research on enzymes that show activity at low temperatures in cold-adaptive organisms. Among the main reasons for this, it is stated that cold-adaptive enzymes have a higher specific activity compared to mesophilous ones at low and moderate temperatures (Antranikian et al. 2005). A reason why they come to the fore is that these enzymes help achieve significant energy savings in practice (Morita et al. 1998).

Today, by-products of the fruit processing industry pose an important waste problem. There is a growing interest in these wastes, though, for the high nutritional values contained in their waste components and for their recyclability. In addition, these wastes can be used as food additives and supplements. Furthermore, these waste products can be used as a substrate in the prepared media. As a matter of fact, more and more international studies have been focusing on issues such as clean energy generation, waste use and recycling.

This study used the *Sarocladium strictum* (Top ekinküfü) (Güner et al.2020) T_4 isolate and apricot pulp as a substrate for the production of polygalacturonase. To save time, workload and electricity, the study was conducted in a non-sterile medium and under non-sterile culture conditions.

Materials and Methods

Preparing suitable growth medium and isolating active pectinase-producing microorganisms in cold

10-20 different soil samples were taken from the orchards within the borders of Erzurum province. Dilution series of 10^{-1},10^{-2}, $l0^{-3}$ and 10^{-4} were prepared with 0.9% sterile physiological saline (FTS). From each of the samples, 5 g\L Pectin, 1 g\L; KH_2PO_4, 1 g\L; $(NH_4)_2S0_4$, 0.75g\L; $MgSO_4$, 0.015g\L; $FeSO_4$, 0.15g\L; $CaCl_2$, 0.25g\L; NaH_2PO_4, 20 g\L petri dishes were prepared in accordance with the Agar medium. By the help of a sterile 0.1 swab, these were spread in the petri dishes, and left for incubation in the refrigerant at +4°C. Apricot pulp was used as a solid substrate for the development of pectinase-producing microorganisms. Apricot pulp was supplied as a by-product from a fruit juice factory in the Aegean Region and dried for 24 hours in an incubator at 60°C. It was then ground and used in the analyses.

Identifying Pectin Lyase and Polygalacturonase Activities of the Isolates

For the polygalacturonase activity determined by editing the method in the study by Patil et al. (2006); 0.7 mL Acetate Buffer (pH 5.5 and 0.1 M), 2 mL 0.5% pectin solution and 1 ml enzyme sample were incubated at 45°C for 30 minutes. The reducing sugars were determined by the dinitro salicylic acid (DNS) method, which used galacturonic acid as reference. The DNS solution was prepared by mixing 1% DNS, 0.2% phenol, 0.05% sodium sulfite, 1% sodium hydroxide, 30% sodium potassium tartrate and by adding pure water to reach the amount of 100 ml. The mixture was incubated at 45°C for 30 minutes, and then 0.1 mL was taken from it. Later, 1.9 mL distilled water and 2 mL DNS were added to it. The mixture was kept in a boiling water bath at 90°C for 20 minutes and the final volume was completed to 15 ml. After cooling, the absorbance was read against the blind solution at a wavelength of 550 nm. The amounts of galacturonic acid were calculated using a calibration curve. Figure 1 shows the calibration graph obtained by reading the absorbance of galacturonic acid solutions prepared at certain concentrations from a galacturonic acid solution of 50 mmol/L (Tepe, 2012) at 550 nm.

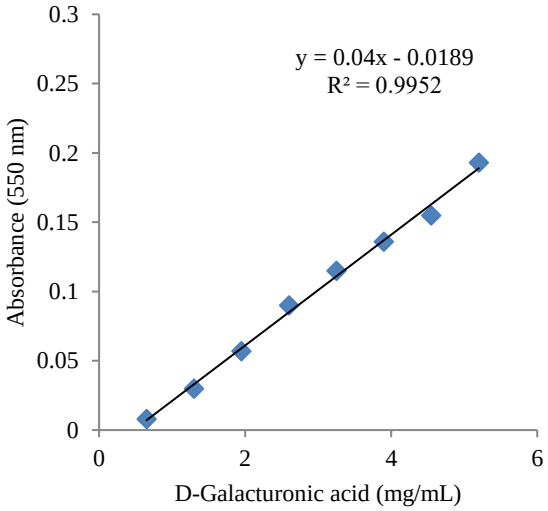

Figure 1. D-galacturonic acid standard curve graph

Calculation of Enzyme Activity

By applying the absorption values at 550 nm to the d-galacturonic acid regression equation, the amount of d-galacturonic acid released by the enzyme in one minute was determined in terms of µmol/minute (Debinga et al. 2005). One unit of enzyme activity is the amount of enzyme that forms 1µmol of D-galacturonic acid from pectin in one minute under standard conditions. The amount of reducing sugar found using the standard D-galacturonic acid curve was calculated by adding it to the equation given below (U/mL):

Total Unit = mg/ml reducing sugar × 100/t × MG

mg/mL: The amount of D-galacturonic acid corresponding to the absorbance of the samples
t : Incubation period (min)
MG : Molecular weight of the released D-galacturonic acid

The fungal biomass was found by measuring the amount of N-acetyl glucosamine released by acid hydrolysis from the fungal kit in the solid fraction (Nimnoi and Lumyong 2011; Velmurugan et al. 2011). For this, a sample (10mk) was taken from the culture and centrifuged at 5000 rpm for 10 minutes. Then, 1 ml concentrated H2S04 (sulfuric acid), and acetylacetone reagent (1 mL) were added respectively to the resulting 0.5g solid fraction. This mixture was incubated in a boiling water bath for 20 minutes. Later, it was cooled at room temperature. Followed by this step, 6 mL Ethanol and 1 mL Ehrlich reagent were added to the mixture. After this mixture was incubated at 65°C for 10 minutes and cooled again, its optical density was found as 530 nm. N-acetyl glucosamine (Sigma-Aldrich) was used as standard.

The cell biomass (g/L) was calculated using the formula indicated below:

Cell biomass (g/L) = (N-acetyl glucosamine (g/L).100)/8.3

The degree of contamination that was likely to occur in the culture medium during the experiments were shown according to microscopic examinations as follows: no contamination was indicated by (-), low contamination by (+), moderate contamination by (++) and high contamination by (+++). The conditions under which less or no contamination was observed and maximum enzyme activity was achieved were identified and used in the next step.

Results and Discussion

Significant polygalacturonase activity was obtained in only 5 of the 34 isolates tested in the study. Erzurum Technical University's Department of Molecular Biology and Genetics made the molecular diagnosis of the T4 isolate, which showed the best polygalacturonase activity among all the isolates used in the study, and identified it as *Sarocladium strictum* T4.

Optimization of polygalacturonase activity with the T4 isolate

The effect of substrate amount on enzyme activity

Polygalacturonase activities in different concentrations of apricot pulp used instead of pectin were identified according to absorbance values of 550 nm after 72 hours at 15°C, pH 6 and 170 rpm mixing speed. As the initial substrate concentration increased, enzyme production increased, and at the point where the number of enzyme molecules was low relative to substrate molecules, the increase in substrate concentration with the formation of an enzyme-substrate complex did not affect the speed. No substrate inhibition was observed in the analyzed range. The maximum polygalacturonase activity was found as 397.4 U/L at 50 g/L and was used as the initial substrate amount in subsequent experiments. The results manifest that the presence of pectic substances in the fermentation medium affects enzyme production. Pectin does not cause inhibition on microorganism reproduction or enzyme activity. In the same vein, Abbasi and Fazaelipoor (2010) found that 50 g/L pectin concentration does not cause inhibition on polygalacturonase activity.

The effect of pH on enzyme activity

The activity of the polygalacturonase enzyme between pH 3-8 at every other unit was found according to the absorbance values of 50g/L substrate (apricot pulp) at 15°C and 170 rpm mixing speed, at 550 nm after 72 hours, and the optimum pH, at which it was active, was determined to be 5 and the enzyme activity was 405.7 U/L. It is also stated in the literature that the optimum pH for the reproduction of most fungi and the production of pectinase group enzymes varies in the 5.0-7.0 range.

The effect of temperature on enzyme activity

Under standard test conditions with 5-25°C temperature and fixing at PH 5, the effect of temperature on pectinase activity was tested by 50g/L substrate (apricot pulp) and mixing at a speed of 170 rpm tested. According to the absorbance values at 550 nm after 72 hours, the maximum PG activity was reached at 15°C and was determined as 406.3 U/L. The fact that *Sarocladium strictum* also showed polygalacturonase activity at low temperature indicates that the enzyme is cold-adaptive.

Table 1. Polygalacturonase activity of isolates (Duncan test)

Isolates	PG Activity (U/L) Biomass (g/L)
T1	301.0^{b} 6.590^{c}
T2	284.4^{c} 6.867^{b}
T3	85.20^{e} 4.373^{e}
T4	354.4^{a} 7.349^{a}
T5	188.0^{d} 5.181^{d}

*The difference between the averages denoted with the same letters in the same column is not statistically significant (P<0.05).

Table 2. The effect of the initial substrate concentration on PG activity (Duncan test)

Initial Substrate Concentration (Apricot pulp) (g/l)	PG Activity Biomass Contamination (U/L) (g/L)
5	350.0^{d} 7.351^{h} ++
10	360.8^{cd} 7.599^{g} ++
20	367.3^{bcd} 7.768^{f} ++
30	378.7^{bcd} 7.926^{e} ++
40	386.4^{bc} 8.168^{d} ++
50	397.4^{ab} 8.314^{a} ++
60	396.4^{ab} 8.283^{b} ++
70	395.7^{ab} 8.273^{b} ++
80	395.1^{ab} 8.241^{c} ++
90	394.9^{ab} 8.274^{b} ++
100	394.7^{ab} 8.276^{b} ++

*The difference between the averages denoted with the same letters in the same column is not statistically significant (P<0.05).

Table 3. The effect of pH on enzyme activity (Duncan test).

pH	PG Activity Biomass Contamination (U/L) (g/L)
3	17.00^{f} 1.349^{e} -
4	108.0^{e} 2.636^{d} -
5	405.7^{a} 8.331^{a} -
6	397.0^{b} 8.302^{a} ++
7	297.9^{c} 6.987^{b} +++
8	183.1^{d} 3.899^{c} +++

*The difference between the averages denoted with the same letters in the same column is not statistically significant (P<0.05).

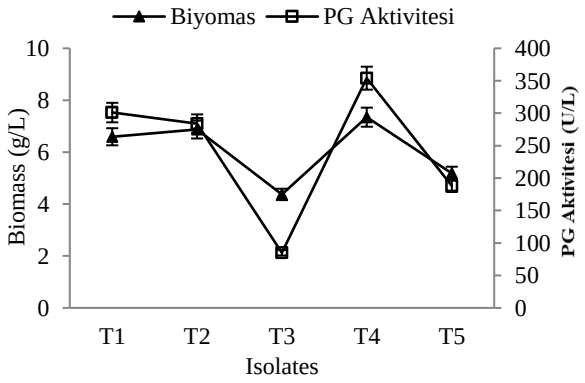

Figure 2. The polygalacturonase activity of the isolates used (Culture conditions: PG activity after 72 hours at 15°C, 5g/L KP, pH 6 and 170 rpm shaking incubator)

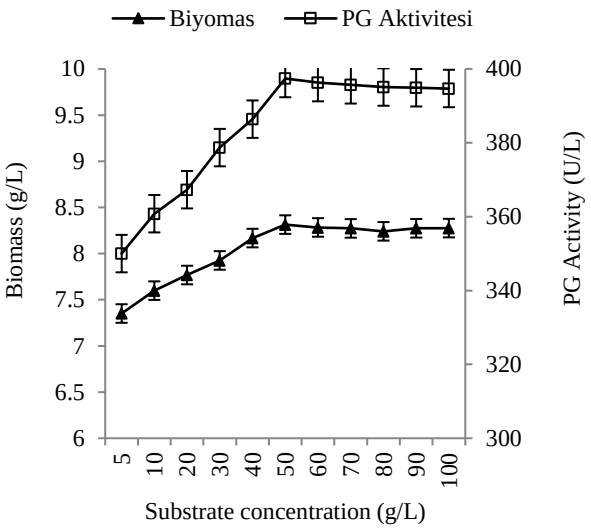

Figure 3. The effect of substrate amount on enzyme activity

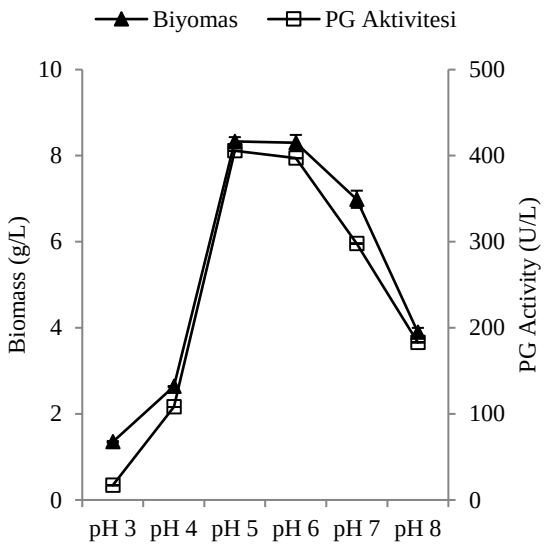

Figure 4. The effect of pH on enzyme activity

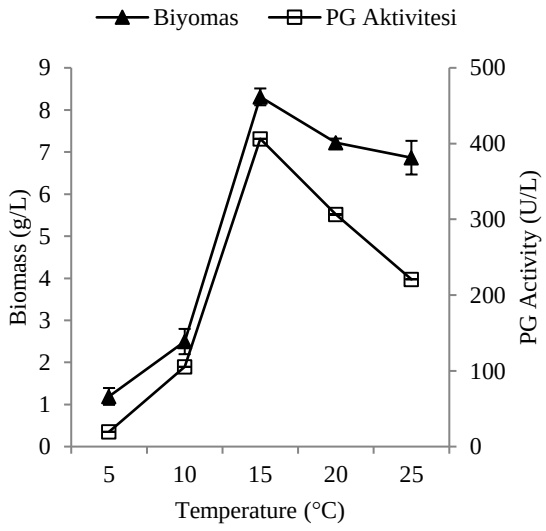

Figure 5. The effect of temperature on enzyme activity

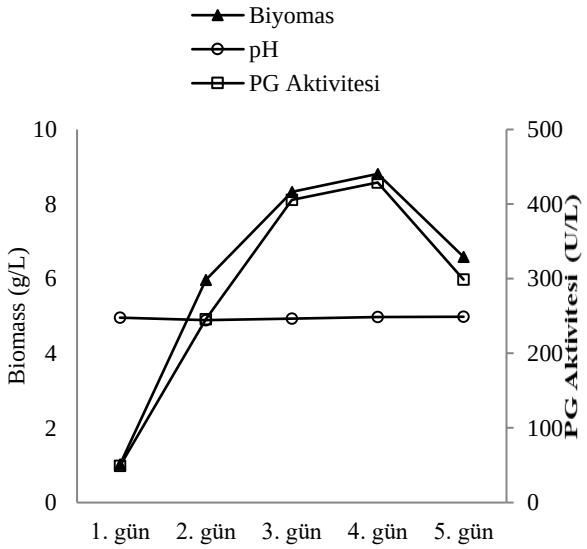

Figure 6. The effect of incubation period on enzyme activity

Table 4. The effect of temperature on enzyme activity**

Temperature (°C)	PG Activity Biomass Contamination (U/L) (g/L)
5	19.50^{e} 1.192^{e} -
10	105.3^{d} 2.496^{d} -
15	406.3^{a} 8.310^{a} -
20	306.4^{b} 7.219^{b} -
25	220.9^{c} 6.865^{c} -

*The difference between the averages denoted with the same letters in the same column is not statistically significant (P<0.05). **(Duncan test)

Table 5. The effect of incubation period on enzyme activity**

Incubation period (hour)	PG Activity Biomass Contamination (U/L) (g/L)
24	49.20^{e} 1.024^{e} -
48	245.7^{d} 5.970^{d} -
72	405.5^{b} 8.332^{b} -
96	429.0^{a} 8.810^{a} -
120	298.7^{c} 6.582^{c} -

*The difference between the averages denoted with the same letters in the same column is not statistically significant (P<0.05). **(Duncan test)

Considering this property, it can be said that it has a high potential to be used in especially the detergent industry where low temperature application is targeted.

The effect of incubation period on enzyme activity

To determine the incubation period that the enzyme can show maximum activity, the activities of how 50g/L substrate (apricot pulp) were evaluated for five days at 15°C, pH 5 and at a mixing speed of 170 rpm at 550 nm absorbance values. The maximum polygalacturonase activity was found as 429,0 U/L on the 4th day (after 96 hours). After this time, a decrease in PG activity was observed in the media. In addition, the ambient pH was checked during this 5-day period, and no significant changes were observed between the measured pH values.

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