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Evaluation of pectinesterase enzyme extracted from Date Palm fruits cv. Zaghloul

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Abstract

The idea of this research is to maximize benefits of ripe date palm fruits (cv. Zaghloul) by using it as a source of pectin esterase which is of prime importance for food industry. Pectin esterase was extracted using sodium chloride and its specific activity was determined. The crude pectin esterase specific activity was17849.52Umg^{-1} protein. Partial purification of the crude enzyme took place with acetone and ethanol to determine the enzyme optimum temperature and pH. The used purification technique resulted in about2.5-fold purification with 20% acetone resulting in 4127.70Umg^{-1} protein specific activity and16.64% recovery. The same purification fold was obtained with ethanol resulting in4135.44 Umg^{-1} protein specific activity and 5% recovery. The optimum temperature was 40°C and optimum pH was 8 for the partially purified enzyme. It can be concluded that the quality of pectiesterase enzyme extracted from ripe Zaghloul dates is comparable with that extracted from apple and orange and has specific activity less than apple (30848.90 Umg^{-1} protein) and more than orange (12736.00 Umg^{-1} protein).

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Introduction

Date palm fruits passes through five developmental stages until maturity. These stages are Hababouk, Kimri, Khalal, Rutab, and Tamar. Each stage is characterized by distinct physical and chemical changes. Fruits of Zaghloul cultivar, the target of this study, are freshly consumed at the Khalal stage. The post-harvest shelf life of many of fruits and vegetables is very limited due to their nature which mostly tends to spoilage resulting from many factors (Kumar, 2015). And due to the high production potential of dates worldwide, it may not always be possible to locally consume all the freshly-harvested produce or export it. New opportunities has been opened to turn the surplus production into value-added products such as syrup, juice anda number of bioactive compounds that can be extracted from some of the by-products. Theadded industrial value could compensate for the economic loss either from fruits' under-grading or its deterioration (El Hadrami *et al*., 2012).

Enzymes are globular proteins acting alone or in larger complexes. Over half of the amount of the enzymes that are used industrially being produced from fungi and yeast, over a third are from bacteria with the remainder amount divided between animal and plant sources. Plant and animal tissues contain more potentially harmful materials than microbes, including the phenolic compounds from plants and endogenous enzyme inhibitors. Attempts are being made to overcome some of these difficulties by the use of animal and plant cell culture.

Pectin is a natural renewable polysaccharide characterized as an emulsifier and/or thickener in commercial applications. Its consumption has proved to reduce blood cholesterol levels by liberating short-chain fatty acids under the effect of microorganisms in the large intestine and colon. On this basis, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recommended pectin as a safe additive with no limit on acceptable daily intake (Tiwari *et al*., 2011). Pectinases are (EC, 3.1.1.11)from the important industrial enzymes that are widely used to break down pectin polysaccharide found in plant cell walls and speed up fruit juice extraction from puree` and improve the final product quality (Sáenz *et al*., 2000). It is particularly used in extraction and increasing the yield of fruit and vegetable juices, controlling cloud stability in juices and extraction of pigments and food colorings (Duvetter *et al.* 2009). This enzyme is widely distributed in higher plants and mainly found in different fruits. It has been purified and characterized from several fruit sources including tomato, orange, papaya, apple, kiwi, grapefruit and mandarin (Julio Montañez Sáenz *et al.* 2000).Several factors affect the rate at which enzymatic reactions proceeds; temperature, pH, enzyme concentration, substrate concentration, and the presence of any inhibitors or activators. Enzyme's activity decreases significantly outside its optimal temperature and pH. The rate of most enzyme-catalyzed reaction increases with the increase in temperature, on the other hand many enzymes are adversely affected by high temperatures, the reaction rate increases with temperature to a maximum level and suddenly decrease with further increase of temperature (Worthington enzyme manual, 1988). The optimum activity for commercial pectinase is between 45 – 55°C. Also extremely high or low pH values generally result in complete loss of activity for most enzymes. The optimum pH varies from one enzyme to another and for pectinase it falls between 3.0 and 6.5 (Dhembare *et al*, 2015).

Due to the importance of date palm as an economic crop and the importance of pectinestearse (PE) role in the food processing industry lies the overall objective of this study in which the activity of PEenzyme extracted from date palm fruits cv. Zaghloul was evaluated and compared to that extracted from other fruit sources as a trial to maximize the benefits of Zaghloul fruits at the beginning of the Rutab stage which is not suitable to be consumed as edible.

Materials and methods

Samples

Apple (cv. Delicious), orange (cv. Navel or late maturing), dates (cv. Zaghloul) and unpollinated date

(without pollination) fruits were purchased from the local market. The Zaghloul sample was at the beginning of the Rutab stage. All samples were stored at -20°C two days before analysis and used frozen.

Chemicals

All used reagents and solvents were analytical grade. Potassium phosphate monobasic, bovine serum albumin and bromothymol blue were products of Sigma Aldrich, potassium phosphate dibasic of Alliance Bio, sodium chloride of Alpha, EDTA of Riedel-deHaën, hydrochloric acid of Fischer, c ommassie of Acros and pectin was a product of Roth.

Equipment

Cooling centrifuge, Heraeus 18000 RPM UV/V spectrophotometer, Thermo spectronic (He□iosγ)

Enzyme extraction

Pectinesterase (PE) was extracted according to the method of Körner B. *et al*, (1980). The pulp of fruit was separated carefully from the peel and ground with 0.25M NaCl solution (1g with 5ml extracting agent) using a mortar and pestle for 3 min. Crude PE was extracted by mixing the homogenate at pH 7.0 for 2hr.

The pH was maintained by addition of NaOH. The homogenate was filtered through filter paper and the extract was centrifuged at 4500rpm and 4°C for 15 min to remove the solid particles.

Estimation of soluble protein

The soluble protein in PE crude extract was quantified by Bradford method (1976) using a calibration curve made with bovine serum albumin (BSA) as a standard.

Pectinesterase (PE) assay

PE activity

The activity of PE was quantified by the continuous spectrophotometric method of Hagerman and Austin (1986). At first all of the solutions of the reaction (pectin, indicator dye, water) were adjusted to pH 7.5 with concentrated (2 N) NaOH just before the reaction start.

The reaction mixture consisted of 200 µl crude enzyme, 2ml citrus pectin solution (0.5 g in 100ml of 0.15M NaCl) , 400 µl bromothymol blue (0.01 g in 100ml of 20mM phosphate buffer pH 7.5). The difference in absorbance at 620 nm at 0min and at 1min was recorded for calculation. One unit of PE activity is defined as a change in absorbance of 0.001 units per minute and one mL of enzyme extract.

$$Activity of \text{PE} = \frac{\Delta A/\text{min}}{0.001} \times \frac{1}{0.2}$$

PE specific activity

Enzymes specific activity was calculated by the following formula:

$$\text{Enzyme specific activity} = \frac{\text{Enzyme activity}}{\text{Soluble Protein (mg)}}$$

Partial purification of the crude PE enzyme

Crude PE was partially purified by fractional precipitation with acetone and ethanol separately as the method of Munir *et al.* (2015) to study the effect of different purifying agents on soluble protein, enzyme activity and consequently enzyme specific activity. The enzyme solution was treated with increasing volumes of acetone or ethanol (cooled at -5°C one day before use). Each volume of cold acetone or ethanol was added slowly, with stirring, to the cold enzyme solution (kept in an ice-salt bath) until the required concentration was reached.

After removing the precipitated fraction by centrifugation for 10 min in a cooling centrifuge, further acetone or ethanol was added to the supernatant fluid and the process repeated to obtain several enzyme fractions at 20, 30, 40, 50, 60, 70, 80 and 90%. The test was carried out on the precipitate from each fraction to choose the best concentration of each purifying agent which gives the highest enzyme's specific activity.

Estimation of optimum pH and temperature

The activity of the partially purified PEenzyme was tested in pH range (6-9) and temperature range (20 –

70°C) to determine both temperature and pH optimum for the enzyme reaction.

Statistical analysis

The collected data were means of three replicates and were analyzed using SPSS statistical software (IBM SPSS Statistics, version 23). The treatments (means) and the differences were compared using one way analysis of variance (ANOVA), Post hoc LSD test was also performed at p≤0.05

Results and discussion

Apple, orange, Zaghloul date and un pollinated Zaghloul date fruits were evaluated for their pectin esterase (PE) enzyme activity.

The specific activity of PE extracted from Zaghloul dates ranked the second position after apple and before orange while that extracted from unpollinated Zaghloul dates ranked the last position.

The recorded PE specific activity values are placed in Table (1) descendingly.

Table 1. Total soluble protein, activity and specific activity of PE extracted from different fruit sources.

PE source	Total soluble protein (mg)	PE activity (IU/ml enzyme)	PE specific activity (Umg-1 protein)
Apple	0.14695	4533.33	30848.90
Date (Zagloul)	0.46873	8366.67	17849.52
Orange	0.20415	2600.00	12736.00
Unpollinated Date (Zaghloul)	0.43293	2033.33	4696.65
Mean	0.31319	4383.33	13995.75
LSD (p≤0.05)	0.14	6698.01	

Table 2. Acetone fractional precipitation of the PE enzyme extracted from Zaghloul date fruits.

Acetone Conc. (%)	Protein content of fraction (mg)	Recovered protein (%)	Total activity fraction (IU)	Recovered activity (%)	Specific activity of fraction (Umg-1 protein)	Purification fold
None	61.97	100.00	104583.33	100.00	1687.74	1.00
20	4.22	6.80	17399.97	16.64	4127.70	2.45
30	1.73	2.80	2456.14	2.35	1416.61	0.84
40	8.92	14.39	6628.50	6.34	743.43	0.44
50	6.21	10.02	16273.61	15.56	2620.94	1.55
60	1.45	2.34	6118.55	5.85	4215.19	2.49
70	2.96	4.78	266.68	0.25	90.03	0.05
80	2.17	3.51	2484.49	2.38	1143.08	0.68
90	2.06	3.32	5693.04	5.44	2767.67	1.64
Total of fractions	29.72	47.96	57320.96	54.81	1928.80	1.14
LSD (p≤0.05)	4.98		43845.09			

The relation between PE activity and specific activity was demonstrated by Fig.2. Depending on the results achieved for soluble protein and enzyme activity; Zaghloul dates was the choice for acetone and ethanol fractionation.

Fractionation with acetone

The results concerning the fractional precipitation of PE enzyme extracted from Zaghloul date fruits with acetone are shown in Table (2). The protein level of the acetone precipitated fractions showed that the 40% fraction possessed the highest protein (8.92 mg) and considerable activity (6628.50 IU) which amounted to 14.39& 6.34% of the original protein and activity respectively.

Although the 20% fraction was not the richest in its protein content (4.22 mg), it afforded the highest activity units (17399.97 IU) which recovering 16.64% of the original with the high purification level (2.45-fold).

Fractionation with ethanol

The fractional precipitation results of PE enzyme extracted from Zaghloul date fruits with ethanol as shown in Table (3) indicated that the 50% fraction recorded the highest protein content (10.37 mg) with considerable activity (7675.84 IU) which recovered 16.73&7.34 % of the original respectively. The 20% fraction showed the highest activity (12963.22 IU) with 12.40% recovery and 1.79 purification fold.

Table 3. Ethanol fractional precipitation of the PE enzyme extracted from Zaghloul date fruits.

Ethanol Conc.(%)	Protein content of fraction (mg)	Recovered protein (%)	Total activity of fraction (IU)	Recovered activity (%)	Specific activity of fraction (Umg-1 protein)	Purification fold
None	61.97	100.00	104583.33	100.00	1687.74	1.00
20	4.30	6.93	12963.22	12.40	3016.84	1.79
30	0.74	1.20	2176.36	2.08	2922.02	1.73
40	8.23	13.28	5674.57	5.43	689.58	0.41
50	10.37	16.73	7675.84	7.34	740.39	0.44
60	1.61	2.61	3036.91	2.90	1880.97	1.11
70	3.02	4.87	5572.63	5.33	1846.22	1.09
80	1.27	2.04	5233.42	5.00	4135.44	2.45
90	3.84	6.19	8846.27	8.46	2306.55	1.37
Total of fractions	33.37	53.85	51179.21	48.94	1533.61	0.91
LSD (p≤0.05)	5.67		43253.87			

Among all of the 16 precipitated fractions by acetone and ethanol, the 60% acetone fraction possessed the highest specific activity (4215.19 Umg^{-1}protein) with the highest purification fold (2.49) compared to the 80% ethanol fraction which possessed a relatively high specific activity (4135.44 Umg^{-1}protein) with a considerable purification fold (2.45). But both of the 20% acetone and ethanol fractions are still promising as they possessed relatively high specific activities with comparable purification folds.

Table 4. Activity (IU/ml enzyme) corresponding to each reaction pH.

Reaction pH	Activity (IU/ml enzyme)
6.0	3178.13
6.5	8742.68
7.0	13734.87
7.5	19209.01
8.0	32302.82
8.5	11539.65
9.0	1410.14
Mean	12873.90
LSD (p≤0.05)	12409.64

Table 5. Activity (IU/ml enzyme) corresponding to each reaction temperature (°C).

Reaction temperature (°C)	Activity (IU/ml enzyme)
20	13256.92
30	19209.01
40	20206.92
50	16966.52
60	4711.10
70	1840.76
Mean	12,698.54
LSD (p≤0.05)	14220.70

Comparing the different acetone and ethanol fractions of PE enzyme in terms of specific activity (Umg^{-1} protein) was demonstrated in Fig. 3.

The effect of different pH levels on PE enzyme activity was studied and results were illustrated in Table 4 and Fig. 4. PE enzyme activity increases with the increase in pH level between 6 and 8 while beyond pH 8 the activity starts to decrease.

The highest or optimum enzymatic activity recorded was 32302.82 IU/ml enzyme at pH 8.

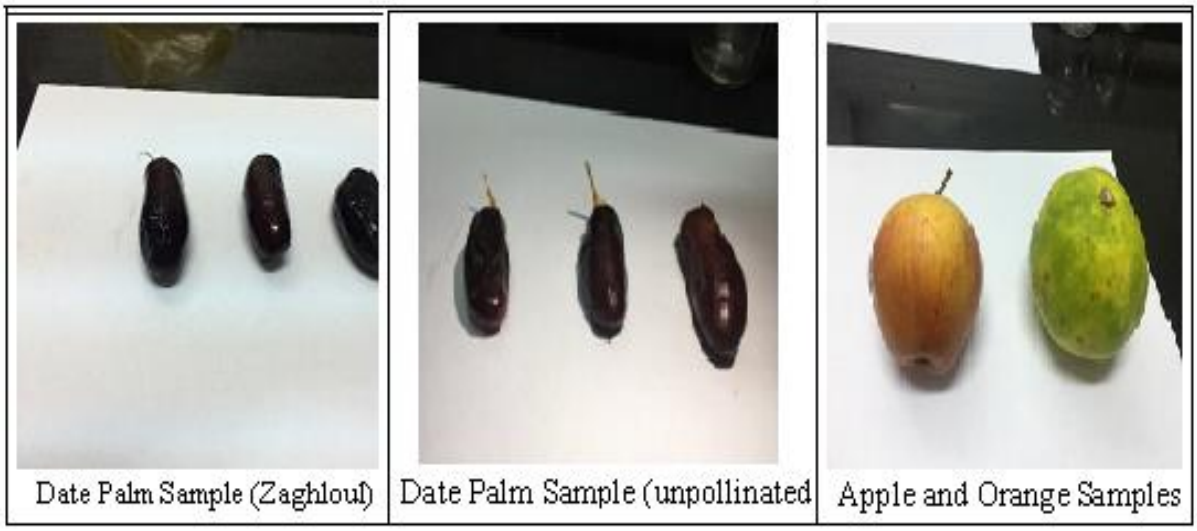

Fig. 1. Samples used in Pre-extraction.

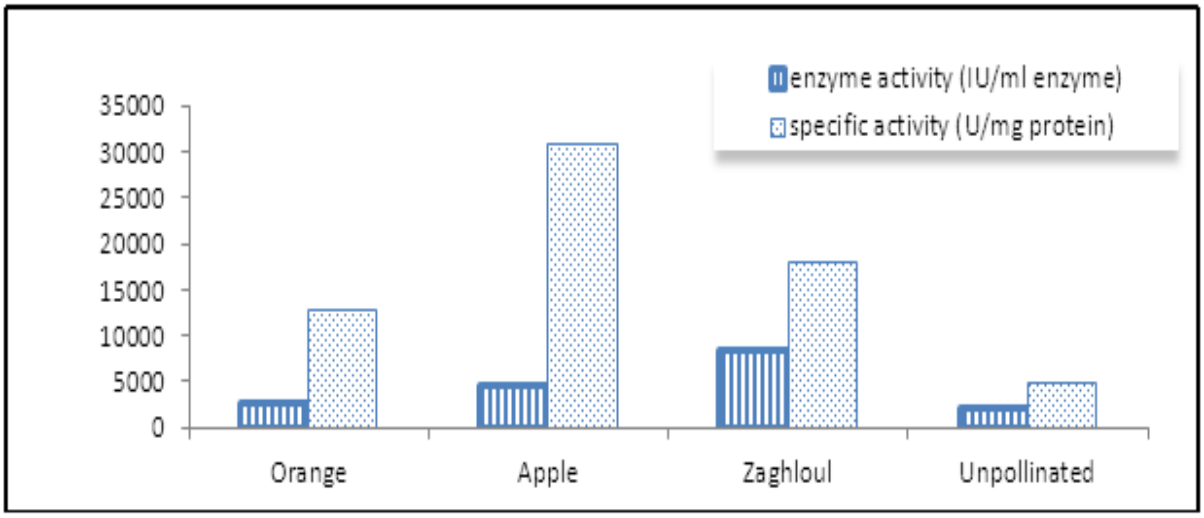

Fig. 2. Enzyme activity and specific activity of PE in four types of fruits.

This finding is in line with that reported by Körner *et al.* (1980) who found that the optimum pH for PE extracted from orange was 7.5 and Ulgen *et al.* (1993) who implied that there was no PE activity below pH 3.5. The same optimum pH values was reviewed by Duvetter *et al.* (2009) for banana was between 6-8, for carrot was 7-8, for lemon and orange was 5-9, for tomato was 5-10 and for plums and strawberry was 7-7.5

In addition, the PE activity was evaluated with different degrees of temperature in the range 20-70°C as shown in Table 5.

Reaction pH plays a major factor in the activity of enzymes. On the light of the results mentioned above PE enzyme activity decreases with increasing pH from 6 to 6.5 then increases with increasing pH from 6.5 to 8 and started to decrease again with increasing pH

from 8 to 9 affirming the fact that extremely high or low pH values generally result in complete loss of activity for most enzymes. The optimal pH for PE enzyme activity was 8 at which the highest activity was detected (32302.82 IU/ml enzyme) which differs from Duvetter *et al.* (2009) who reported that in tomato based products, maximal PE activity can be attained at pH 7.2

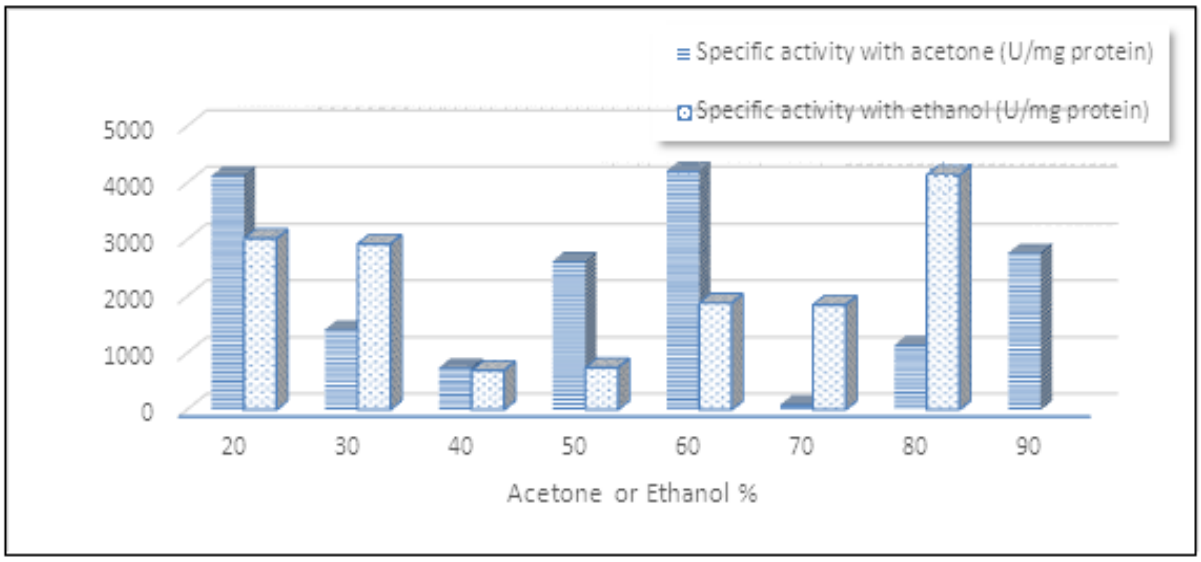

Fig. 3. Comparison of PE specific activity vs. acetone and ethanol fractions.

The other crucial factor affecting enzyme's activity is temperature. All enzymes have temperature ranges for their activity but there are certain temperatures where they work optimally.

The optimum temperature for PE enzyme extracted from Zaghloul dates was 40 °C compared to 60 °C reported by Körner *et al.* (1980) for PE extracted from orange, and 55 °C reported by Duvetter *et al.* (2009) for PE extracted from tomato. Duvetter *et al.* (2009) reported a 2-6 fold increase in PE catalytic activity by increasing the temperature from 20 to 55 °C which is in contrast with our results that recorded only about 34% increase in PE activity with increasing temperature from 20 to 40 °C and about 90% decrease with increasing temperature from 40 to 70 °C.

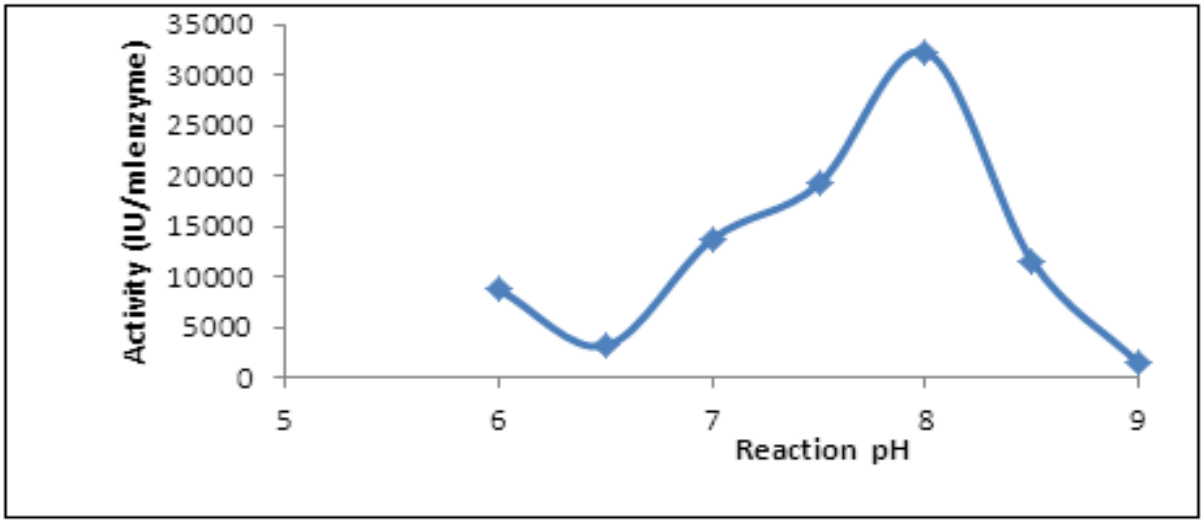

Fig. 4. PE activity vs reaction Ph.

Conclusion

The present work draws attention to a novel source of pectinesterase which may have the potential to become important and economic raw material for food processing industries. Results revealed that PE enzyme extracted from date palm fruits (cv. Zaghloul) can compete with that extracted from citrus sources and commercially used on the basis of its specific activity reporting 17849.52 Umg^{-1} protein. Fractional distillation of Zaghloul date fruits with 20% acetone or ethanol resulted in a partially purified PE enzyme with specific activity 4127.70 and 3016.84 Umg^{-1} protein, respectively. The maximum catalytic activity of PE enzyme is achieved at pH 8 and reaction temperature 40 °C based on these results we can conclude that PE enzyme extracted from Zaghloul dates is unstable at high pH and temperature degrees. It is recommended to make industrial trials using pectiesterase enzyme extracted from ripe Zaghloul date fruits on small scale and compare it with usual plant pectiesterase sources. Also other types of date palm fruits can be evaluated for their pectinesterase enzyme activity.

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