

Full Length Research Paper

# Purification and characterization of pectinmethylesterase from *Aspergillus repens* isolated from cultivated soil

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*Aspergillus repens* isolated from cultivated soils released pectinmethylesterase (PME) into the liquid culture medium during growth. The enzyme preparation was partially purified by ammonium sulphate precipitation and dialysed. The ammonium sulphate-dialysate fraction of the enzyme was separated by molecular exclusion and ion exchange chromatography. The molecular weight of the enzyme was found to be 141,300 daltons. The optimum temperature for PME activity was 30°C and most active at pH 6.5. The activity of the enzyme was stimulated by Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> and Zn<sup>2+</sup>, while EDTA, PbCl<sub>2</sub>, HgCl<sub>2</sub> and IAA inhibited enzyme activity. The activity of the enzyme increased with increase in substrate concentration reaching maximum at 4 mg/ml. The Lineweaver-Burk plot for the hydrolysis of pectin indicated approximately 1.3 mg/ml. These qualities could be explored during the industrial applications of this enzyme.

**Key words:** Cultivated soils, pectinmethylesterase, temperature, enzyme, fraction.

## INTRODUCTION

Soils are particulate materials of the outer crust of the earth surface formed through the continuous weathering of the underlying parental rocks (Brady and Weild, 1999; Adekayode, 2003). Soil constitutes substratum on which plants and other vegetation grow, providing essential nutrients required for their survival. Also, soil harbours quite a number of living organisms of which microorganisms is not an exception (Ijah and Abioye, 2003).

Pectinmethylesterase (Pectinesterase) belongs to the carboxylester hydrolysis. It deesterifies pectin and pectinic acid to produce methanol and pectic acid (Rose 1980; Da Silva et al., 1997). Pectinmethylesterase are formed by higher plants, moulds, yeasts and bacteria not by animals (Forgarty and Kelly, 1983). However, recent concern for microbial pectinmethylesterases is on the increase. The high specificity of pectinmethylesterases toward the methylester of pectic acid lend impetus to this

enzyme. Enzyme deesterification of pectin precedes in linear fashion along the substrate chain length (Alana et al., 1990; Casida, 1991). Pectinmethylesterases productions are widely distributed among the various groups of microorganisms (Olsson, 1990; Odotola and Ikenebomeh, 1997; Arotupin, 2007).

The commercial applications of pectinmethylesterases particularly from fungi deserve an urgent attention. This work focused on the purification and to determine some properties of the purified enzyme from *Aspergillus repens* isolated from cultivated soils.

## MATERIALS AND METHODS

### Organism

The *A. repens* used for this work was isolated from cultivated soils of Teaching and Research Farm of Federal University of Technology, Akure, Nigeria. The organism was routinely grown and maintained on acidified-streptomycin potato dextrose agar slants at 40°C as stock. The ability of the fungus to produce PME was ascertained by the methods of Kimural et al. (1993) and Andro et al. (1984). The medium contained pectin, 1 g; NH<sub>4</sub>NO<sub>3</sub>, 1 g; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.05 g;

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CaCl<sub>2</sub>. 2H<sub>2</sub>O, 0.01 g and distilled water 1000 ml at pH 5.6. The ability of the fungus to produce pectinmethylesterase was determined by the growth of the fungus on pectin-mineral medium and the enzyme assayed.

#### Medium preparation and inoculation for production of pectinmethylesterase

This was carried out in 250 ml conical flask containing 50 ml of the basal medium of Olutiola and Akintunde (1979). Spores of 48 h-old culture of the fungus were harvested by a slightly modified method of Akinyosoye and Akinyanju (1989) and used to inoculate the basal mineral medium.

#### Enzyme assay

A modification of the titrimetric determination of carboxyl group liberation method of Hills and Mohem (1947) was used for the assay of pectinmethylesterases (PME) activity. The reaction mixture contained 5 ml of 0.5% w/v pectin (sigma) in 0.1 M NaCl. The pH was adjusted to 7.0 with 0.01 N NaOH. At zero time, 1 ml of the enzyme solution (crude enzyme) was added and the reaction mixture incubated at 25°C for 1 h. The reaction mixture was then titrated to a pH of 7.0 with 0.01N NaOH. A unit of PME activity was defined as the amount of enzyme in 1 ml of the reaction mixture that removed one micromolecules of the methoxyl groups per minute at 25°C

#### Preparation of enzyme for column chromatography

Dialysis of the crude enzyme was carried out using acetylated cellophane tubing prepared from Visking tubing (Gallenkamp) as described by Whitaker et al. (1963). The protein content in the preparation was precipitated with ammonium sulphate (analytical grade) according to Olutola and Akintunde (1979).

#### Separation of enzyme on Sephadex G-100 chromatography column

A vertical glass tube chromatography column of 1.5 × 70 cm (Pharmacia Sweden) was used. Sephadex G-100 was employed for the fractionation of the enzyme. The column was eluted with 0.05 M citrate-phosphate buffer (pH 6.0), containing NaN<sub>3</sub>. Proteins of known molecular weight used in calibration include catalase (mol. Wt., 2400,000); haemoglobin (mol. Wt., 68,000), egg albumen (mol. Wt., 45,000), myoglobin (mol. Wt., 17,000) cytochrome C (mol. Wt., 12,400) (Olutiola and Cole, 1980). The void volume was determined with blue dextran. Five-milligrams each of the proteins were dissolved in 10 ml of 0.05 M C-P buffer, pH 6.0 and applied to the column. Fractions of 5 ml per tube were collected and protein content of each eluted fraction measured at 280 nm on a UV spectrophotometer.

#### Application of the enzyme to Sephadex G-100 column

Ten milliliters of the ammonium sulphate-dialysate enzyme concentrate was applied to the column eluted with buffer as described by Olutiola and Ayres (1973). Fractions of 5 ml per tube were collected and the protein content determined with UV spectrophotometer at 280 nm as for column calibration. In fractions in which protein was detected by this method, protein determination was repeated by the method of Lowry et al. (1951). These fractions were also analysed for pectinmethylesterase.

#### Fractionation by ion-exchange chromatography

The SP Sephadex C-50 column was prepared like the Sephadex G-100 column, except that that a shorter column (2.5 × 40 cm) was employed. Fractions which exhibited appreciable enzyme activity after gel filtration were pooled and concentrated. The enzyme concentrate was made up with 0.2M C-P buffer (pH 6.0) to 10 ml and applied to the column of SP Sephadex C-50 that had been eluted with the same buffer containing 0.2M KCl. Fraction of 5 ml per tube was collected and the protein content measured as earlier described. Pectinmethylesterase activity of each of the fraction was determined as earlier described under enzyme assay.

#### Effect of physicochemical factors on purified enzymes

The effect of temperature on the enzyme activity was determined by incubating the reaction mixture at different temperatures ranging from 10-60°C at 5°C interval for 1 h. The residual enzyme activities were determined for each temperature of incubation.

#### Temperature stability of the enzyme

The influence of temperature on the stability of the enzyme was determined. This was done by subjecting an aliquot of the purified enzyme to a temperature of 60°C in water bath for period ranging from 0, 5 to 45 min at interval of 5 min. The activities of the heated enzyme was measured by incubating the enzyme substrate mixture at 25°C for 1 h as earlier described for enzyme assay.

#### Effect of pH on enzyme activity

The effect of pH on the enzyme activity was determined by incubating 5% pectin with 0.02 M C-P buffer of pH ranging from 2.0 to 10.0. The pH of substrates was adjusted accordingly with 0.01 M HCl and 0.01 M NaOH as appropriate. The pectinmethylesterase activity was then assayed as previously described.

#### Effect of substrate concentration on enzyme activity

The effect of substrate concentration on the enzyme activity was determined by incubating various concentrations of pectin ranging from 0.5 to 5 mg/ml for 1 h at 25°C. The pectinmethylesterase activity was determined as earlier described.

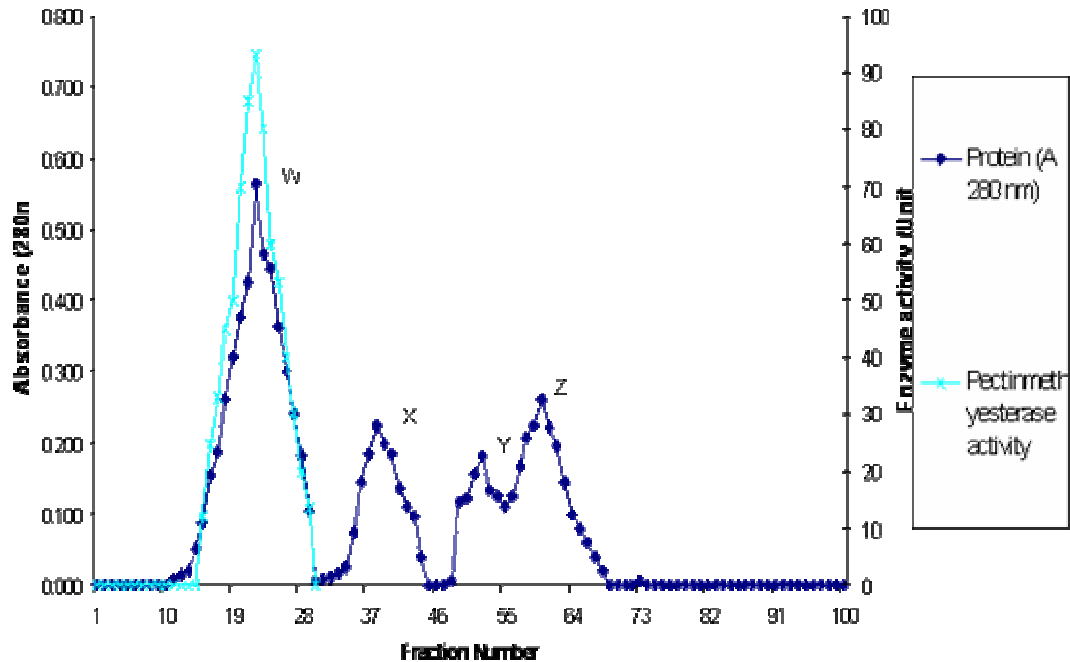
#### Effect of chemical agents on enzyme activity

The effect of some cations and chemical compounds at different molar concentration on the enzyme activity was determined. In this case, concentrations of 5 to 35 mM of K<sup>+</sup>, Na<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> and Zn<sup>2+</sup> were employed. The concentrations of 1 to 14 mM of ethylenediaminetetraacetic acid (EDTA), mercuric chloride (HgCl<sub>2</sub>), lead chloride (PbCl<sub>2</sub>) and iodoacetic acid (IAA) were employed for chemical compounds. The pectin-substrate was initially incubated with each test chemicals at 4°C for 35 min before being used for the enzyme assay.

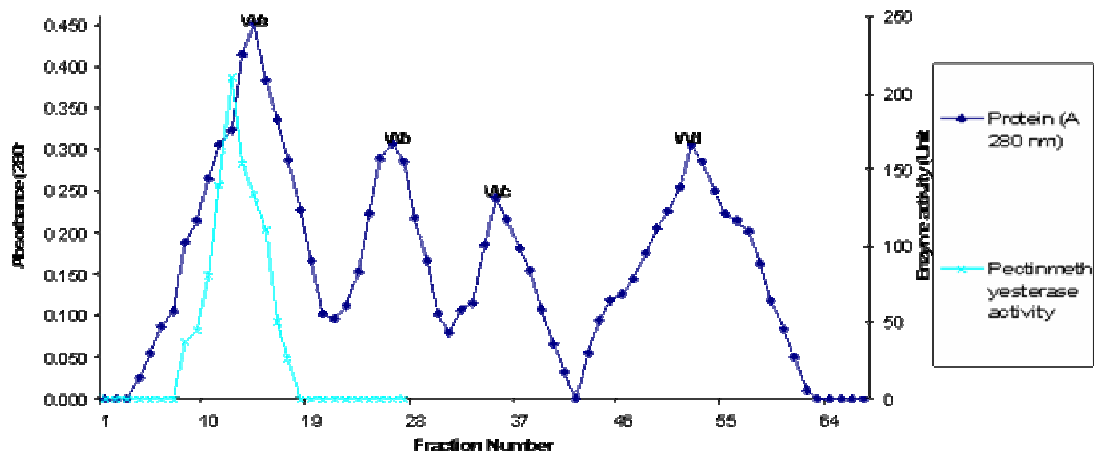
## RESULTS

### Fractionation on sephadex G-100

The ammonium sulphate-dialysate fraction on Sephadex G-100 tend to produced four absorption peaks designa-



**Figure 1.** Gel Sephadex (G-100) separations of protein in concentrated extract of *A.repens* and enzymatic activity of the fractions.



**Figure 2.** Separation by ion exchange chromatography (SP Sephadex C-50) of protein extracted from *A.repens* and previously purified by gel filtration.

ted as W, X, Y and Z (Figure 1). The major peak W component of *A.repens* exhibited pectinmethylesterase activity, while other peaks X and Z had no PME. The molecular weight of this component from the elution volume was approximately 141,300 daltons.

#### Fractionation on SP sephadex C-50

Further fractionation of the components of peak W of *A.repens* from the gel filtration on SP Sephadex C-50

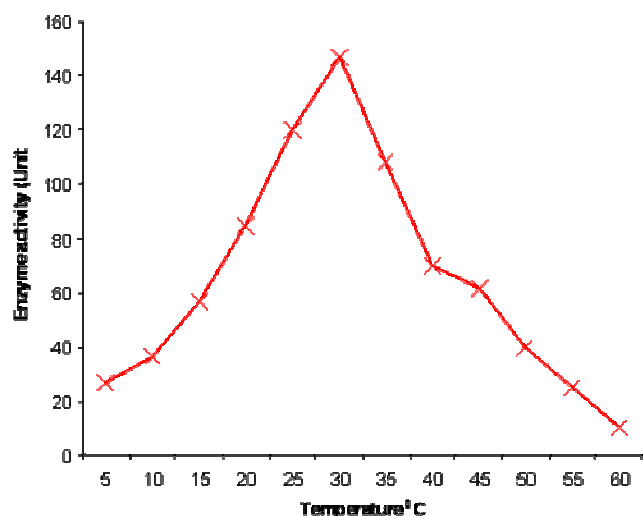
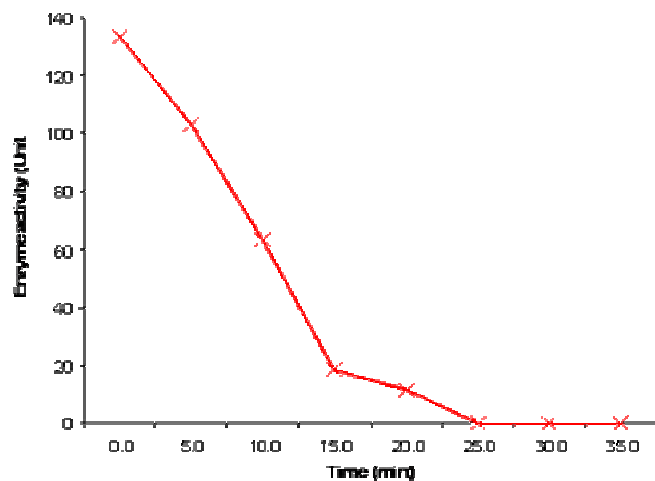
yielded four distinct peaks of absorption shown as Wa, Wb, Wc and Wd, respectively (Figure 2). Only the components of peak Wa exhibited pectinmethylesterase activity, while those of Wb, Wc and Wd lack such enzymatic activity.

#### Purification of pectinmethylesterase from *A.repens*

The ion-exchange chromatography fraction gave the purification fold of approximately 8 (Table 1).

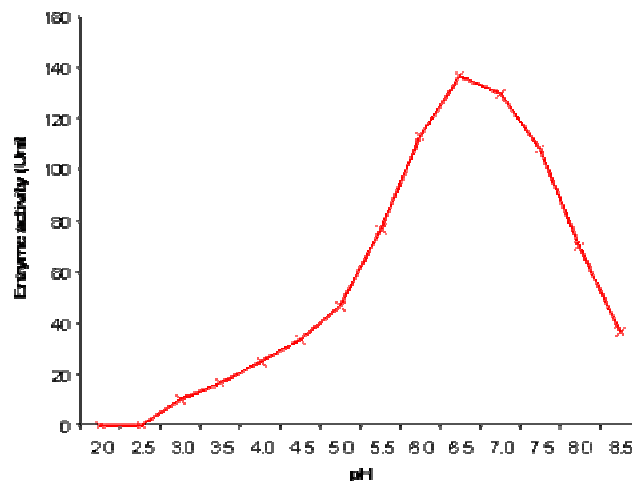
**Table 1.** purification of pectinmethylesterase (PME) from *A. repens*.

Fraction	Total PME	Total protein (mg)	Specific activity (u/mg)	Purification	Yield
Crude	3960	4850	0.82	1	100
(NH <sub>4</sub> ) <sub>2</sub> S0 <sub>4</sub>	2250	1100	2.05	2.5	56.82
Sephadex G-100	1133	460	2.46	3.0	28.61
SP Sephadex C-50	850	130.5	6.5	7.93	21.47

**Figure 3.** Effect of temperature on the purified (SP Sephadex C-50 fraction) pectinmethylesterase activity of *A. repens*.**Figure 4.** Effect of duration of heating (60°C) on the purified (SP Sephadex C-50 fraction) pectinmethylesterase activity of *A. repens*.

### Effect of temperature, heat and pH on the purified enzymes activity

The optimum temperature for the activity of pectinmethylesterase was 30°C (Figure 3). However, within 10 min of heating, approximately 50% loss of enzyme activity was

**Figure 5.** Effect of pH on the purified (SP C-50 fraction) pectinmethylesterase activity of *A. repens*.

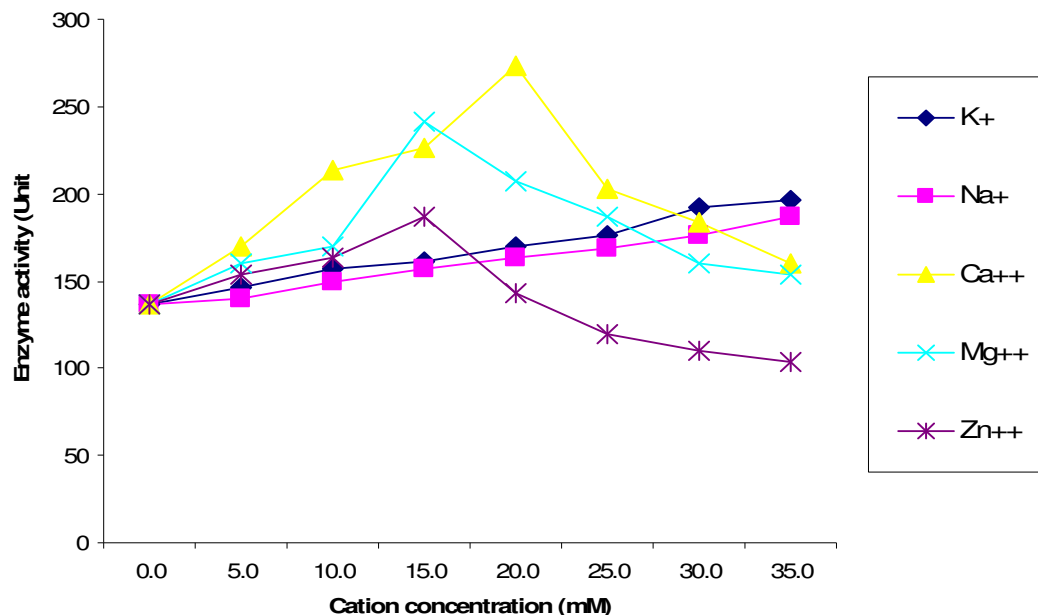
lost. Additional 10min of heating resulted in retention of only 9% enzyme activity, and further heating resulted in complete loss of enzyme activity (Figure 4). Maximum activity of the enzyme occurred at pH of 6.5 (Figure 5).

### Effect of cations and some chemicals on the purified enzymes

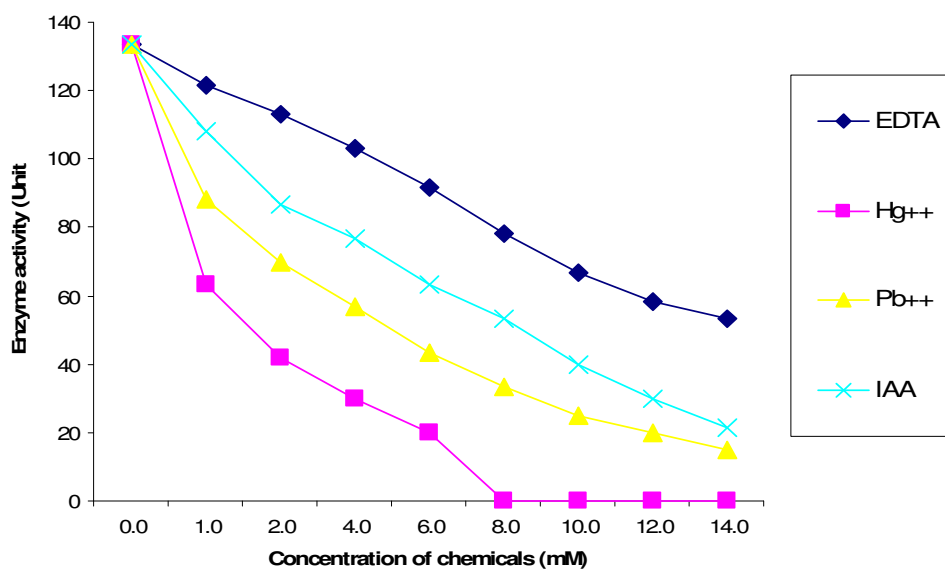
The activity of the enzyme increased with increase in the concentration of K<sup>+</sup> and Na<sup>+</sup>. The concentration of 15 mM was highest for maximum PME activity when Mg<sup>2+</sup> and Zn<sup>2+</sup> were employed, while the highest PME activity of *A. repens* occurred at 20 mM of Ca<sup>2+</sup> (Figure 6). Further increase in their concentrations resulted in the decline of the enzyme activity. All the concentrations of EDTA, Hg<sup>2+</sup>, Pb<sup>2+</sup> and IAA employed were inhibitory to the activity of the PME (Figure 7). The concentration of 1.0 mM of mercuric chloride resulted in approximately 50% loss of enzyme activity, while concentration of 8 mM produced complete loss of enzyme activity.

### Effect of substrate (pectin sigma) concentration of enzyme activity

The activity of the enzyme increased with increase in the



**Figure 6.** Effect of cations on the purified (SP C-50 fraction) pectinmethylesterase activity of *A. repens*.



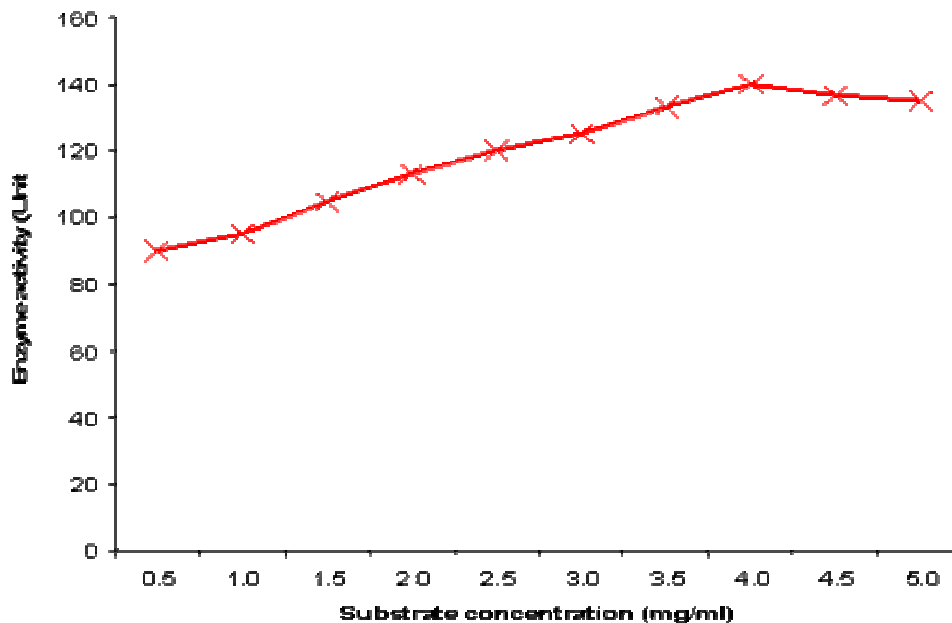
**Figure 7.** Effect of chemical (inhibitors) on the purified (SP C-50 fraction) pectinmethylesterase activity of *A. repens*.

substrate concentration (Figure 8) reaching maximum at 4 mg/ml. The Lineweaver-Burk plot for the hydrolysis of pectin indicated approximately 1.3 mg/ml (Figure 9).

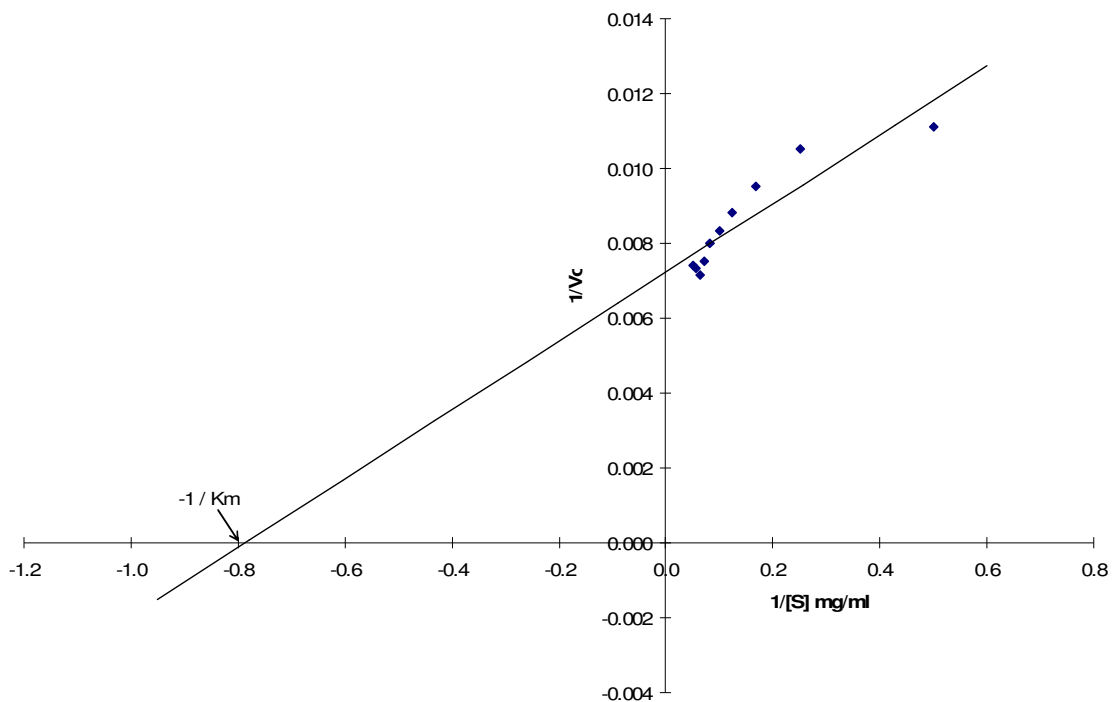
## DISCUSSION

Preliminary investigations revealed that *A. repens* was capable of hydrolyzing pectin. Also, the presence of the pectinmethylesterase (PME) in the culture filterates

further corroborated the production of PME by the fungus. The hydrolysis of pectin by *Aspergillus flavus* and *Aspergillus niger* have been reported (Fredrick et al., 1990; Casida, 1991) and *Fusarium oxysporium* (Odutola and Ikenebomeh, 1997). This observation is in conformity with the discovery of Odutola and Ikenebomeh (1997), Margarita et al. (2000), Kapoor and Kuhad (2002) that pectinases (Pectinmethylesterase) are responsible for the considerably hydrolysis of pectin. The presence of this fungus in the soil no doubt contributed to the rapid degra-



**Figure 8.** Effect of substrate concentrations on the purified (SP C-50 fraction) pectinmethylesterase activity of *A. repens*.



**Figure 9.** Lineweaver-burk plot for the hydrolysis of pectin by the partially purified (SP C-50 fraction) pectinmethylesterase activity of *A. repens*.

dition of plant materials containing pectin in the soil.

In the present study, a molecular weight of approximately 141,300 daltons was obtained for PME from *A.*

*repens*. Different molecular weights have been reported for this particular enzyme isolated from other microorganisms (Oyede, 1998). The difference has been

associated with the type of host cell wall, nature and type of organism used, substrate employed and analytical methods (Oyede, 1998).

The results of this investigation showed that temperatures at which the reaction mixture was incubated greatly affected the activity of the enzyme. The optimum temperature (30°C) of activity exhibited by PME of *A. repens* was documented for PME from *Penicillium citrinum* (Olutiola and Akintunde, 1979). The same optimum temperature was recorded for PME activity of *Erwinia carotovora* and *F. oxysporium* (Odutola and Ikenebomeh, 1997). Therefore, the effect temperature on enzyme activity may be related to the effect of temperature on the velocity of enzyme reaction.

The heating of the enzyme at 60°C resulted in a considerable decrease in its activity. Pectinases from *Aspergillus* strains have been described to be susceptible to denaturation at temperature (heating) about 50°C (Barley and Pessa, 1990; Galiotou-Panayotou et al., 1997).

Generally, enzyme activities are almost dependent on changes with the pH of the reaction mixture which not only influence the enzyme activity, but also affect the Km and Vmax (Murray et al., 1990).

The concentrations of cations used in this study exerted their effects on pectinmethylesterase activity. In this investigation, specific concentrations of cations were stimulatory, while others were inhibitory to enzyme activity (Figure 6). However, the stimulation of pectinylases activities by K<sup>+</sup>, Mg<sup>2+</sup>, and Ca<sup>2+</sup> have been documented (Sakamoto et al., 1994; Famurewa et al., 1993; Sakai et al., 1993). In this case, metal ion may form an integral part of the active enzyme or it may combine with the substrate to give the true substrate of enzyme (Conn and Stumph 1989; Murray et al., 1990).

Notably, the concentrations of EDTA, Hg<sup>2+</sup>, Pb<sup>2+</sup> and IAA employed in this investigation were inhibitory to the activity of pectinmethylesterase (PME) produced by *A. repens*. Obineme et al. (2003) suggested that EDTA acted by chelating Ca<sup>2+</sup> with a resultant loss of catalytic activity of the enzyme. Also, the inactivation of the enzyme activity by IAA could be as a result of the alteration in the pH of the reaction mixture. Arotupin (1991) reported the inhibition of enzyme activity due to change in pH either to higher or lower values.

Substrate concentrations have a marked effect on the PME activity from *A. repens*. The result of the study agreed with the findings of Odutola and Ikenebomeh (1997) on the activity of polygalacturonase and PME from *E. carotovora* and *F. oxysporium*. This increased activity with increase in substrate concentration may be attributed to the effective binding of the substrate to the active site. Murray et al. (1990) documented the fact that further increase in substrate concentration above the optimum level will not produce any increase in the enzyme activity since no enzyme molecule will be available to react with the substrate.

The apparent Km value pectin hydrolysis fall within the range reported by Oyede (1998) and Ajayi et al. (2003). The Km indicated the concentration of substrate to fill the half active sites of an enzyme. It is also a measure of strength of the enzyme-substrate (ES) complex. In fact, Lineweaver and Jansen (1951) reported that a high Km value indicates weak binding and vice versa. Therefore, this PME with low Km value have strong affinity for the substrates.

In this investigation, *A. repens* isolated from soil elaborated considerable pectinmethylesterase activity. The characteristics exhibited by this particular enzyme (purified) would no doubt be vital information for fruit processing and wine industries during clarification.

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