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PURIFICATION AND CHARACTERISTICS OF POLYHYDROXY ALKANOATE DEPOLYMERASES FROM *EIKENELLA CORRODENS*, *KLEBSIELLA PNEUMONAE* AND *ALCALIGENES FAECALIS*

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ABSTRACT

In a previous screening study for polyhydroxy alkanoate (PHA) degrading activities , three isolates were obtained and characterized as *Eikenella corrondens, Klebsiella pneumoniae* and *Alcaligenes faecalis* . In the present study, PHA depolymerases from these three isolates were purified by DEAE anion exchange chromatography and by chromatofocusing (isoelectric focusing) after precipitation from the crude enzyme preparations by ammonium sulfate. The molecular weights for the three enzymes were determined by gel permeation sodium dodecyl sulphat (SDS) polyacrylamide gel eclectrophoresis .

INTRODUCTION

Polyhydroxybutyrate (PHB) and polyhydroxy alkanoate are regarded as very promising candidates for biodegradable thermoplastics which do not pollute the environment [1] . The extracllular PHA depolymerases in bacteria play a crucial role in clearing PHA from the environment [2] . Extracellular depolymerases from several aerobic bacteria capable of degrading PHAs have been isolated and investigated [2,3] .

In a previous study three bacterial isolates capable of degrading PHB/V yielded from a screening study and were identified as *Eikenella corrodens, Klebsiella pneumoniae* and *Alcaligenes faecalis*. In the present study, the PHA depolymerases were further purified and characterized.

MATERIAL AND METHODS

Enzyme :

Enzyme production cultures [4] at the late stationary phase were centrifuged at 10.000 g for 10 min and the supernatant was concentrated 15 times using Amicon ultrafilter (MW cut off 10.000) under pressure. The concentrated culture supernatant was brought to 40% w/v saturation with solid ammonium sulfate . After 30 min of stirring, the formed precipitate was recovered by centrifugation at 7000 g for 10 min and redissolved in 5mM phosphate buffer (pH 7.5). The enzyme preparation was concentrated by ultrafiltration, as above, and loaded onto a 4 m x 9 cm DEAE anion exchange resin HPLC column (Waters Associations Milford, MA) that had been preequilibrated with 50 mM phosphate buffer (pH 7.5) . The enzyme was eluted with a linear gradient of NaCl (1M) in the same buffer with 1 ml/min flow rate for 30 min. Fractions were collected , concentrated by ultrafiltration and assayed for depolymerase, and esterase activities and for protein contents.

Assay for depolymerase and esterase activities :

Depolymerase and esterase activities were assayed as previously described by **Yousef et al., (1998)** [4] .

Determination of protein contents :

The Bio-Rad protein assay, based on dye-binding (Coomassie brilliant blue) was used. The dye reagent (Bio-Rad) was diluted in deionized water (1:4) and filtered through Whatman No. 1 filter paper prior use in the assay. Lyophilized bovine serum albumin (BSA) was used for making standard with a concentration range from 1 to 25 μg/ml. To 0.8 ml of the protein, 0.2 μg/ml of the dye reagent was added and the O.D. was measured at λ 595 nm.

SDS- polyacrylamide gel electrophoresis :

The method of **Laemmli (1970)** [5] with 12.5% w/v gel was used for crude and pure enzyme preparations. Protein denaturation was carried out by heating for 3 min at 100°C in the presence of 1 volume of denaturing buffer (0.05% w/v bromophenol blue , 2% w/v SDS, 1% v/v 2-mercaptoethanol, 10% v/v glycerol in electrophoresis buffer) . Protein was stained with 0.25% v/v coomassie brilliant blue in 35% v/v methanol and 10% v/v acetic acid. As a molecular weight marker, an electrophoresis calibration kit containing β-galactosidase, fructose phosphate kinase, pyruvate kinase, fumarase, lactic dehydrogenase and triosephosphate isomerase (Sigma Chemicals) were used.

Chromatofocusing (isoelectric focusing) :

Separation of protein was carried out at pH gradient according to their isoelecteric point (pI). The separation was performed using a Rotofor cell (Bio-Rad) which is a preparative scale, free solutions, isoelectric focusing apparatus that provides an alternative for protein separation and purification. Rotofor cells incorporates a cylindrical focusing chamber with an internal ceramic cooling fingers. Separation occurs within the annulus formed by the inside wall of the chamber and the cooling fingers. A 60 ml sample is focused in 4h with 12 watts of power. Rotation at 1 r.p.m. around the focusing axis stabilizes against connection and gravitational disturbance. A series of 19 parallel microfilament polyester membranes divide the focusing chamber into 20 discrete

compartments each holding one fraction. After focusing, the solution in each compartment is rapidly collected without mixing , using harvesting apparatus supplied with the unit. Pure enzyme preparation should be desalted by dialysis or ultrafiltration prior to ampholyte addition to ensure that the normal pH range ampholyte will extend over the full length of the focusing chamber. The electrolyte for the anode is usually 0.1 M H_3PO_4 and the electrolyte for the cathode is 0.1 M NaOH. Ion exchange membranes are used in the Rotofor cell to separate the sample from the electrolyte while allowing the current to pass through and set up the pH gradient. After separation, the pH of each of the 20 fractions was measured and tested for the depolymerase and esterase activity. In addition to the components provided, operation of the Rotofor cell requires a power supply, recirculating coolant, a vacuum source, a 60 cc syringe, biolyte ampholytes, and 20, 12 x 75 mm culture tubes. There are two electrode assemblies which hold the cathode and anode electrolyte solutions and provided electrical contact between the focusing chamber and the power supply.

Determination of hydrolytic products of HB/V :

Gel permeatation chromatography (GPC) was employed for the determination of hydrolytic products of PHB/V by the enzyme. Sterile 15 x 125 mm test tubes coated with PHB/V 15.6 polymer (from chloroform solution) were prepared. The pure enzyme (2 ml) was added to the tubes and incubated at 20°C in rotating drum for 2 days . The tube contents were frozen, lyophilized and chromatographic samples were prepared. Controls of polymer and enzyme were included. The lyophilized samples were extracted with 5 ml THF at room temperature, filtered through 0.45 μm Millipore filter and 10 μl samples were chromatographed through 60 x 0.75 cm Slyragel column 300 A° (Waters Association Milford, MA, USA) using THF as eluent. The molecular weight distribution after degradation was recorded and the areas of the residual polymer peak were compared with control samples treated similarly.

RESULTS

The PHA depolymerases of *E. corrodens, K. pneumoniae* and *A. faecalis* isolate were separated using DEAE anion exchange HPLC after precipitation from the culture suppernatants by ammonium sulfate The separation steps with specific activity and purity of the enzymes are shown in Table 1. Figures 1, 2 and 3 show the separation profiles for the enzyme by DEAE anionic exchange PHLC.

After being purified, the MW of the depolymerases were determined using SDS - PAGE and were estimated to be 50.000, 45.000 and 47.000 Da for the enzymes of *E. corradens, K. meumoniae* and *A. faecalis,* respectively.

The isoelectric points for the three purified enzymes were determined by chromatofocusing method with linear pH gradient from 3 to 11. The results in Figure 4, 5, and 6 showed pI values of 7.2, 7 and 7.5 for *E. corrodens, K. pneumoniae* and *A. faecalis,* respectively. The peaks of depolymerase activities were found at fractions 10, 12 and 14 for *E. corrodens, K. pneumoniae* and *A. faecalis,* respectively. These fractions contained the esterase activities .

To determine the hydrolytic products of PHV/V 15.6 using the purified depolymerases, the decrease in MW of the polymer was followed by gel permeation chromatography.

Table (1) : Purification of depolymerase enzyme.

Purification	Total activity (u/ml)	Protein (mg/ml)	Activity specific (u/ml)	* Recovery (%)	** Purification fold
		E. corrodens			
1. Conc. crude enzyme	100	1.9	52	100	1
2. Amm. sulfate precipitate	65	0.8	81.3	65	1.7
3. DEAE anion exchange fraction	43	0.356	123	43	2.4
		A. faecalis			
1. Conc. crude enzyme	140	1.67	83.8	100	1
2. Amm. sulfate precipitate	110	1.1	100	78.6	1.2
3. DEAE anion exchange fraction	80	0.55	145	57	1.8
		K. pneumoniae			
1.Conc. crude enzyme	70	0.9	77.7	100	1
2.Amm. sulfate precipitate	55	0.6	91.6	78.56	1.2
3. DEAE anion exchange fraction	20	0.15	133	28.5	1.7

* Based on total activity
** Based on specific activity

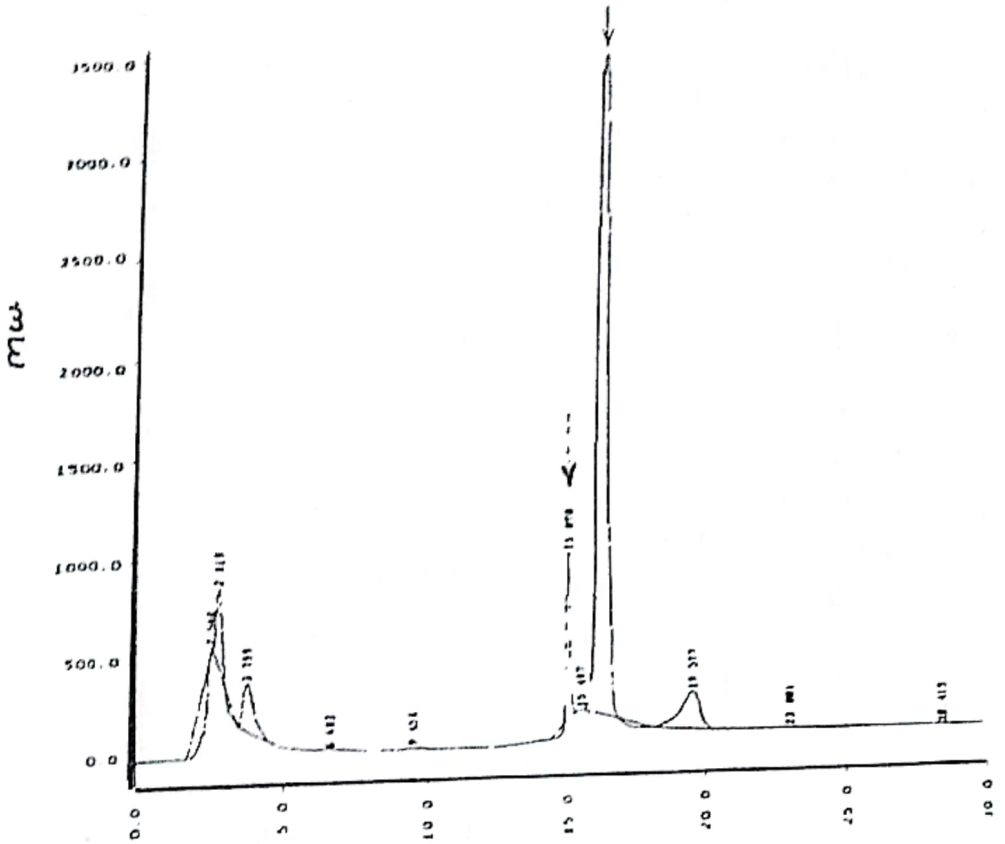


Fig. (1) : Separation of *E. corrodens* depolymerase on DEAE - anion exchange HPLC

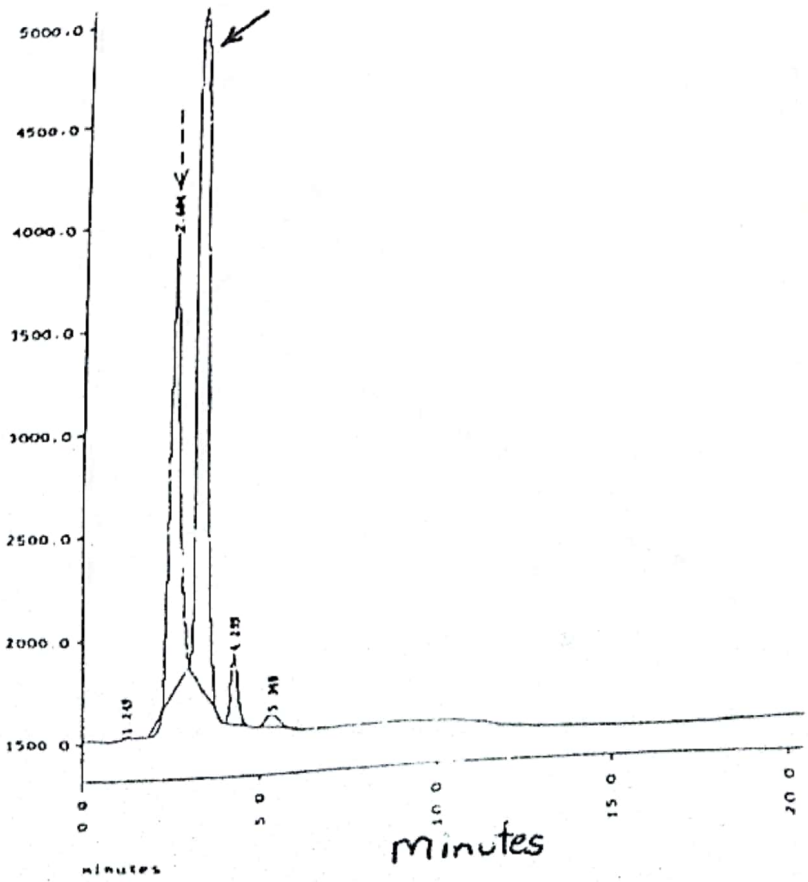


Fig. (2) : Separation of *K. pneumoniae* depolymerase on DEAE - anion exchange HPLC.

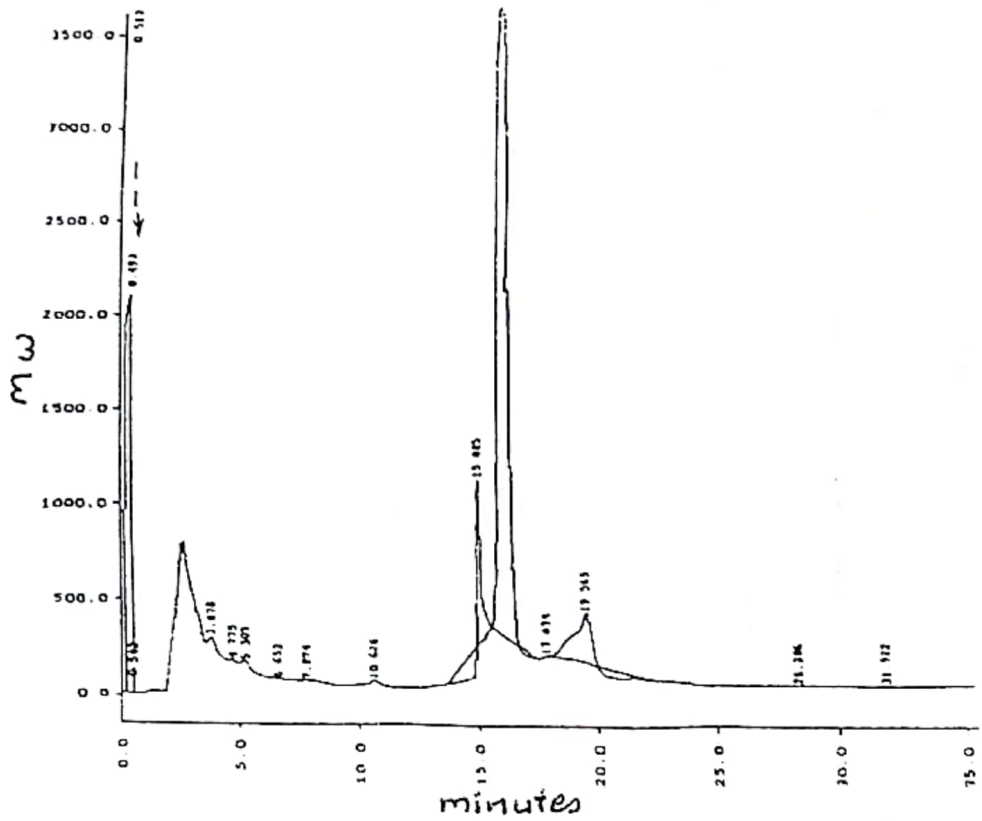


Fig. (3) : Separation of *A. faecalis* depolymerase on DEAE - anion exchange HPLC.

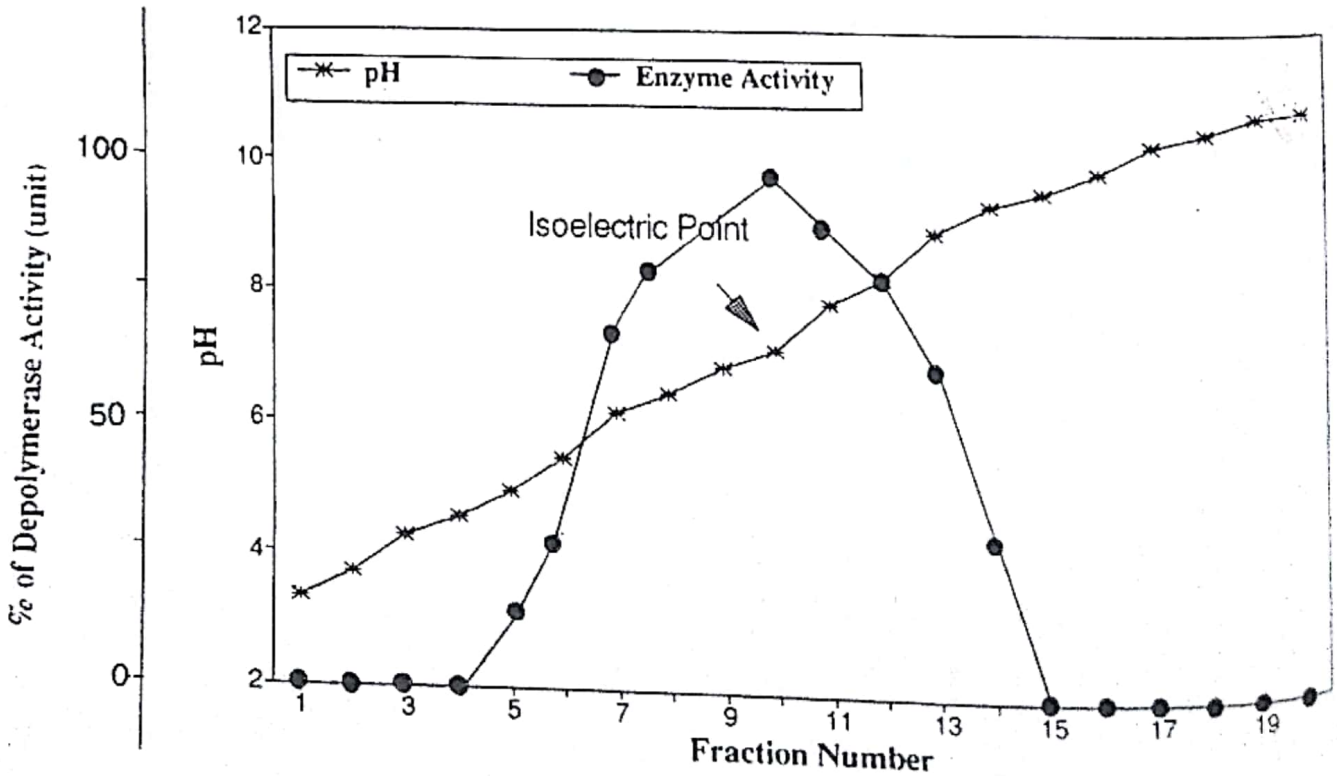


Fig. (4) : Isoelectric point of *E. corrodens* depolymerase.

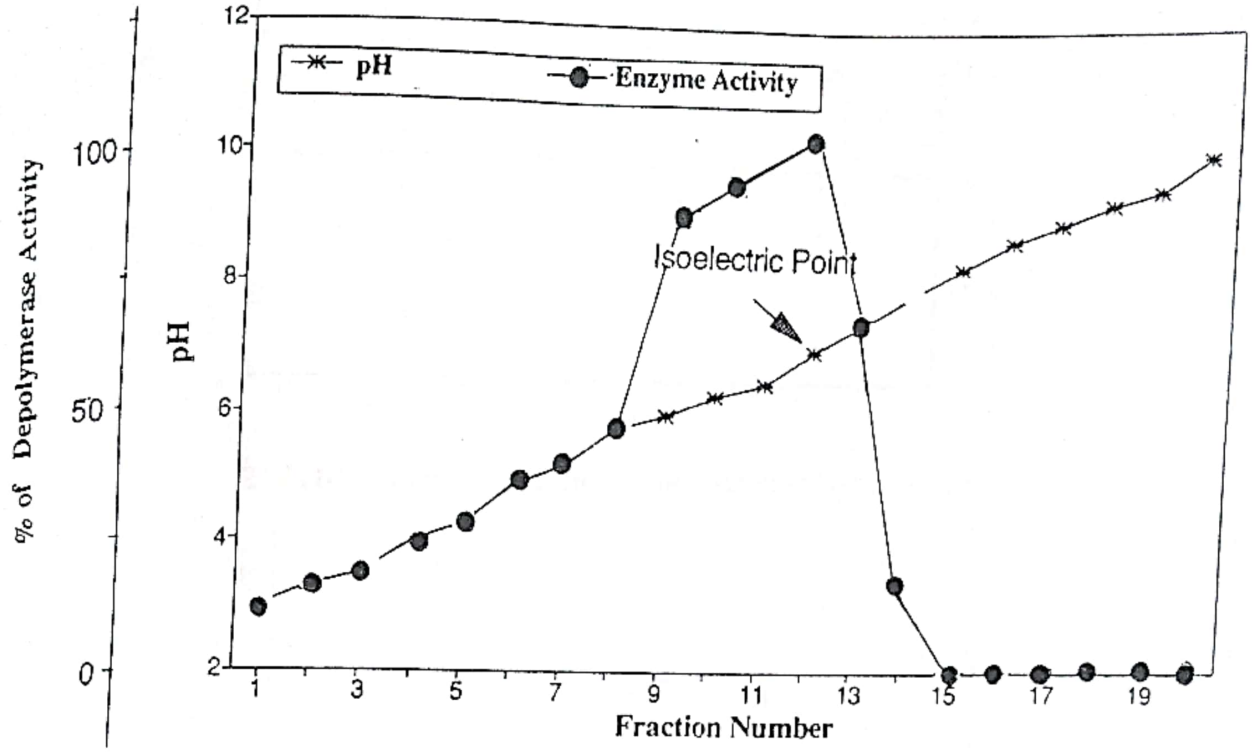


Fig. (5) : Isoelectric point of *K. pneumoniae* depolymerase.

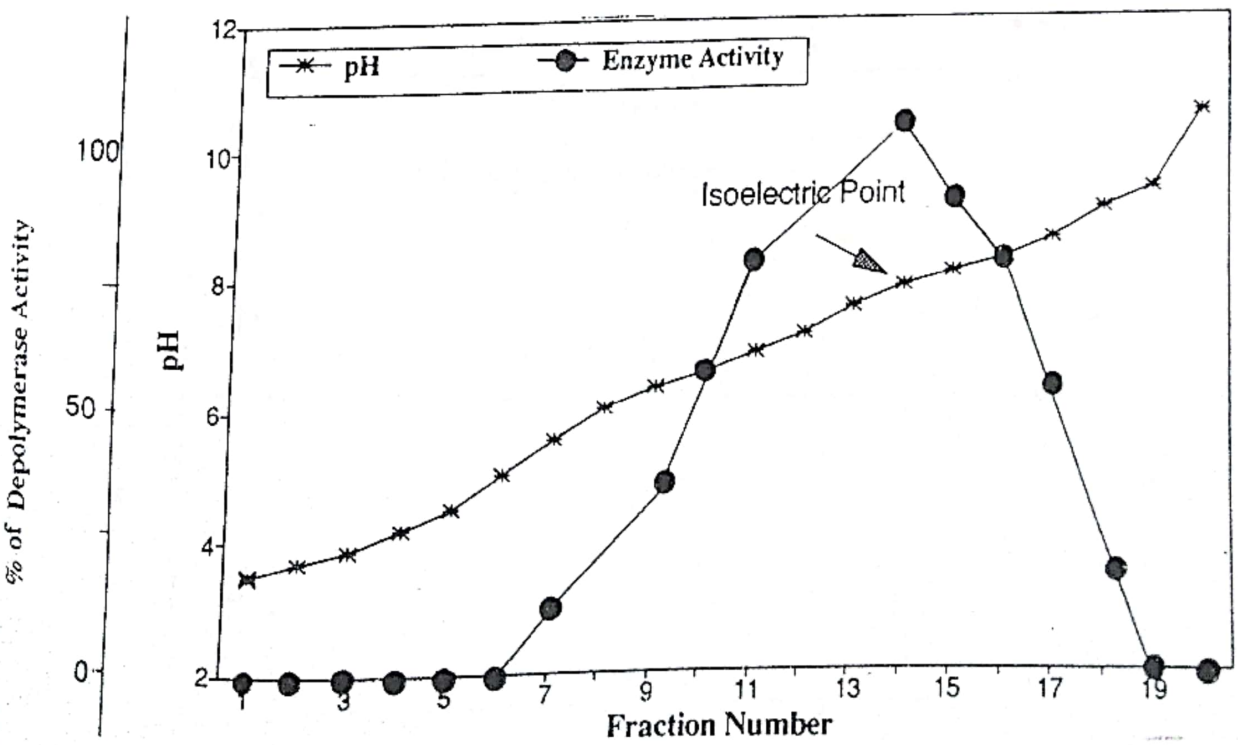


Fig. (6) : Isoelectric point of *A. faecalis* depolymerase.

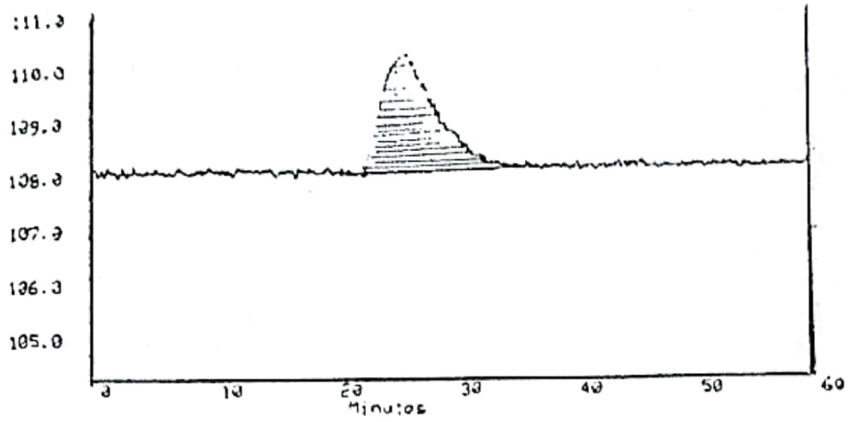
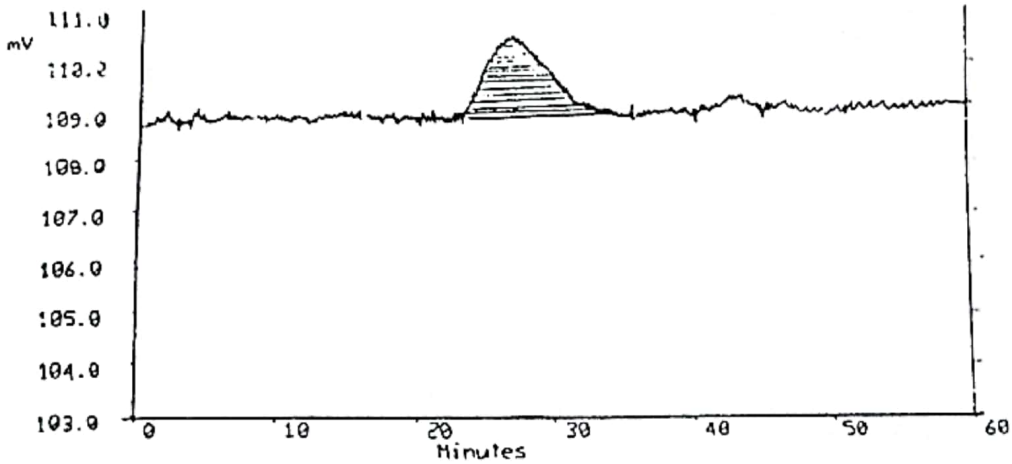
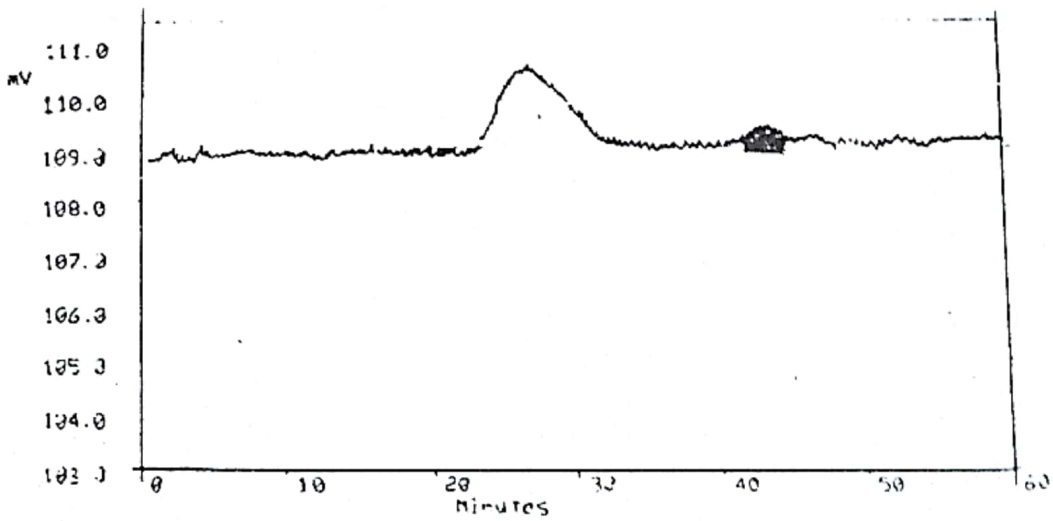


Fig. (7): Gel permeation chromatography of PHB /V15.6



Peak Molecular Weight 152500 Da



Peak Molecular Weight 167 Kda

Fig. (8) : Gel permeation chromatography of degraded PHB/V by *E. corrodens* depolymerase.

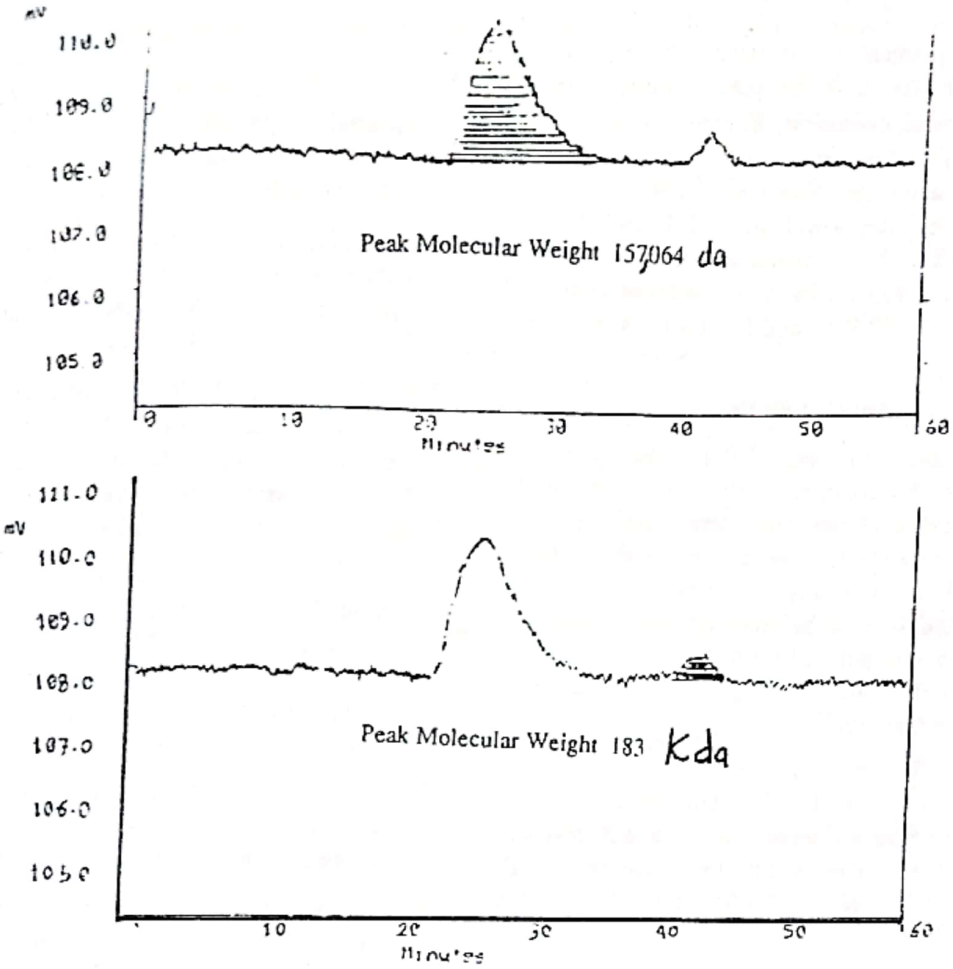


Fig. (9) : Gel permeation chromatography of degraded PHB/V by *K. pneumoniae* depolymerase

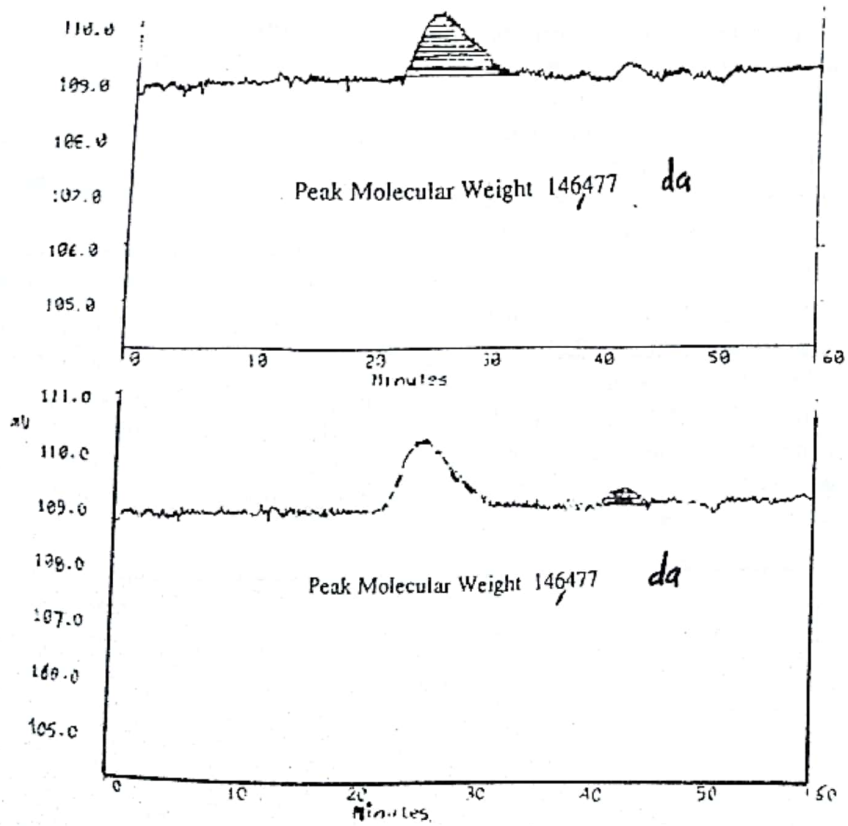


Fig. (10) : Gel permeation chromatography of degraded PHB/V by *A. faecalis* depolymerase.

The gel permeation chromatography results for the control polymer, and the polymer treated with depolymerases of *E. corrodens*, *K. penumoniae* and *A. faecalis* are presented in Figures 7, 8, 9 and 10, respectively . The polymer with MW of 490117 Da (490 Kda) yielded components with MW of 152500 (152.5 Kda) and 167 Da by *E. corrodens* enzyme , 157064 (157.06 Kda) and 183 Da by *K. pneumoniae* enzyme and 14677 Da (146.77 Kda) and 187 Da by *A. faecalis* enzyme .

DISCUSSION

DEAE anion exchange HPLC was used for purification of the enzymes. Only one active peak having PHA depolymerase and esterase activities was shown for each enzyme. The active peak fractions showed no PCL degrading activities. These results suggest that the three depolymerases are esterases. A single enzyme was purified from *A. faecalis* T1 [6] while the activity from *P. lemoignei* was fractionated into two active fractions [7].

The MW of the purified enzyme were determined by SDS PAGE. The purified PHB depolymerases from a *Comamonas sp.* and *A. faecalis* were 54,000 Da (54 Kda) and the two isoenzymes of *P. lemaignei* had MW of 54,000 (54 Kda) and 58,000 Da (58 Kda) [2, 8].

The isoelectric points of the three enzymes lies near to neutrality. These pI are close to the PHA depolymerase of *Cryptococcus* (7.5) are different from that of the 5.5 value *Pseudomonas* [9].

For the determination of hydrolytic products, the high MW starting material was converted to low MW products by the depolymerases. The appearance of single peak, for each enzyme , with MW slightly higher than hydroxybutyric acid (126 Da) and hydroxy valeric acids (146) concurrently with little shift in the position and distribution of the polymer confirms the degradation of the polymers.

However, the MW of the hydrolytic products were not coincide with that expected from hydrolysis of the ester linkage which would be 126 for monomer, 252 for dimer and 378 for trimer. This difference could be attributed to calibration uncertainty and the cleavage at the ester linkage can not be ruled out . The presence of simple peak supports the role of esterase in degradation.

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تنقية وتعريف انزيمات الديبوليمراز المحللة للبولي هيدروكسي الكانوات من اللايكنيلا كورودينز و كليبسلا نيمونيا والكاليجينز فيكاليز

نهال السيد يوسف ، همت كمال عبداللطيف ، فتحي محمد السيد سري ، كاميرون جي . أ*

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يعتبر التحلل الميكروبى للبوليمرات طريقة أمنه للتخلص من نفايات البلاستيك دون الاضرار بالبيئة وقد زاد الأهتمام باستخدام البولى استر المكون من هيدروكسى الكانوات . ولذلك فى هذا البحث تم عزل وتنقية وتعريف ٣ سلالات بكتيرية محللة لهذا البوليمر وهى اللايكنيلا كورودينز و كليبسلا نيمونيا والكاليجنيز فيكاليز.
تفرز البكتريا المعزولة انزيمات الديبوليمراز والاستراز المحللة للبولى هيدروكسى الكانوات خارجها فى الوسط الغذائى وذلك عند تنمية البكتريا على وسط غذائى بسيط يحتوى على ١٪ من البولى استر كمصدر للكربون .
تم تعيين فاعلية انزيمات الديبوليمراز المحللة للبولى هيدروكسى الكانوات بتحديد النقص فى عكار البوليمر نتيجة للتحلل بواسطة الانزيم ووجد أن هذه الانزيمات لها نقطة توازن ايونى من ٧ - ٧ر٥ وزن جزئى من ٤٥ إلى ٥٠ كيلو دالتون .