CHARACTERIZATION AND IMMOBILIZATION OF PARTIALLY PURIFIED POLYGALACTURONASE FROM ASPERGILLUS NIGER (SA6) FOR INDUSTRIAL USE

By

BUGA, MOHAMMED LAWAL

DEPARTMENT OF BIOCHEMISTRY
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

May, 2008

CHARACTERIZATION AND IMMOBILIZATION OF PARTIALLY PURIFIED POLYGALACTURONASE FROM *ASPERGILLUS NIGER* (SA6) FOR INDUSTRIAL USE

By

BUGA, MOHAMMED LAWAL B.Sc (UDUS 1987), M.Sc (Jos,1992).
Ph.D/Sci./31586/2001-2002

A DISSERTATION SUBMITTED TO THE POSTGRADUATE SCHOOL,
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

May, 2008

DECLARATION

I hereby declare that this dissertation entitled "Characterization and Immobilization of Partially Purified Polygalacturonase from *Aspergillus niger* (SA6) for Industrial Use" has been written by me in the Department of Biochemistry under the supervision of Dr. S. Ibrahim and Prof. A.J.Nok. The information derived from the literature has been duly acknowledged in the text and a list of reference provided.

No part of this dissertation was previously presented for another degree at any University.

BUGA, MOHAMMED LAWAL

DATE ----------------------------

CERTIFICATION

This dissertation entitled "Characterization and Immobilization of Partially Purified Polygalacturonase from *Aspergillus niger* (SA6) for industrial use" by Buga, Mohammed Lawal meets the regulation governing the award of the Degree of Doctor of Philosophy of the Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

....

Dr S. Ibrahim DATE---------------------

(Chairman Supervisory Committee)

....

Prof. A .J. Nok (MFR) DATE-------------------------

(Member Supervisory Committee)

....

Dr. S. Ibrahim DATE-------------------------

(Head, Dept. of Biochemistry)

…..

Prof. S. A. Nkom DATE-----------------------------

(Dean, Postgraduate School

DEDICATION

This work is dedicated to my parents Alhaji Buga and Hajiya Fati, my wife Hajiya Zaliha and my children Nafisa, Abdulrahman, Usman and Khadija

ACKNOWLEDGEMENT

I wish to thank the Almighty Allah for making it possible for me to undertake this programme. My profound and sincere appreciations go to my supervisors Dr Sani Ibrahim and Professor Andrew J. Nok for their excellent supervision, unflinching support, encouragement and guidance throughout the period of this study. They have been a source of inspiration to me, through their constructive critisms and painstaking reviews of this work.

I am very grateful to the management of the Raw Materials Research and Development Council, (RMRDC), Abuja for sponsoring this programme. I sincerely wish to thank Professor Wolfgang Buckel of the Philipps University, Marburg, Germany for hosting me and providing laboratory facilities and reagents to undertake part of this research work. My thanks also go to Professors. G. H. Ogbadu and J.I Okogun of the Sheda Science and Technology Complex, Abuja and National Institute For Pharmaceutical Research and Development, Abuja respectively for allowing me to use their facilities to conduct part of this work.

I wish to thank the Postgraduate Coordinator, Dr Miss E.Onyike, the Seminar Coordinator, Dr H. C. Nzelibe, Malam Balarabe Sallau, Malam Shehu Isah and Mr. G. C. Njoku for their useful advices. My sincere thanks also go to all the academic and technical staff of the department for their moral support.

I also wish to thank my Director, Dr Ado K. Abdullahi and Deputy Director, Mr. E.A. Asanga for their support and encouragement. I wish to thank Mr. Mike Dimkpa for typing of this work. I wish to thank my wife, Hajiya Zaliha, my children Nafisa, Abdulrahman, Usman and Khadija for their patience and support, my brothers Alhaji Garba Buga, Engr. Ibrahim Buga, Nasiru Buga and Tukur Buga for their moral support. Lastly but not the least my sincere appreciation go to Sani Mohammed Kwaido (Hakimi) , Garba Abdullahi Kangiwa Alhaji Usman Hassan, Engr. Garba Ladan , Dr Dan-Azumi Ibrahim and others too numerous to mention for their moral support.

ABSTRACT

Polygalacturonase (PG) was isolated from *Aspergillus niger*(*A. niger*) SA6. The enzyme was partially purified and characterized. It was then immobilized by entrapment using calcium alginate. The enzyme showed two bands on SDS-PAGE suggesting the presence of an endo and exo PG with apparent molecular weights of 35KDa and 40KDa respectively. The PG was purified 9 fold with a yield of 0.18 % and a specific activity of 246 μmole/min/mg. The K_M and V_{max} values of the enzyme were 2.74 mg/ml and 0.78μmole/min/mg respectively. The optimum temperature and optimum pH of the enzyme were 40°C and 4.5 respectively. The native PG was found to be more stable to temperature changes than to pH changes. Both the V_{max} and K_M of the native PG increased in the presence of EDTA and the following divalent cations Mg^{2+},Ca^{2+} and Mn^{2+}. The increase was more pronounced in the presence of Ca^{2+} ion. The activation energy (Ea) of native PG was 259.19 Cal/degree/mole. The Dixon Plot of the enzyme gave pKa values of 4.9 and 5.6 suggesting Glutamic acid and histidine at the active site of the enzyme. The

apparent K_M and V_{max} of the immobilized PG were 11.1mg/ml and 1.65 μmole/min/mg respectively. The optimum pH and optimum temperature of the immobilized PG were 4.5 and 40°C respectively. Immobilized PG was more resistant to changes in pH than to temperature. The activity of the immobilized PG reduced to 34.56 % and 14.81 % of the initial activity in the second and third catalytic cycles respectively. The half-life of the enzyme at 40°C and the activity lost per minute on thermal storage were 10 minutes and 0.0213 μmole of D- galacturonic acid. These findings present novel information on the physico- chemical characteristics of the native and immobilized Polygalacturonase isolated from a local strain of *Aspergillus niger* (SA6).

TABLE OF CONTENT

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

List of Tables

List of Figures

List of Plates

List of Appendices

Abbreviations And Definitions

ADI- Acceptable Daily Intake

APA- Amino Penicillanic Acid

APS- Ammonium Per Sulphate

BSA- Bovine Serum Albumin

DE- Degree of Esterification

DEAE- Di Ethyl Amino Ethyl

DNS- Dinitro Salicylic Acid

DP- Degree of Polymerization

ETA- Enzyme Technical Association

FPLC- Fast Performance Liquid Chromatography

GRAS- Generally Recognized As Safe

HM- High Methoxyl

IPPA- International Pectin Producers Association

JECFA-Joint Expert Committee on Food Additives

KDa- Kilodalton

LM- Low Methoxyl

PCR- Polymerase Chain Reaction

PG- Polygalacturonase

PI- Iso-electric Point

SA6- Samaru Aspergillus Number 6

SDS-PAGE- Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SDS- Sodium Dodecyl Sulphate

TEMED- Tetra Methylene Diamine

U – Enzyme Unit

WHO- World Health Organisation

Enzyme Unit= One unit of Polygalacturonase activity is defined as one µmole of D-galacturonic acid released from Pectin/minute at 40°C and pH 4.5

Specific Activity = Units of the Enzyme/Total mg protein

Total Activity=Specific Activity x Total mg protein in fraction

Purification Fold= Specific Activity of a given fraction/Specific activity of the crude preparation

%Recovery= Total Activity of a given fraction/Total Activity of the crude preparation

CHAPTER ONE

1.0 INTRODUCTION

Pectinases are a group of enzymes (EC. 3.2.1.15 and EC. 3.2.1.67) endo and exopolygalacturonase, Polymethylgalacturonate lyase (EC. 4.2.2.10) and Pectin esterase (EC. 3.1.1.11) that can degrade pectin containing substrate or modify it during fruit ripening. Pectinases are produced by fungi, yeast, bacteria, protozoa, insects, nematodes and plants (Whitaker, 1991; Ahmed, *et. al.,* 1997; Solis *et. al.,* 1997).

They are widely used in the food industry to improve cloud stability in fruits and vegetable nectars (Baker and Bruemmer, 1972; Gupta *et. al.,* 1993). Pectinases are also used in the modification of pectins, processing of natural fibres in textile industry (Akin *et al* 2001; Baracat, et. *al*., 1991; Sawada and Ueda, 2001;Csiszar ,et .al., 2001 and haze removal from wines (Rombouts and Pilnik, 1978; Bauman, 1981; Kilara 1982).

Production of pectinases can be done by both solid state cultures and submerged fermentation techniques. (Meyrath and Volavsek, 1975, Akinola and Olatunji, 2000, Sebastian *et.al*.,1996;

Ronald *et. al* .,1997; TuttoBello and Mill, 1961). However, production of pectinase by *Aspergillus* strains was observed to be higher in solid state fermentation than in sub-merged process (Solis-Pereira, *et. al*., 1996; Maldonado *et. al*., 1998; Acuna-Arguelles, *et. al.,* 1995). Genetic expression of pectinases from *Aspergillus niger* in solid state and sub-merged fermentations was found to be dependent on physiological conditions such as composition of culture media, pH, temperature and duration of cultivation (Sebastian, *et. al.,* 1996; Natalia, et. al., 2004)

Solid state fermentation is generally defined as the cultivation of micro organisms on solid materials in the absence or near absence of free water (Sanzo, *et. al.,* 2001). This process has several advantages over sub-merged fermentation including the ability to reach high product concentrations and the production of less liquid effluents, although the control of pH, temperature and oxygen tension can be difficult (Cannel and Moo-Young, 1980;Costa, *et al.* 1998; Castilho, *et. al.,* 2000).

Aspergillus niger is the main micro organism used for the production of pectinases, and has been found to be safe owing to its wide use in the food industry (http://www.epa.gov/biotech-rule/pubs/fra/fra006.htm).

Pectin, the natural substrate of Pectinase is synthesized in the golgi bodies of plants and forms a matrix in which the hemicellulose polysaccharides of the plant cell are embedded. In fruits, it is broken down by pectinase to pectinic acid and finally pectic acid. During this chemical breakdown the fruit gets softer (ripens) as the cell wall degenerates (McGraw, 1987).

Enzyme immobilization in the food processing industry helps in the development of continuous process, allowing more economic organization of the operations, automation and decrease of labor. Immobilized enzymes give products that are relatively pure, which is an important factor especially in food processing and pharmaceutical industries where contamination could be of serious toxicological, sensory or immunological consequences. The other advantages of immobilization are greater control over reactions and high volumetric productivity (Hartmier,1988). Quite a number of techniques and supports both synthetic and natural are available for immobilization of enzymes.

The choice of support and/or technique depends on the nature of the enzyme, its substrate and ultimate application.

Nigeria imported food processing enzymes to the tune of N1,764,717,474 in 2001 to service the food and beverage processing industries (National Bureau of Statistics, 2001). Enormous amount of foreign exchange was lost in this regard. There is no enzyme production plant in Nigeria (Bello, 2000) and in most parts of the Country fruits are produced in abundance but not processed. Consequently, spoilage is high due to inadequate local processing and preservation. Attempts have been made to immobilize commercial pectinase preparations on various supports for fruit processing (Sohel *et. al.,* 2007; Deviet *et. al.,* 2004) and endo- Polygalacturonase(Pier *et. al.,* 2004). However, despite its potentials, immobilization of Polygalacturonase isolated from a local strain of *Aspergillus* (SA6) has not been attempted.

Therefore, there is the need to produce Polygalacturonase locally so as to conserve the nation's scarce foreign exchange. Also, it is desirable to purify and characterize the locally isolated Polygalacturonase since commercial pectinases are often mixtures of cellulases, hemicellulases and proteases hence, they are poorly characterized. Similarly there is the need to immobilize and characterize the enzyme. This would provide empirical data for future research and applications.

It is against this background that this study was designed in order to isolate, partially purify, characterize and immobilize Polygalacturonase from a local strain of *Aspergillus niger* (SA6). This is for possible use in fruit processing industries.

The specific objectives of this study are:

1) To partially purify and characterize Polygalacturonase from *Aspergillus niger* (SA 6).
2) To immobilize the enzyme.
3) To characterize the immobilized Polygalacturonase and compare its activity with that of the native enzyme.
4) To evaluate its suitability or otherwise for use in fruit processing.

CHAPTER TWO:

2.0 LITERATURE REVIEW

2.1 PECTINASES

Pectinases, also called pectinolytic enzymes are a group of enzymes endo and exopolygalacturonase, (EC. 3.2.1.15 and EC. 3.2.1.67)., Polymethylgalacturonate lyase (EC. 4.2.2.10) and Pectin esterase (EC. 3.1.1.11) that can degrade pectin containing substrate or modify it during fruit ripening. They are widely used in the food industry to improve cloud stability in fruits and vegetable nectars (Baker and Bruemmer, 1972; Gupta *et. al.,* 1993). Pectinases are produced by fungi, yeast, bacteria, protozoa, insects, nematodes and plants (Whitaker, 1991; Ahmed, *et. al.,* 1997; Solis *et. al.,* 1997). Pectinases are known to belong to the family 28 in the classification of glycosyl hydrolases (Pikersgill *et. al.,* 1998). They are classified according to their way of action on the galacturonan part of the pectin molecule. Commercial pectinase is often used with cellulases, hemicellulases and proteases. A large variety of pectic enzymes are commercially available in both liquid and solid forms. Production of pectinases can be done by both solid state cultures and submerged fermentation techniques. (Meyrath and Volavsek, 1975, Akinola and Olatunji, 2000, Sebastian *et.al*.,1996; Ronald *et. al* .,1997; TuttoBello and Mill, 1961).

Pectinases show different substrate specificity. Beside the main pectin degrading enzymes, protopectinases which convert insoluble protopectin into soluble pectin have also been found (Iguchi, *et. al.,* 1997). However, due to high sequence homology with endo polygalacturonases this class of enzymes most probably represents certain types of polygalacturonases.

2.2 CLASSIFICATION OF PECTINASES

Pectinases are classified according to the way they act on the galacturonan part of pectin molecule. Pectin methylesterase, de-esterify pectin to produce pectic acid and methanol, Polygalacturonase split glycosidic linkages next to free carboxylic groups by hydrolysis. Those enzymes that have a terminal mechanism of action are distinguished by the prefix ‘exo’ while those with random mechanism of action are distinguished by the prefix” endo”

Pectin lyase split glycosidic linkage next to free carboxylic group by β-elimination (Sarkanen, 1991, Pilnik and Voragen, 1993).

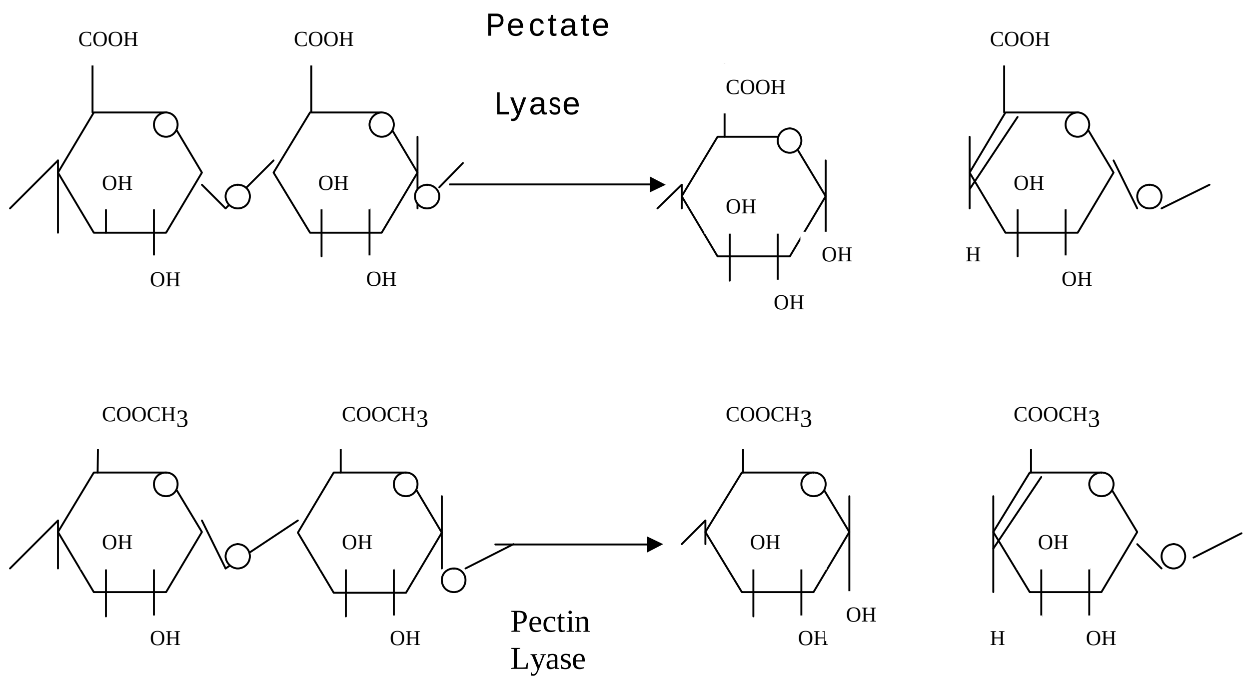


Figure 2.1 Alpha-1,4 glycosidic Bond Cleavage of Pectin Molecule by Pectate lyase and Pectin lyase.

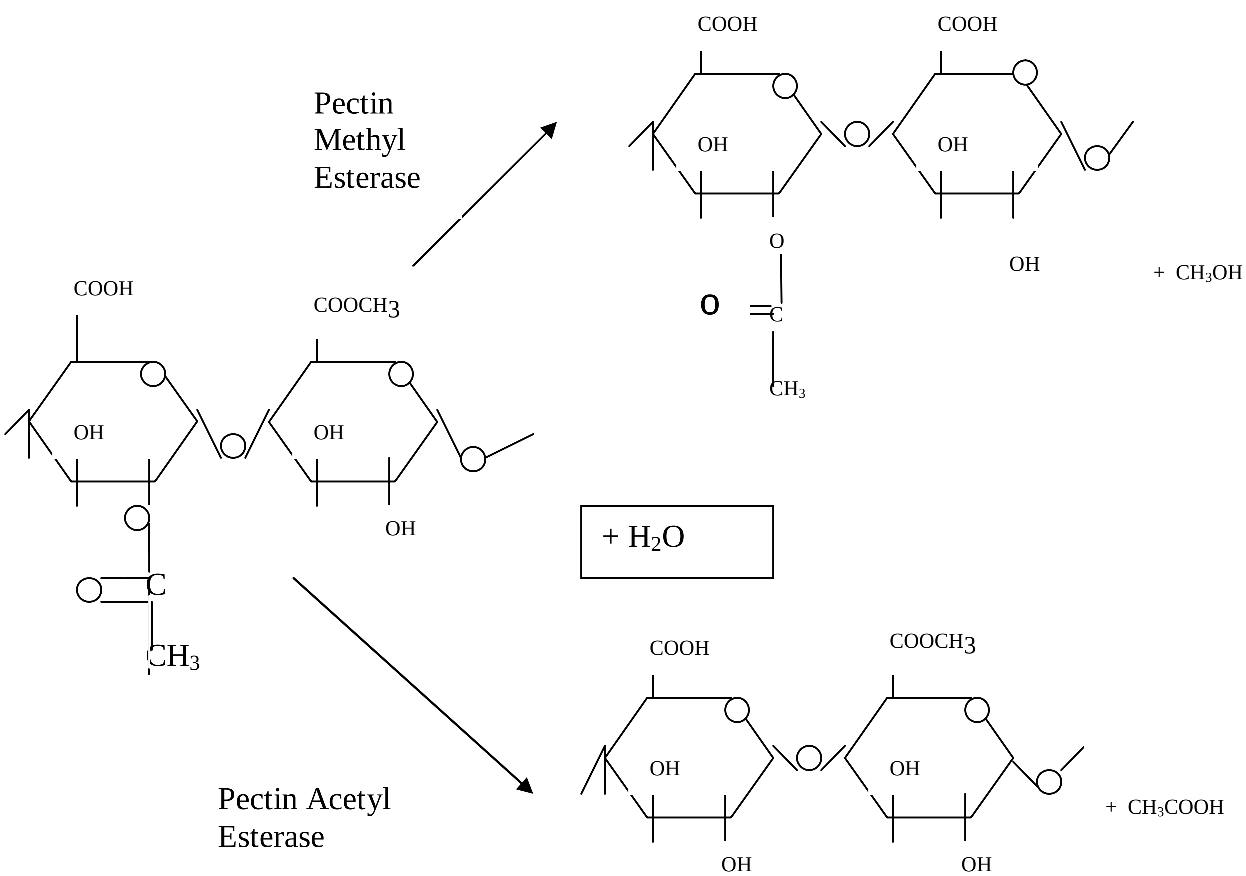


Figure 2.2 De-esterification Reactions of Pectin by Pectin Methyl and Acetyl Esterase

2.3 POLYGALACTURONASES

Polygalacturonases (EC. 3.2.1.15 and EC. 3.2.1.67 for endo and exopolygalacturonase can be divided into three groups. Endopolygalacturonases randomly attack the alpha 1,4 glycosidic bond of pectin chain producing galacturonic acid oligomers, exopolygalacturonase type 1 hydrolyses D-galacturonic acid from the non-reducing end, and exopolygalacturonase type II releases di-galacturonate from the non-reducing end of polygalacturonic acid. Endopolyglacturonases and exopolygalacturonases of type 1 were identified in fungi. To date only two exopolygalacturonases of type II were isolated from bacteria Erwinia Chrysanthemi, EC16 and 3937 strains (He and Collmer, 1990; Shevchik *et. al.,* 1999).

Polygalacturonases contain an 8-10 turn right handed parallel β-helix domain with two loop regions forming a tunnel – like substrate binding cleft. Studies have shown that the active site of polygalacturonases enables two conserved aspartates and lysines at its centre to act as catalytic residues. Arginines and lysines line the sides of the substrate-binding cleft, making the overall electro-static potential in the substrate binding cleft positive. In some of the enzymes, a histidine residue near the active site is believed to influence its catalytic efficiency. The conserved aspartates may play a role as catalytic base and proton donor in the reaction mechanism (Pikersgill, *et. al* .,1998).

Suryakant *et. al.,* (2001) has also isolated and characterized a single extra-cellular endopolygalacturonase from *Fusarium moniliforme* with tryptophan residue involved in substrate binding and an Arginine residue at or near the active site possibly involved in extended binding of the substrate. A carboxylate and a histidine residues involved in catalysis.

2.3.1 POLYGALACTURONASE ENCODING GENES AND FAMILIES

Kester and Vsser, (1990) isolated five different endopolygalacturonases and one exopolygalacturonase from a commercial pectinase K2B 078 from *Aspergillus niger*. The five polygalacturonases were purified to homogeneity and their physico-chemical characteristics suggested that they may be encoded by five different genes. Two of the genes PGI and PGII were found to be the most abundant. Bussink *et. al.,* (1990) also isolated the PGII encoding gene (pgaII) from *Aspergillus niger* using reverse genetics. In the same vein, Bussink, *et. al.,* (1991) isolated pga I gene. Studies have also shown that in a search for new endopolygalacturonase encoding genes in *Aspergillus niger* N400 genomic library was screened using a pga II derived probe. In addition to pga I and pga II, five different pga II related – classes of hybridizing phages (A-E) were identified, indicating the presence of an endo-polygalacturonase-encoding family in *Aspergillus niger*. Bussink,et. al.,(1992) isolated the third PG encoding gene pgaC using molecular analysis.

Apart from the *Aspergillus niger* pga genes, PG-encoding genes were isolated from other *Aspergilli* using PCR heterologous screening of a DNA library with a fungal pga probe (Table 1) it was shown that the structural features of the pga genes among the different *Aspergilli* are highly conserved. This is supported by a similar size of the enzymes (362-383) amino acids encoded and by the exon-intron organisation.

The endopolygalacturonase encoding genes isolated from Aspergilli so far contain one to three introns, which interrupt the coding region at identical positions (Lucie, 2000)

Table 2.1 Some PG-Encoding Sequences From Various Aspergilli as Present in the Nucleotide Sequence/Protein Data Bases

Organism	Gene	Enzyme purified	GeneBank/ EMBL Accession Number	Swiss Port/PIR/GP accession number	PDB. Crystal Data	Length (a.a)	MWt (Dalton)	Gene type
A.acculeutus	pgal	-	AF054893	-	-	378	38533	-
A. flavus	PecA PecB	- -	U05015 U05020	P41749 P41750	- -	363 366	37464 37877	II II
A- niger	PgaI	+	X58892	P26213	-	368	38108	II
	PgaII	+	X58893	P26214	ICZF	362	37684	I
	PgaC	+	X64356	Q12554	-	383	40502	III
A. Oryzae	pgaA	+	D14282	P35335	-	363	37520	II
	pgaB	+	AB007769	-	-	367	37864	II
A Parasiticus	PecA	-	U17167 L23523	P49575	-	363	37506	II
A. Tubingensis	PgaII	-	X58894	P19805	-	362	37512	I
A.niger RH5344		+	X52902 X54146	-	-	362	37512	I

Source: Lucie (2000).

+ indicates that the protein was isolated and characterized. The amino acid composition of the primary sequence is deduced from the pga coding region based on the sequence homology and allows to calculate the molecular mass of the protein.

aa = amino acid

From table 2.1 it can be seen that three genes I II & C were isolated and characterized from *Aspergillus niger*. Ten different polygalacturonase encoding genes were isolated from six different *Aspergilli* with a molecular weight range of 38108-40502 Dalton and amino acid residues of 368-383. The table shows the number and diversity of PG encoding genes in *Aspergillus species*. Other pga genes isolated included that from yeasts *Kluyveromyces Marxianus* (Siekstele, *et. al.,* 1999) and *Saccharomyces cerevisiae* (Blanco, *e.t al.,* 1998) different species of penecillium (*P. expansum*,, http:11 atmb.cnrs-mrs.fr/-pedro/cazy/ghf 28.html) *P. janthinelum* (Ishida *et. al.,* 1997), and *P. o losonii* , http://afmb. cnrs-mrs.fr/pedro/ CAZY/ghf 28. htm) and a number of phytopathogenic fungi such as *Sclerotinia sclerotiorum* (Fraissinet *et. al.,* 1995) and *Botrytis cinerea*

(Wubben, *et. al.,* 1999) in which an endopolygalacturonase encoding gene family was identified.

In bacteria the length of PGS ranges from 312 amino acids in *Agrobacterium tumefaciens* (Rong *et. al.,* 1991) to 529 in *Pseudomonas salanacearum* (Huang and Schell 1990). It was shown that plant PG's contain from 365 in *Lycopersicum esculeatum* to 514 amino acid residues in *Cryptomeria Japonica* (Hong and Tucker, 1998 ;Komiyama *et. al.,* 1994)

2.3.2 PHYSICO – CHEMICAL CHARACTERISTICS OF SOME FUNGAL POLYGALACTORONASES

Using recombinant DNA technology, polygalaturonase from *Aspergillus* have been successfully over produced and purified to homogeneity.The *Aspergillus niger*RH5344 endopolygalacturonase was expressed under the alcohol dehydrogenase promoter in *S. cerevisiae* yielding 180mg/L of the Protein (Long and Looman,1995) and a relatively high level of expression.

Table 2.2 **Physico-Chemical Characteristics of some fungal polygalacturonases**

Source of Enzyme	Gene isolated	Type of activity	PI	T_{opt}	A_{spec} U/mg Protein	pH_{opt}	K_M (mg/ml)
A. niger 1[a] *11*[a] *111*[a]	- - -	ENDO ENDO ENDO	3.8 - 4.5	- - -	- - -	4.0 4.5 5.5	- - 1.7
A. niger 1[a] *11*[a]	- -	ENDO ENDO	- -	- -	81 44	4.1 3.8	- -
A. niger	-	ENDO	-	-	75	5.0	0.54
A. Japonocas	-	ENDO	-	-	1362	4.5	-
Botrytis Cinerea	-	ENDO	-	-	2049	4.0	1.2
Trichoderma 1[b] *Koningii 11*[b]	- -	ENDO ENDO	6.41 6.57	- -	- -	5.0 5.0	0.8 0.85
Verticillium albo-artrum	-	ENDO	-	-	-	6.5	1.5
A. niger 1 *II* *C*	+ + +	ENDO ENDO ENDO	3.2 – 3.5 4.6 – 5.9 -	- - -	550 2760 24.9	4.2 4.2 4.2	<0.15 <0.15 <0.15
A..Oryzae A *B*	+ +	ENDO ENDO	- -	45°c 55°c	- -	5.0 5.0	- -
A Carbonarius I *II* *III*	- - -	- - -	- - -	55°c 50°c 55°c	640 7000 550	4.0 4.1 4.3	- - -
A. Tubingensis	+	EXO	3.7-4.4	-	255/262	4.2	3.1/3.2
N. Crassa	-	ENDO	-	45°c	299	6.0	5.0
Alternaaria Alternata	-	ENDO	8.8	-	12.4v	5.0	0.1
Fusarum Oxysporum	-	EXO	6.4	-	8.8	5.0	-

a =multiple forms **b**= 1so enzymes, glycoproteins **V**=specific activity in Viscometric units

T_{opt} = Optimum temperature, **pH opt** = Optimum pH **A_{spec}** = specific activity

Source: Lucie (2000).

From the data in table 2.2 it can be deduced that fungal polygalacturonases are generally active under acidic conditions with temperature optimum of about 40°C to 50°C.

Michaelis constant values for polygalacturonic acid range from <0.15 to 5mg/ml and the specific activities of 8.8 to 7000 U/mg of protein. Polygalacturonases usually retain a low level of enzymatic activity when pectin with degree of esterification (DE) greater than 50 % used as substrate. De Vries and Visser, (2001) also reviewed physico-chemical properties of some fungal endopolygalacturonases as presented in table 2.3.

Table.2.3 Physico-Chemical Properties of Endopolygalacturonase fron Aspergillus niger

A.niger strain and type of enzyme	Enzyme	M.Mass (KDa)	pH Opt	P1
Endopolygalacturonase				
A. niger	E1	35	4.1	-
A. niger	E2	80	3.8	-
A. niger	Endo – 1	55	4.9	3.2-3.5
A. niger	PLB	40	8.5-9.0	5.0-9.0
A. niger	PL1	37.5	-	3.65
A. niger	PL II	37.5	-	3.75
A. niger	PLY A.	43	7.5 – 8.5	-

pH opt = Optimum pH, **P^I** = Iso electric point.

Source: De vries and Visser (2001)

From table 2.3 it can be shown that except for PLB & PLYA which have a pH optimum in the alkaline region all other endopolygalacturonases operate in acidic condition with optimum pH ranging from 4.1 – 4.9. The molecular masses of the Endopolygalacturonases range from 35 – 80 KDa while the iso electric point is from 3.2 – 9.0. These values indicate some variations in the physico-chemical properties of fungal endopolygalactuonases from different sources. Although fungal polygalactoronases share a high degree of sequence identity, their hydrolysis rates and mode of action on pectin and on oligomeric substrates can differ remarkably. This has been demonstrated (Heinrichova and Rexova – Benkova, 1976; Rexova – Benkova, 1973) using oligogalacturonides of different chain length (n= 2-6) and analyzing their reduced equivalents.

The degradation of modified pectins by polygalacturonases of different origin revealed that methylesters and acetyl groups limit their action to different extent (Pasculli, *et. al.,* 1991). Detailed kinetic studies of the *Aspergillus niger* polygalcturonases PGI II & C revealed some important differences among the members of the family (Benen, *et. al*.1999). While all the enzymes are composed of at least seven identified active subsites, they differ in the bond cleavage frequencies on galacturonic acid oligomer and in the level of pectin degradation. These results are important for understanding the in vivo behaviour of the individual members of the endopolygalacturonase-encoding gene family of the fungus.

2.4 PRODUCTION OF PECTINASES

Pectinases have been produced from *Aspergillus* species using both solid state cultures and sub-merged fermentation techniques (Meyrath and Volavsek, 1975, Akinola and Olatunji, 2000; Sebastian, *et. al.,* 1996, Ronald, *et. al.,* 1997; Tuttobello and Mill, 1961). Solid state fermentation is generally defined as the cultivation of micro organisms on solid materials in the absence or near absence of free water (Sanzo, *et. al.,* 2001). This process has several advantages including the ability to reach high product concentrations and the production of less liquid effluents, although the control of pH, temperature and oxygen tension can be difficult (Cannel and Moo-Young, 1980; Costa, *e.t al.* 1998; Castilho, *et. al.,* 2000).

Production of pectinase by *Aspergillus* strains have been observed to be higher in solid state fermentation than in sub-merged process (Solis-Pereira, *et. al*.,1996; Maldonado *et. al* .,1998; Acuna-Arguelles, *et. al.,* 1995). Several bacteria and fungi are known to produce a wide array of pectinases with varying physico-chemical properties (Alkorta, *et. al.,* 1998).

Genetic expression of pectinases from *Aspergillus niger* in solid state and sub-merged fermentations is dependent on physiological conditions such as composition of culture media, pH, temperature and duration of cultivation. It was observed that *Aspergillus niger* produce high amounts of pectinase in solid state fermentation on pectin within 1 – 2 days after inoculation and the synthesis of enzymes was less sensitive to carbon repression compared to sub-merged fermentation (Solis-Pereira, et. al., 1993; Maldonado and Saad, 1998). Complex substrates such as citrus pectin (Galiotou, *et. al.,* 1993; Galiotou, *et. al.,* 1997) and wheat bran (Cavalito, *et. al.,* 1996) have been observed to favor polygalacturonase production at lower pH (3-4) with variation in the composition of the pectinolytic enzymes produced. Maldonado and Saad, (1993) reported that pectin, polygalacturonic acid and galacturonic acids induced synthesis of pectin methyl esterase and polygalacturonase in *Aspergillus niger* by increasing transcription, whereas glucose repress the synthesis of both enzymes by a carbon repression mechanism that occurs at the translational level. Pectinase production by different fungal strains using solid state fermentation is presented in table 2.4.

Table 2.4. Pectinase Production by Different Fungal Strains using Solid State Fermentation.

Strain	PL (U/g)		PG (U/g)	
	A	B	A	B
Moniella SP. SB9	16800.0	18740.0	3.2	21.0
Penicullium Sp EGC5	2540.0	9250.0	8.7	11.0
P. Viridicatum RFC 3	Nd	656.0	1.5	19.0
P. glabum F1	51.0	Nd	14.4	5.1
Penicillum SP. RFC2	10002.0	Nd	7.2	10.2
P. Italicum	876.0	Nd	16.0	16.0
P. Citrinum	602.0	Nd	7.8	3.0
Curvularia inequalis Emp. 11-1	372.0	Nd	2.0	15.0
Curvularia SP. 5.11.1	Nd	Nd	2.0	3.0
Aspergillus Sp. EG66F	894.0	83.0	10.2	15.0
Aspergillus SP. EGC4	330.0	1518.0	2.4	11.4
A – niger	39.0	288.0	3.6	9.0
Thermoascus SP. 179.5	368.0	222.0	27.0	34.0
Aureobasidium SP. RE	Nd	81.0	1.0	9.0
Phanero Chaetes SP. 291	Nd	720.0	6.0	21.0
Cladosporium SP RFCI	Nd	56.0	8.4	11.0

Medium A = Sugarcane bagasse 90 % Wheat bran 10 %

Medium B = Wheat bran 90% sugarcane bagasse 10%

Nd = Not detected.

Source: Natalia, *e.t al.,* 2004.

2.5 INDUSTRIAL APPLICATION OF PECTINASES

The first use of pectinase in fruit juice processing dates back to the 1930's (McMillan and Sheiman, 1974; Nelson, 1991). Steady supplies of purified standardized pectic enzymes have been commercially available for about fifty years. Commercially produced pectinase is often a mixture of cellulases, hemicellulases and proteases, which are available both in liquid and solid forms.

Aspergillus niger is the main micro organism used for the production of pectinases in the industry because it has been found to be safe over the years (Canal-LLauberes,1993; Gramp and Urlaub, 1984). Pectinases are used in the food industry to improve cloud stability of fruits and vegetable nectars (Baker and Bruemmer, 1972; Gupta, *et. al.,* 1993). Pectinases are also used in the modification of pectins, processing of natural fibres in textile industry (Akin *et al* 2001;Baracat, *et. al* ., 1991; Sawada and Ueda, 2001;Csiszar ,et .al., 2001 and haze removal from wines (Rombouts and Pilnik, 1978; Bauman, 1981; Kilara 1982). In determining the standard of natural juice, juice extracted using pectinase is usually considered as minimally processed(Haight and Gump, 1995). Pectinases account for 10 % of the global industrial enzymes production (Stutzenberger, 1992). It has been self declared “Generally Recognized As Safe” (GRAS) by the Enzyme Technical Association (ETA, 1999).

2.6 PECTIN

Pectin was first isolated in the 1820s and the first production of liquid pectin extract was recorded in 1908 in Germany. The process later spread rapidly to the United States, where a classic patent (US Patent 1,082,682,1913) was obtained by Douglas(http://www.ippa.info/history-of-pectin.htm)

In recent years the center of production has moved to Europe and citrus producing countries such as Mexico and Brazil. Pectin is one of the most versatile stabilizers. It’s gelling, thickening and stabilizing attributes makes it an essential additive in the production of many food products such as jams and fruit jellies (http://www.ippa.info/applications-for-pectin.htm). Medically, it alienates symptoms of sore throat and diarrhea. Pectin is a heteropolysaccharide found in the middle lamella and the primary cell wall of high plants. It functions as a "glue" that holds the other cell wall polysaccharides such as cellulose and hemicellulose and the hydroxyproline-rich glycoprotein (extension) together. Two anti parallel pectin chains can be condensed in the cell wall by crosslinking with Ca^{2+} ions to form "junction zones" Carpita and Gibeaut, (1993) reported that in some species pectins may be cross-linked to other pectins or non-cellulosic polysaccharides by ester linkages with dihydroxycinnamic acids such as deferulic acid. Pectins are found in all stages of development of plants. The amount depends on the maturity, degree of ripeness, variety and storage conditions after harvesting (Reed, 1975). Pectins have been given an Acceptable Daily Intake (ADI) of "Not specified" by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) and are listed on that basis in the Codex General Standards on Food Additives (http//www.ippa.info/safety.htm).

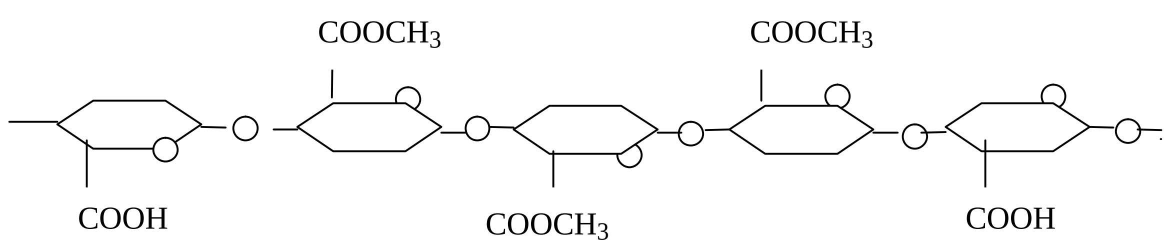
2.7 SOURCES OF PECTIN

Commercially, the primary source of pectin is the peel of citrus fruits such as lemon, orange lime, grape etc. The secondary source is the apple pomace and sunflower heads (McGraw , 1987). It is extracted by adding hot acid to dissolve the pectin followed by its precipitation with ethanol, methanol or propanol Pectins are synthesized in the golgi bodies of Plants and forms a matrix in which the hemicellulose polysaccharides of the plant cell are embedded. It is broken down by pectinase to pectinic acid and finally pectic acid. During this chemical breakdown the fruit gets softer (ripens) as the cell wall degenerates (McGraw , 1987).

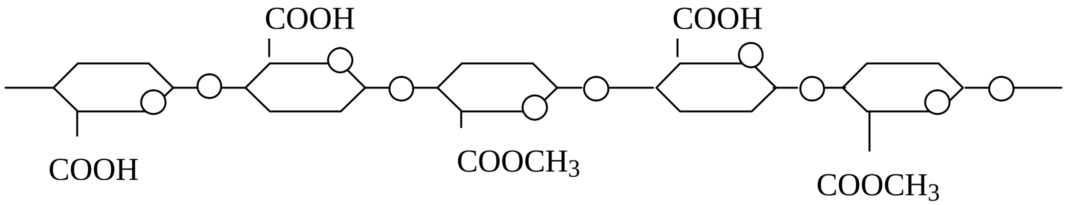
2.8 PHYSICO-CHEMICAL PROPERTIES OF PECTIN

Pectin is structurally a heteropolysaccharide with smooth regions of alpha-1,4 linked D-galacturonic acid residues of which approximately 70 % of the carboxylic groups are methyl esterified. Beside these homogalacturorian residues, the polysaccharide also contains highly branched regions with rhamnose and D-galacturonate as the alternating constituents in which rhamnose can be found to be highly substituted with galactose or long branched arabinose side chains. The D-galacturonate moieties can be methyl esterified, o-acetylated or substituted with xylose.

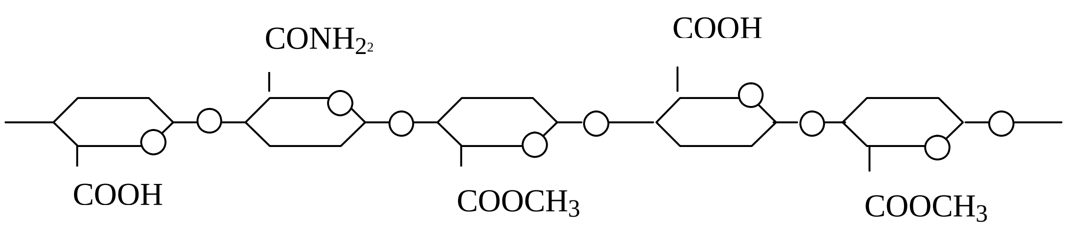
Pectins range in molecular size from 30,000 – 300,000 Dalton. The gel forming properties of pectins are largely dependent on their methyl ester groups. Their classification is thus based on degree of methylation. Those with methyl content of 70 % and above are called high methoxyl or (rapid set) pectins, while those with methyl content of less than 50 % are called low methoxyl pectins. Intermediate values of 60 – 65 % are characteristic of slow set pectins. Pectins can be treated with ammonia to produce amidated pectins



High Methoxyl Pectin (HM- Pectin)



Low Methoxyl Pectin (LM-pectin)



Amidated pectin

Source: http://www.ippa.infor/types-of-pectin.htm

2.9 INDUSTRIAL APPLICATIONS OF PECTIN

Pectins have a broad range of applications such as gelling agent, thickener, texturizer, emulsifier, fat or sugar replacer in low calories foods and as a component of many medicines. Pectins are used in many industries because of their ability to form gels.

Pectin is also used in medicine because of its anti diarrhea effect, for lowering of blood cholesterol levels and as a natural prophylactic substance against poisoning with toxic cations. Because of its biodegradable and recyclable property, pectin films are being used beyond food and pharmaceutical industries.

The degree of polymerization (DP) and the degree of methyl esterification (DE) determine the condition of use. Based on the number of the methyl esterfied galacturonic acid residues pectins are classified for use as low and high methyl esterified pectins. The low methyl esterified (LM) pectin is obtained by treatment of high methyl esterified (HM) pectin with mild acidic or alkaline conditions. Treatment of LM pectins with ammonia yield amidated pectins. The degree of methylation determines the gelling properties of pectin. In LM pectins the gel is formed in the presence of Ca^{2+} ions over a wide pH range (1.0 – 7.0) or higher (http: //www.herc.com/foodgums/pectin). In the HM pectin gelling requires formation of interaction zones by hydrogen bonds and hydrophobic interactions of the methyl ester groups among the molecules (Thakur, *et. al.,* 1997). This reaction requires a pH of around 3.0 and the presence of sucrose at concentrations exceeding 55 % by weight. Thus, the textural properties of pectin gels are determined by their degree of esterification, the sugar composition of the side chains, the degree of acetylation, amidation and cross-linking of pectins.

Based on these properties about 80 % of the world production of HM pectin is used in the manufacturing of jams and jellies. Other applications are in the preparation of fruit drink concentrates and fruit juices. HM-pectin has been shown to prevent the formation of casein clumps in acidified dairy products at pH below the iso-electric pH (4.6). The LM-Pectin is used in combination with carrageenan to produce sugar free jams for diabetics and in fruit preparation for yoghurt. The Ca^{2+} ion requirement of the LM pectin is used in gelation of fruit and milk deserts (http/www.herc.com/foodgums/pectin).

2.10 ASPERGILLUS SPECIE

The origin of the name *Aspergillus* (rough head) dates back to the 18th century, when Micheli described a mould with conidiophores and heads bearing chains of spores Micheli (1729). It took almost 200 years before a first taxonomic key for the classification of the aspergilli appeared(Thom and Church ,1926). The major criteria for the classification of *Aspergillus* isolates were

(1) Colour of conidial heads

(II) Stalk surface and colour

(III) Shape of vesicules and

(iv) Absence or presence of metulae.

This criteria allowed the separation of 13 groups among the isolates; *Aspergillus niger* was classified as one of the black *Aspergilli* which also contained metulae and phialides. However, imperfect filamentous fungi such as the black *aspergilli* exhibit a high degree of variability in their morphological characteristics often caused by the external growth conditions (Samson , 1994). In the past, this led to frequent changes of specie names and re-classification of individual isolates. Table 5 shows the changes in the classification of the genus *Aspergillus* based on the morphological criteria since 1945.

Table 2.5 Over view of the classification of the black Aspergilli based on Morphological criteria.(1945-1994)

The names in parentheses represent the synonyms.

Thom & Raper 1945	Raper& Fennel 1965	Al- Musallam 1980	Samson 1994
-	*A. tubigensis*	-	*A. tubigensis*
A-niger	*A-niger* *(A fumaricus)*	*A-niger & varicant* *A-foetidus*	*A-niger*
A. foetidus	*A. foetidus* *Var. pallidus (subfuscus) var. Accidus*		-
A. awamori	*A. awamori* *A. miyakoensis A. luchuensis*	- -	-
A. miyakoensis	-	-	-
A. Phoenicis	*A. phoenicis*	-	-
A. Pulvurulentus	*A. pulvurulentus*	-	-
A-niger mut. Cinnamomeus	-	-	-

A-niger mut. Schiemanni	-	-	
-	*A. ficuum*	-	-
-	*A. ellipticus*	*A. elipticus*	-
-	*A. heteromorphus*	*A. heteromorphus*	*A. ellipicus*
A. artroporpureus	-	*A. helicotrix*	*A. heteromorphus*
A. fonsecaus	-	-	-
A. fumaricus	-	-	-
A. carbonarius	-	-	-
A. luchuensis	*A. carbonarius*	*A. carbonarius*	-
A. Japonicus	-	-	*A.carbonarius*
A. Violaceus fuscus	*A. Japonicus (A. nanus)*	*A. Japonicus var. Japonicus var. Aculeatus*	-
-	-	-	*A. Japonicus*
A. nanus	*A. acuteatus (A. violaceus-fuscus)*	-	-
A. Subfuscus	-	-	-
	-	-	-
		-	-

Source: Lucie, 2000

It is evident from table 2.5 that the *Aspergillus* group is a very diverse group with wide ranging isolates. It is note worthy however, that there seems to be consensus on the key members of the group by all the researchers. These members are *Aspergillus niger*, *Aspergillus carbonarius* and *Aspergillus Japonicus* and to a lesser extent *Aspergillus foetidus*, *Aspergillus ellipticus*, and *Aspergillus heteromorphus*.

A more sophisticated means of classification of fungi has been attempted. This method which employs molecular biological techniques in taxonomic classification focus on nucleotide base to base molar percentage, DNA-DNA complementarity, ribosomal RNA sequence comparison and restriction fragment length polymorphism. Kozowski and Stepien (1982) studied restriction digests of mitochondrial DNA and found that all the *Aspergillus* groups examined are related.

2.11 ASPERGILLUS NIGER

Raper and Fennel, (1965) designated 15 species as comprising the *Aspergillus niger* grouping, which includes all the aspergilli with black conidia. The major distinction separating *Aspergillus niger* from other species of *Aspergillus* is the production of black or very dark brown spores from biseriate phialides Raper and Fennel, (1965). Other features include the smooth and generally colorless conidiophores. *Aspergillus niger* isolates grow slowly on Czapek agar. These physical characteristics such as spore colour and rate of growth on defined media are subject to change, especially under extended pure culture or selection and mutation.

Reports associating *Aspergillus niger* with infectious disease in healthy individuals are uncommon, although *Aspergillus niger* is a recognized opportunistic pathogen.

2.12 INDUSTRIAL APPLICATION OF *ASPERGILLUS NIGER*

The primary uses of *Aspergillus niger* are for the production of enzymes and organic acids by fermentation. The history of safe use of *Aspergillus niger* is due to its use in the food industry for the production of many enzymes such as pectinases, alpha-amylases, amyloglucosidase, cellulases, lactase, invertase, and acid proteases (http://www.epa.gov/biotech-rule/pubs/fra/fra006.htm). Citric acid fermentation using *Aspergillus niger* is carried out commercially in both surface culture and sub-merged processes (Kubicek and Rohr, 1986).

Due to its ease of visualization and resistance to several anti fungal agents, Aspergillus *niger* is being used to test the efficacy of preservative treatments (Jong and Grantt, 1987). It has also been shown to be sensitive to micronutrient deficiencies making it possible for its use in soil testing. *Aspergillus niger* is also being considered to perform certain enzymatic reaction that are very difficult to accomplish by strict chemical means such as specific additions of steroids and other complex rings (Jong and Gautt, 1987).

2.13 ENZYME IMMOBILIZATION TECHNIQUES

Enzyme immobilization techniques have been broadly classified into four namely entrapment, covalent binding, cross-linking and adsorption. However, economy of the process depends on productivity which is the activity integrated over operational time. Enzymes can be immobilized in a continuous or batch process.

The main advantages of continuous process over a batch process are the ease of automation and control. The enzyme , stability and volumetric activity are some of the variables that are routinely evaluated to indicate the effectiveness of a proposed immobilization technique. As in any heterogenous catalysis system, both external and internal diffusion of substrate and product can greatly affect the reaction rate. Also due to mass transfer effects, particle shape, particle size, pore size, enzyme loading per particle and substrate flow rate can all affect the reaction rate.

The mass transfer limitation arising from immobilization is manifested in an increase in the apparent value of the Michaelis-Menten constant (K_M), thus the value of this constant as compared to the corresponding intrinsic value of the soluble enzyme is often recognized as a good indicator of the extent of mass transfer resistance. (http.//www.glue.umd.edu/-nsw/ench485/lab76-htm). Thus, it is important to recognize the difference between intrinsic and apparent parameters. Constants such as temperature and pH optima for immobilized enzymes may be different from those of the soluble enzymes. The presence of positive or negative charges in the matrix can lead to a shift in the optimum pH. Similarly, the binding of enzymes can restrict their movement leading to a shift in the temperature optimum.

The immobilized enzyme must remain stable with respect to disturbances in the environment and quickly recover with minimum disruption in productivity. If loss of activity is recorded due to such disturbance it may necessitate repacking of the entire column.

2.13.1 ENTRAPMENT

Enzymes have been entrapped using natural polymers such as agar, agarose and gelatine through thermo reversal polymerization and in alginate and carrageenan by ionotropic gelation (Tampion and Tampion, 1987; D'Souza 1989; Mosbach 1974). Some synthetic polymers such as poly uretheane, pre polymers and polyacrylamide have also been investigated (D'Souza and Nadkarni ,1981; Deshpande and D'Souza, 1988). The most widely used matrix for entrapment is polyacrylamide which is applicable more in waste treatment and fabrication of analytical devices. However, because of its toxicity polyacrylamide may not be a useful support in the food industry.

Many entrapment methods used currently are based on the physical occlusion of enzyme molecules within a "caged" gel structure such that the diffusion of the enzyme molecules to the surrounding medium is restricted. A highly cross linked gel has a fine "wire mesh" structure and can effectively hold smaller enzymes in the cages.

The degree of cross-linking depends on the condition at which polymerization is carried out. However, due to statistical variation in the mesh size some of the enzyme molecules gradually diffuse toward the outer shell of the gel and eventually leak into the surrounding medium. Furthermore, because an immobilized enzyme preparation is used for a long period of operation there is also gradual loss of intrinsic activity even for the best method, necessitating eventual repacking of the entire system. (http://www.glue.umd.ench.edu/-nsw/ench485/lab13.htm).

Apart from leakage, the other problem associated with entrapment method is the mass transfer resistance to substrates, products and incubators. Again, because the average diameter of a typical bead of enzyme impregnated gel is much larger compared to the average diffusion length, substrate can not diffuse deep into the gel matrix. Also the diffusional resistance encountered by product molecules can sometimes cause the product to accumulate near the center of the gel leading to product inhibition.

2.13.2 COVALENT BINDING

Though it is not a good technique for immobilization of cells, covalent binding has been extensively used for immobilization of enzymes. The functional groups extensively investigated are amino, carboxyl and the phenolic group of tyrosine (Hartmier, 1988; Kolot, 1981). Covalent binding has also been extensively investigated using inorganic supports (Husain and Jafri, 1995) and synthetic membranes (Weetall, 1985; Hicke *et. al.,* 1996). In this method, enzymes are covalently linked to support material through functional groups that are not involved in catalysis. It is therefore necessary to carryout immobilization in the presence of competitive inhibitor of the enzyme so as to protect the active site.

The covalent binding should be optimized so as not to alter the conformational flexibility of the enzyme. Some of these problems are solved using carbohydrate moiety in the bond formation since number of industrially useful enzymes are glycoproteins and their carbohydrate moiety is not involved in the catalysis. Functional aldehyde group can be introduced in a glycoprotein by oxidizing the carbohydrate moiety by periodate oxidation without significantly affecting the enzyme activity. The enzyme could then be covalently linked to a support containing an alkylamine group through schiffs base reaction. Enzymes such as glucose oxidase, peroxidase, and invertase have been immobilized using this technique. (Kolot ,1981; D'Urso and Fortier, 1996; Melo and D' Souza ,1992).

2.13.3 CROSS – LINKING

Immobilizing enzymes through chemical cross-linking using homo and hetero-bifuntional cross-linking agents has been demonstrated (Hartmier, 1988). In view of its generally recognized as safe (GRAS) status, glutaraldehyde, which interacts with amino groups through a base reaction has been well investigated. It is relatively cheap, highly efficient and stable (Nakajima *et al* 1993). Some enzymes or cells have been cross-linked in the presence of an inert protein such as gelatin, albumin and collagen (D'Souza 1989; Kolot, 1981).

Studies have also shown that raw hen-egg white is an economical and novel proteinic support rich in lysozyme for the immobilization of enzyme or non viable cells either in a powder (Reichin, 1980;

D'Souza and Nadkarni, 1981; D'Souza, *et. al*.,1985), bead (Kaul, *et. al.,* 1984) or highly porous foam (D'Souza, *et. al*.,1985; Marolia and D'Souza, 1993) form, using glutaraldehyde as cross-linker.

2.13.4 ADSORPTION

Adsorption is the simplest technique of immobilizing enzymes which does not grossly alter the activity of the bound enzyme. For the enzymes immobilized through ionic interactions adsorption and desorption of the enzyme depends on the basicity of the ion exchanger. A dynamic equilibrium is observed between the adsorbed enzyme and the support which is often affected by the pH as well as the ionic strength of the surrounding medium.

This kind of reversibility of binding has been used for the economic recovery of the support in the industry, especially for the resolution of racemic mixtures of amino acids using amino acid acylase (Hartmier 1988, Tampion and Tampion 1987). A variety of commercially available ion-exchangers have been investigated (Kolot, 1981). Polyethylenimine has been used for imparting polycationic characteristics to many of the neutral supports based on cellulose or inorganic materials (D'Souza

,1983). Enzymes with low iso-electric point (PI) such as invertase, urease, glucose oxidase, catalase and other enzymes have been bound through adsorption followed by cross-linking on polyethylenimine-coated supports (D'Souza, 1983; Bahuleker, *et. al* .,1991; Godbole, *et. al.,* 1990; Kamath, *et. al.* 1988). Varieties of biospecific interactions for the reversible immobilization of enzymes by adsorption has also been investigated. Such enzymes as acetylcholine esterase, ascorbic acid oxidase, invertase, peroxidase, and glucose oxidase have been immobilized by biospecific-reversible immobilization on lectin bound supports (Viera, *et. al.,* 1988, Mattiasson, 1982). Sankaran, *e.t al.,* (1989) and Sada, *et. al* .,(1987) reported immobilization of enzymes through hydrophobic interaction which is of great significance because unlike ionic binding, hydrophobic interactions are usually stabilized by high ionic concentrations, thus enabling the use of high concentrations of substrates as desired in an industrial process without fear of desorption.

Table 2.6: Support Materials for Enzyme Immobilization

Inorganic	Organic	Biological
Alumina	Activated clay	Cellulose+ derivatives +
Nickel Oxide	Polystyrene	Dextran + derivatives
Stainless Steel	Acrylics	Agarose + derivatives
Glass	Nylon	Starch + derivatives
Silica	Polyacrylamide	Alginate

Source: Bickerstaff, 1987

2.14 APPLICATION OF IMMOBILIZED ENZYMES

Tanabe Seiyaku Company of Japan used the first immobilized enzyme for the resolution of chemically synthesized amino acids. Amino acid acylase is the enzyme which catalyses the deacytylation of the L-form of the N-acetyl-amino acids leaving unaltered the N-acetyl-d amino acid that can be easily separated, racemized and recycled. Immobilization techniques used for this process include ionic binding of the enzyme to DEAE-Sephadex and its subsequent entrapment as micro droplets of its aqueous solution into fibers of cellulose triacetate. Also, Rohm GmbH of Germany has immobilized this enzyme on macro porous beads made of flexi glass-like material (Yu and Chang, 1982; Chibata ,1980).

Sharma and Messing, (1980) have reported the application of glucose isomerase in the conversion of glucose syrups into high fructose syrups. This is an important process in the food industry. Application of this technology has gained considerable importance especially in non-tropical countries that have abundant starch raw materials. Invertase is the first reported enzyme in an immobilized form (Carasik and Carroll ,1983). A large number of invertase systems have been patented (Nelson and Griffin, 1916).

The enzyme aspartase, which catalyses a one step stereo-specific addition of ammonia to the double bond of Fumaric acid to form L-aspatic acid has been immobilized using whole cells of *E.coli*. This process is considered the first industrial application of an immobilized microbial cell. The initial process made use of polyacrylamide entrapment which was later substituted with carageenan treated with glutaraldehyde and hexamethylenediamine.

Tanabe Sayaku and Kyowa Hakko of Japan have used immobilized Fumarase for the production of Malic acid for pharmaceutical use (Chibata ,1980).

In the diary industry immobilized β. galactosidase have been used in the preparation of lactose-hydrolysed milk and whey. Lactose-hydrolysed whey may be used as a component of whey-based beverages, leavening agents, feed stuffs or may be fermented to ethanol and yeast. The first company to hydrolyse lactose in milk using immobilized lactase was Centrale del latte of Milan Italy, utilizing the Snam progetti technology. The process makes use of a neutral lactase from yeast entrapped in synthetic fibers.

Studies have shown that immobilized enzymes have been used to produce 6-aminopenicillanic acid (6-APA) by the deacylation of the side chain in either penicillin G or V using penicillin acylase (Pastore and Moris, 1976). More than 50 % of 6-APA produced in the pharmaceutical industry today is enzymatically produced using immobilized penicillin acylase. Immobilized glucose oxidase can be used in the production of gluconic acid, removal of oxygen from beverages and the removal of glucose from eggs prior to dehydration in order to prevent maillard reaction. It has been demonstrated that glucose can be removed from egg using glucose oxidase and catalase which are co-immobilized either on polycationic cotton cloth or in hen egg white foam matrix (Marolia and D'Souza 1993, Kamath, *et. al.,* 1988).

Lipases are applied in the industry in detergents, leather treatment, controlled hydrolysis of milk fat for acceleration of cheese ripening, hydrolysis, glycerolysis and alcoholysis of bulk fats and oils etc. lipases are spontaneously soluble in aqueous phase but their natural substrates (Lipids) are not. A number of approaches to solve this problem using immobilized lipases have been reviewed (Deshpande and D'Souza 1988). Poulsen ,(1981) has reported that immobilized rennin or other proteases might allow for the continuous coagulation of milk for cheese manufacture. Proteases are key catalysts for the synthesis and modification of peptides. One of the areas in which they can be used in large scale is in the synthesis of peptide sweeteners using immobilized enzymes such as thermolysin (Gill,*et. al*.,1996).

Proteolytic enzymes such as subtilisin, alpha – chymotrypsin, papain, ficin or bromelain which have been immobilized by covalent binding, adsorption or cross-linking are used by Bayer AG of Germany to resolve AN-acyl- DL- phenylglycine ester race mate, yielding N-acyl-D-esters or N-acyl-D-amides and N-acyl-L-acids (Samejima, *et. al* .,1980).

Immobilized enzymes have also been used in the fabrication of bio-sensors. Stable membranes have been prepared by binding glucose oxidase to cheese cloth in the fabrication of glucose biosensor (Turner, *et. al* .,1987, Kumar, *et. al.,* 1992). Immobilized enzymes have also been used in regeneration of enzyme cofactors. Membrane reactors containing continuous cofactor regenerating systems have been used in the large scale production of sugar derivatives and optically active x-hydroxy acids and alpha-amino acids (D'Souza and Melo, 1991).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Refrigerated centrifuge – Sorval RC 5B plus Thermo electron Corporation, U.S.A., Amicon apparatus, Weighing balance – Sartorius Stedim Biotech. Gmbh, Germany, Vertical electrophoresis apparatus, FPLC – Pharmacia FPLC systems, Uppsala, Sweden, Water batch – Menmert, Germany. Chromatography columns – XK16/20, Autoclave – Hiclave – HV 25, Spectrophotometer UV-visible- Kontron Instruments,Uvicon 933 25-1276. Amersham Biosciences Pump P-l 18-1110-91, Vortexing machine – vortex Genie 2, Hirschmann Laborgerate, Shaker – UPM infors AG, CH-4103 Botmingen, Germany

3.1.1 CHEMICALS AND REAGENTS

Hydrochloric Acid, Wheat Germ, Potato Dextrose Agar, Tween 80, Sodium Acetate, Acetic Acid Ammonium Sulphate, Sodium Potassium Tatarate, Alginate, Sephadex G75 , Bovine Serum Abumin, Bio-rad Dye Reagent, D-galacturonic Acid, Dinitro Salicylic Acid (DNS), Molecular Weight Markers, Methanol, Acrylamide, Bis-acrylamide, Tetramethylene Diamine (TEMED), Ammonium Per Sulphate, Coommassie Brilliant Blue R250, Bromo Phenol Blue, Dialysis Membrane β-mercaptoethanol, Tris, Sodium Dipotassium Hydrogen Phosphate, Disodium Potassium Hydrogen Phosphate, Citrus Pectin, Sodium Dodecyl Sulphate (SDS),. Ethylene Diamine Tetra Acetic Acid (EDTA), Magnesium Chloride, Calcium Chloride, and Manganese Chloride were purchased from Sigma chemical company representatives in Germany

All the chemicals and reagents used were of analytical grade.

3.1.2 MICROORGANISM

Aspergillus niger (SA6) strain was collected from the microbial culture bank of Department of Crop Protection, Institute for Agricultural Research (IAR), Ahmadu Bello University, Zaria, Nigeria. The culture was maintained on Potato Dextrose Agar and sub-cultured periodically throughout the duration of the research.

3.2 METHODS

3.2.1 PREPARATION OF MEDIA AND CULTIVATION OF *ASPERGILLUS NIGER (SA6)*

Exactly 200g of potato was sliced,cut and boiled. It was filtered with muslin cloth and the volume made up to 1000ml. Exactly 20g each of Dextrose and Agar were added, boiled and autoclaved at 121^{o}C for 15 minutes. After cooling about 5g of streptomycin powder was added to prevent bacterial contamination .(*http:en.wikibooks.org/Potato Dextrose Media Preparation*)

Cultivation of the *Aspergillus niger (SA6)* was carried out in pre-weighed 250 ml Erlenmeyer flasks covered with aluminum foil containing 5g of wheat germ and 7.5 ml of 0.4M HCl.

The flasks containing culture medium were autoclaved at 121^0C for 15 minutes. After cooling each flask was weighed and water loss during autoclaving was corrected to 1 litre.

3.2.2 INOCCULATION OF *ASPERGILLUS NIGER (SA6)*

Exactly 10 ml of conidial suspension (approximately 10^7 spores/g of dry substrate, which was obtained from a 7–day old agar slant suspended in autoclaved Tween 80 solution was used to inoculate the substrate in a sterile chamber.

After inoculation, the contents were carefully mixed and the flasks placed in a humid cultivation room at 30^0C under static condition for 3 days according to the method of Sebastian, *et al* .,(1996).

3.2.3 PREPARATION OF CRUDE POLYGALACTURONASE FROM CULTURE MEDIUM

After cultivation, solid state cultures were suspended in 40 ml of distilled water stirred for 10 minutes and the pH measured. This was followed by the addition of 40ml of 0.05M acetate buffer pH 5.0 and the mixture left to stand for 10 minutes. The mixture was filtered through a muslin cloth and Whatman No 1 filter paper and the solid material retained on the filter paper was extracted again with 20ml of 0.05M acetate buffer pH 5.0.

Both extracts were pooled, clarified by centrifugation at 5000xg for 30 minutes and brought to a total volume of 100ml with 0.05M acetate buffer. The extract (crude enzyme) was kept at 4^0c until required (Sebastian, *et. al*.,1996).

3.2.4 ULTRA FILTRATION OF CRUDE POLYGALACTURONASE FROM CULTURE MEDIUM

The crude Polygalacturonase was concentrated using Amicon equipment with cellulose acetate membrane In order to remove low molecular weight proteins of 10,000 KDa and below

3.2.5 AMMONIUM SULPHATE PRECIPITATION OF POLYGALACTURONASE FROM CULTURE MEDIUM

The crude Polygalacturonase was precipitated with gentle stirring at 40,50,60,70 and 80 % saturation of solid ammonium sulphate. It was centrifuged at 10,000xg for 15 min. The 70 % precipitate was re-dissolved in 0.05M acetate buffer pH 5.0 and dialysed over night against the same buffer to remove low molecular weight substances and other ions that may interfere with the enzyme activity (Dixon and Webb, 1964)

3.2.6 DETERMINATION OF PROTEIN CONTENT (BRADFORD METHOD)

When protein binds Coumassie Brilliant Blue in Bio-Rad dye its absorbance shifts from 465nm to 595nm.

Twenty micro litre (µl) of the enzyme was mixed with 1ml of Bio-rad dye and vortexed. The mixture was incubated at room temperature for 5 minutes. The absorbance was read at 595 nm using spectrophotometer. The absorbance values were converted to protein concentration by extrapolation from the standard curve (Appendix 7). Aliquots of Bio-rad dye and buffer were used as blank.(Bradford, 1976)

3.2.7 ASSAY OF POLYGALACTURONASE

Exactly 0.1ml of enzyme was mixed with 0.8 ml of citrus pectin substrate and 0.1ml of acetate buffer added to make up the volume to 1ml. The mixture was incubated for 20 minutes at 40^0C. After incubation 1ml of Dinitrosalicylic acid (DNS) was added and the mixture incubated at 90^0C for 5 minutes to develop the colour.

The mixture was cooled and absorbance read at 575 nm using a spectrophotometer. A blank of 1ml of DNS and 1ml of buffer was used to zero the spectrophotometer. The absorbance values were used to calculate the activity of the enzyme using standard curve (Appendix 8). One unit of enzyme activity was defined as the amount of enzyme that produced one µmole of D-galacturonic acid per minute at 40^0C and pH 5.0 (Modified method of Soares, *et. al.,* 1999).

3.3 ENZYME PURIFICATION

3.3.1 ION-EXCHANGE CHROMATOGRAPHY WITH FAST PERFORMANCE LIQUID CHROMATOGRAPHY (FPLC)

Exactly fifteen mililitre of crude enzyme was loaded onto the column with dimension of 15.5cmx1cm. Fifty six fractions of 5ml each were collected at a flow rate of 3 ml per minute. Each fraction was assayed for enzyme activity and protein content . An elution profile was plotted to show active peaks.

3.3.2 GEL FILTRATION (FPLC)

One ml of the sample was gradually injected into the pre-equilibrated column. Filtration was carried out for 2 1/2 hours. Fifty six fractions were collected and protein content and activity were determined. The fractions with high activity were pooled and used for characterization and electrophoresis

3.4 CHARACTERIZATION AND KINETIC STUDIES OF PARTIALLY PURIFIED POLYGALACTURONASE

3.4.1 INITIAL VELOCITY STUDIES

The effect of substrate concentration on the activity of Polygalacturonase was determined by incubating 0.1ml of the enzyme with 1,3,5,7,9,13 and 15 mg/ml of citrus pectin. The V_{max} and K_M of the enzyme were determined using double reciprocal plot (Line weaver and Burk, 1934)

3.4.2 EFFECT OF TEMPERATURE CHANGE ON THE ACTIVITY OF POLYGALACTURONASE

The optimum temperature of the enzyme was determined by incubating the enzyme with citrus pectin at 30^{0}C, 35^{0}C, 40^{0}C, 45^{0}C, 50^{0}C, 55^{0}C and 60^{0}C for 20 minutes and pH 5.0. The activity was then assayed as per the method of Miller, 1959. A plot of temperature versus activity was constructed to obtain the optimum temperature of the enzyme by the method of Stauffer (1969).

3.4.3 EFFECT OF pH CHANGE ON THE ACTIVITY OF POLY-GALACTURONASE

The optimum pH of the enzyme was determined using acetate buffer and a range of pHs-, 3.5 , 4.0, 4.5, 5.0, 5.5 by dispersing the enzyme in 0.05M acetate buffer in the various pHs and then taking 0.1ml of the dispersed enzyme for assay as per the method of Miller, 1959. A plot of enzyme activity versus pH was plotted to determine the optimum pH of the enzyme.

3.4.4 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE of the partially purified Polygalacturonase was carried out as described by Laemli (1970) . Exactly 20 µL of the crude enzyme , partially purified enzyme and molecular weight markers were each treated with 20µL of sample buffer(125mM Tris-HCl buffer, pH 6.8, 4 % SDS, 10 % (V/V) Glycerin, 10 %(V/V) β-Mercaptoethanol, 0.25 % Bromophenol blue) and incubated at 95^{o}C for 5 minutes in a Thermomixer.

The prepared samples were loaded onto the gel (12.5% for both running gel and stacking gel). The electrophoresis was performed at 150 volts for 1$^{1/2}$ hours. After the electrophoresis, the gel was removed from the gel cassette, stained overnight and then de-stained several times with 7 % acetic acid until clear bands appeared. The apparent molecular weight of the enzyme was estimated from the standard marker proteins.

3.5 STABILITY STUDIES ON THE PARTIALLY PURIFIED POLYGALACTURONASE

3.5.1. DETERMINATION OF pH STABILITY OF POLYGALACTURONASE

Aliquots of the enzyme solution (0.1ml) was dispersed in 0.05M acetate buffer pH 3.5, 4.0, 4.5, 5.0 and 5.5; phosphate buffer pH 6.0, 6.5, 7.0, 7.5 and Tris-Hcl pH 7.5, 8.0, 8.5 and 9.0 and maintained at room temperature for 24hrs. Exactly 0.1 ml of the enzyme was used to determine the residual activity of the enzyme at optimum conditions as per the method of Miller 1959

3.5.2. DETERMINATION OF TEMPERATURE STABILITY OF POLYGALACTURONASE

0.1ml of the enzyme solution was incubated at different temperatures 30^{0}C, 40^{0}C, 50^{0}C, 60^{0}C and 70^{0}C for 1 hr. Exactly 0.1ml of the enzyme was withdrawn and assayed for residual activity at optimum conditions.

3.5.3. EFFECT OF pH ON K_M AND V_{max} OF POLYGALACTURONASE

The activity of the enzyme was determined at varying substrate concentration of 1, 3, 5, 7, 9, 13 and 15 mg/ml at different pH values 3.5, 4.0, 4.5, 5.0, 5.5 using acetate buffer. The kinetic constants V_{max} and K_M were determined at each pH value using double reciprocal plot. The pKa values were determined using the Dixon Plot (Dixon, 1953). These values were used to predict the possible amino acids at the active site.

3.5.4. EFFECT OF EDTA AND SOME DIVALENT CATIONS ON THE ACTIVITY OF POLYGALACTURONASE

The enzyme was incubated with 10 mM each of EDTA, Mg^{2+}, Ca^{2+} and Mn^{2+} and citrus pectin). Exactly 0.1ml of the mixture was used to determine the activity of the enzyme at different substrate concentrations as per the method of Miller 1959. The K_M and V_{max} were determined for each substrate concentration.

3.5.5. DETERMINATION OF ENERGY OF ACTIVATION (Ea) OF POLYGALACTURONASE

The enzyme was incubated at 30^0C, 40^0C, 50^0C, 60^0C and 70^0C in a water bath as described by Stauffer and Etson (1969). The various test tubes were cooled to room temperature and the activity of the enzyme determined. An Arrhenius plot of log V_o (enzyme activity) versus reciprocal of Kelvin temperature (^{0}K) was done to determine the energy of activation of the enzyme.

3.6 IMMOBILIZATION OF POLYGALACTURONASE WITH SODIUM ALGINATE

Exactly 2.39µg of the partially purified enzyme was mixed with 4 ml of 3 % sodium alginate solution. The beads were formed by dropping the polymer from a height of approximately 20 cm into an excess (100ml) of stirred 0.2M $CaCl_2$ solution with a syringe and needle at room temperature. The bead size which was controlled by the pump pressure of the hypodermic needle was 2.0 mm in diameter and a volume of 4.18cm^3. The beads were left in the calcium chloride solution for 3 hrs to mature. The entrapped enzyme was used for characterization as previously described for the native enzyme.

3.6.1 CHARACTERIZATION OF IMMOBILIZED POLYGALACTURONASE

3.6.2. INITIAL VELOCITY STUDIES OF IMMOBILIZED POLYGALACTURONASE

The effect of substrate concentration on the activity of immobilized polygalacturonase was determined by incubating the enzyme with 1, 3, 5,7,9,10,13 and 15mg/ml of citrus pectin.

The activity of the enzyme was assayed at each substrate concentration using the method of Miller 1959.

The apparent V_{max} and K_M of the immobilized polygalacturonase was determined using double reciprocal plot (Line-weaver-Burk 1934).

3.6.3. EFFECT OF pH CHANGE ON THE ACTIVITY OF IMMOBILIZED POLYGALACTURONASE.

The optimum pH of the immobilized polygalacturonase was determined using acetate buffer with a range of pH's 3.5-5.5 by putting the enzyme beads in 0.05M acetate buffer prepared at the various pH's and assayed for activity as per the method of Miller 1959. A plot of activity versus pH was made to determine the optimum pH.

3.6.4. EFFECT OF TEMPERATURE CHANGE ON THE ACTIVITY OF IMMOBILIZED POLYGALACTURONASE

The optimum temperature of immobilized polygalacturonase was determined by incubating the enzyme at 30°C, 35°C, 40°C, 45°C,50°C, 55°C and 60°C for 20 minutes and assaying for activity as per the method of Miller 1959.

A plot of temperature versus activity was made to obtain the optimum temperature of the enzyme as per the method of Stauffer 1969.

3.6.5. DETERMINATION OF pH STABILITY OF IMMOBILIZED POLYGALACTURONASE

The enzyme was mixed with 0.05M acetate buffer pH 3.5-5.5, Phosphate buffer p H 6.0-7.5 and Tris-Hcl buffer pH 7.5-9.0 and maintained at room temperature for 24 hours. Exactly 0.1 ml of the enzyme was used to determine the residual activity at 40°C as per the method of Miller 1959.

A plot of activity versus pH was constructed to determine pH stability

3.6.6. DETERMINATION OF TEMPERATURE STABILITY OF IMMOBILIZED POLYGALACTURONASE.

The enzyme was incubated at 30°C,40°C, 50°C, 60°C, and 70°C for 1 hour at pH 4.5. Exactly 0.1 ml of the enzyme was withdrawn and assayed for activity as per the method of Miller 1959. A plot of activity versus temperature was constructed to determine temperature stability.

3.6.7. DETERMINATION OF CATALYTIC ACTIVITY OF IMMOBILIZED POLYGALACTURONASE PER CYCLE

The first activity of the immobilized polygalacturonase was assayed at apparent optimum conditions (pH 5.0 and temperature of 40°C). The same enzyme was subjected to second and third catalytic cycles at 30 minutes interval to determine the catalytic stability of the enzyme.

CHAPTER FOUR

4.0 RESULTS

4.1 PARTIAL PURIFICATION OF POLYGALACTURONASE

The enzyme was purified nine fold with specific activity of 246 μmole/mg and a yield of 0.18 %(Table 4.1)

4.2 AMMONIUM SULPHATE PRECIPITATION PROFILE OF POLYGALACTURONASE FROM CULTURE MEDIUM

The activity of the enzyme at 40, 50, 60 70 and 80 % ammonium sulphate saturation was determined. The highest activity was obtained at 70 % saturation which was 0.78 μmoles /min/mg as shown in figure 4.1. This percentage of ammonium sulphate was used for the precipitation of the enzyme from the culture medium. The least precipitation of the enzyme was recorded at 40 % saturation and beyond 70 % saturation, activity of the enzyme dropped.

Table 4.1 PURIFICATION DATA OF POLYGALACTURONASE FROM ASPERGILLUS NIGER (SA6)

Purification Steps	**Vol. of sample (ml)**	**Total Protein (μg)**	**Total Activity (μmole of D-galacturonic acid/min**	**Specific Activity (μmole of D-galacturonic acid/mg/min**	**Purification Fold**	**% Recovery**
Crude Extract	500.00	8335.00	225.00	26.99	1.00	100.00
Ammonium Sulphate Precipitation	25.00	3415	17.25	5.10	0.19	7.70
Ion-Exchange Chromatography(FPLC) on DEAE Sepharose	15.00	181.95	18.30	100.58	3.72	8.10
Gel Filtration on Sephadex G-75	1.00	1.67	0.41	246.00	9.10	0.18

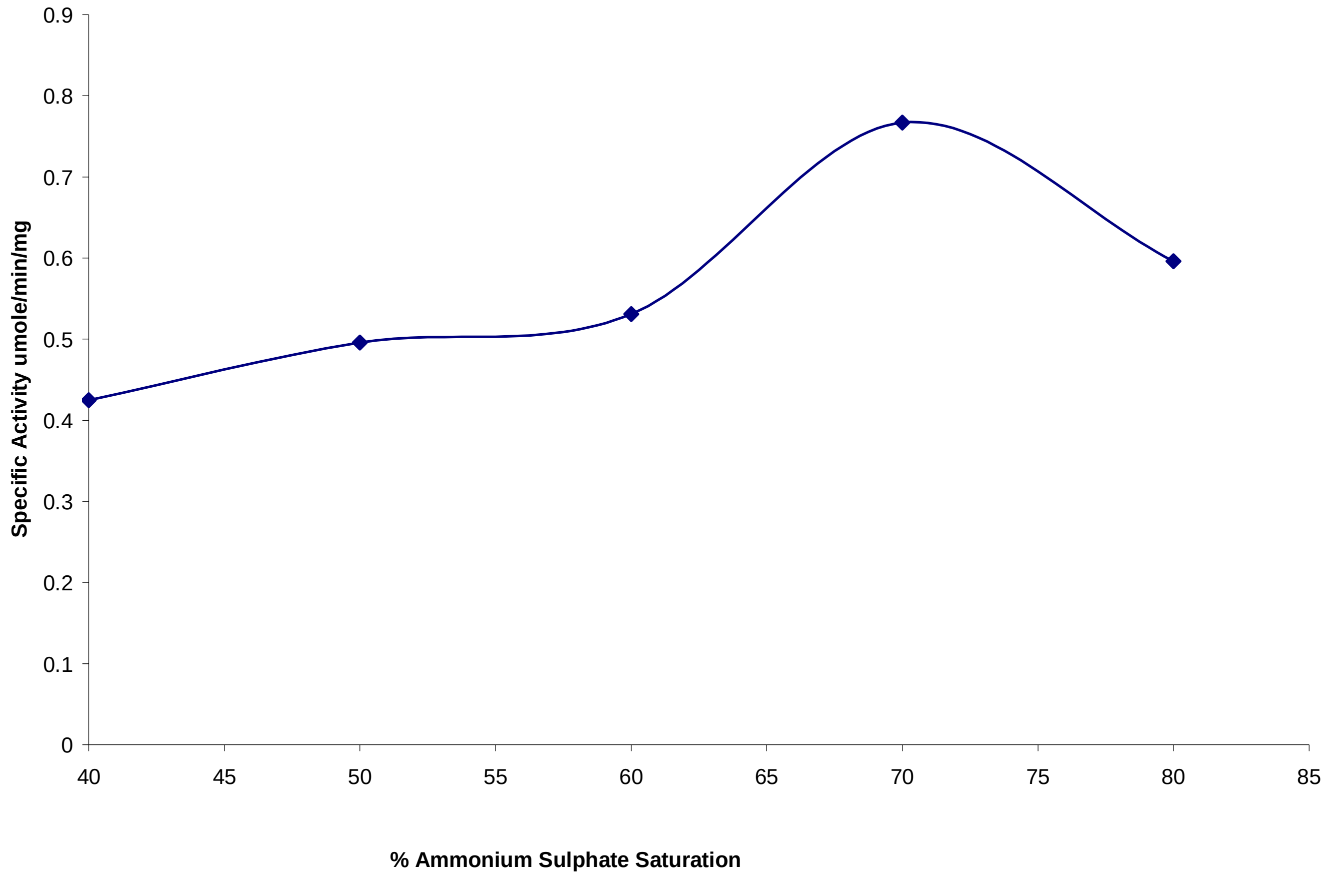


Fig.4.1 Ammonium Sulphate Precipitation Profile of Polygalacturonase

4.3 ION-EXCHANGE CHROMATOGRAPHY (FPLC) PROFILE OF THE POLYGALACTURONASE.

The elution profile of the enzyme on DEAE- sepharose at 0-100% NaCl gradient revealed one active peak with highest activity of 1.8µmole/min/ml(1.1 µmoles /min/mg) obtained in fraction 3 as presented in figure 4.2.

4.4.GEL FILTRATION (FPLC) PROFILE OF THE POLYGALACTURONASE.

Gel filtration of fraction 2 from ion-exchange gave one active peak at fractions 3 and 4. Both fractions contained protein and activity. However, fraction 4 gave the highest activity of 0.9µmole/min/ml (0.51µmoles /min/mg) as presented in figure 4.3.

4.5. SODIUM DODECYL SULPHATE ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) OF THE POLYGALACTURONASE.

SDS-PAGE of fraction 4 from gel filtration revealed two distinct bands with apparent molecular weights of 35 KDa and 40 KDa ,suggesting a dimeric protein (endo and exo)

Polygalacturonase as presented in plate 4.5 a and 4.5b.

The apparent molecular weights of the enzyme were extrapolated from the standard marker proteins.

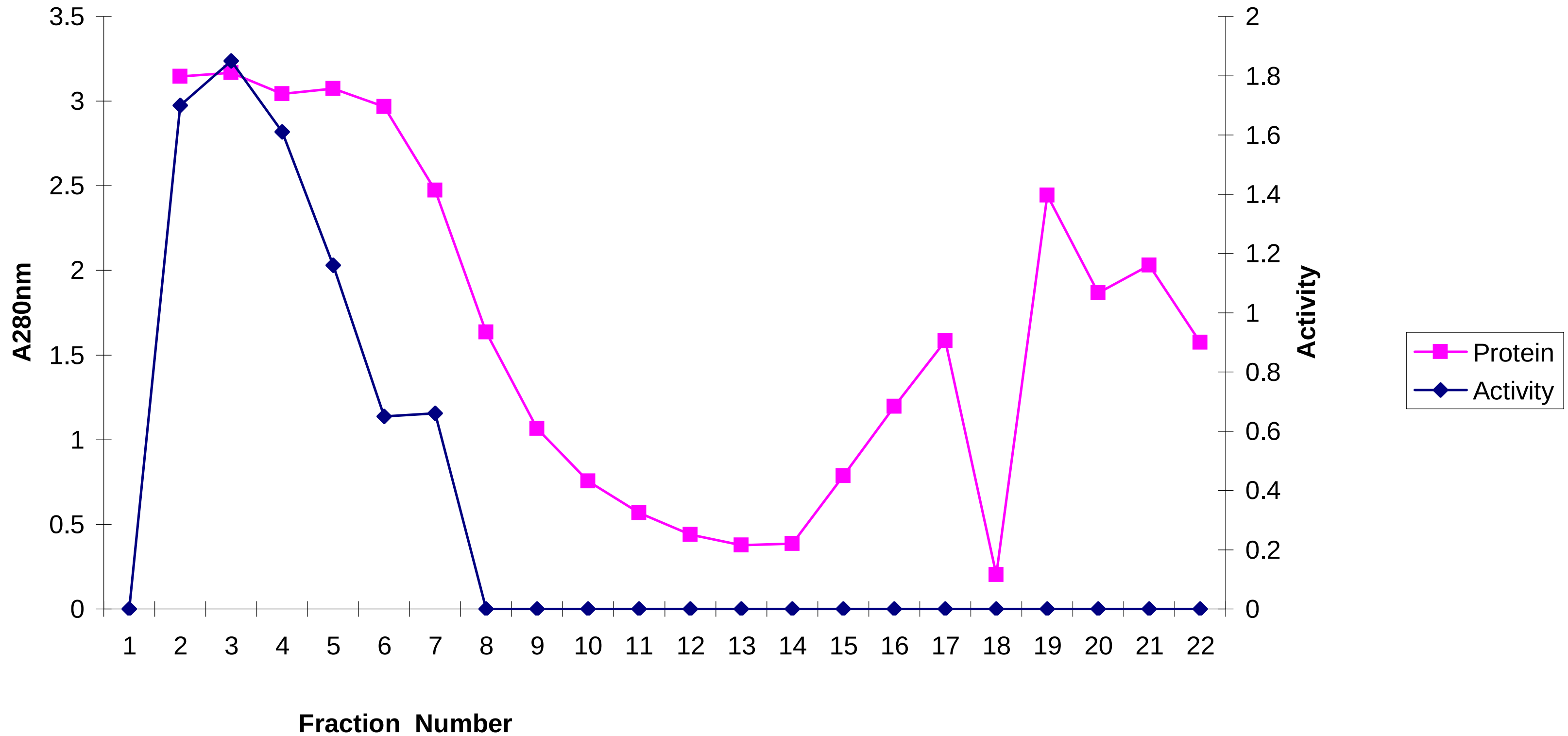


Fig. 4.2 Ion Exchange Profile of Polygalacturonase

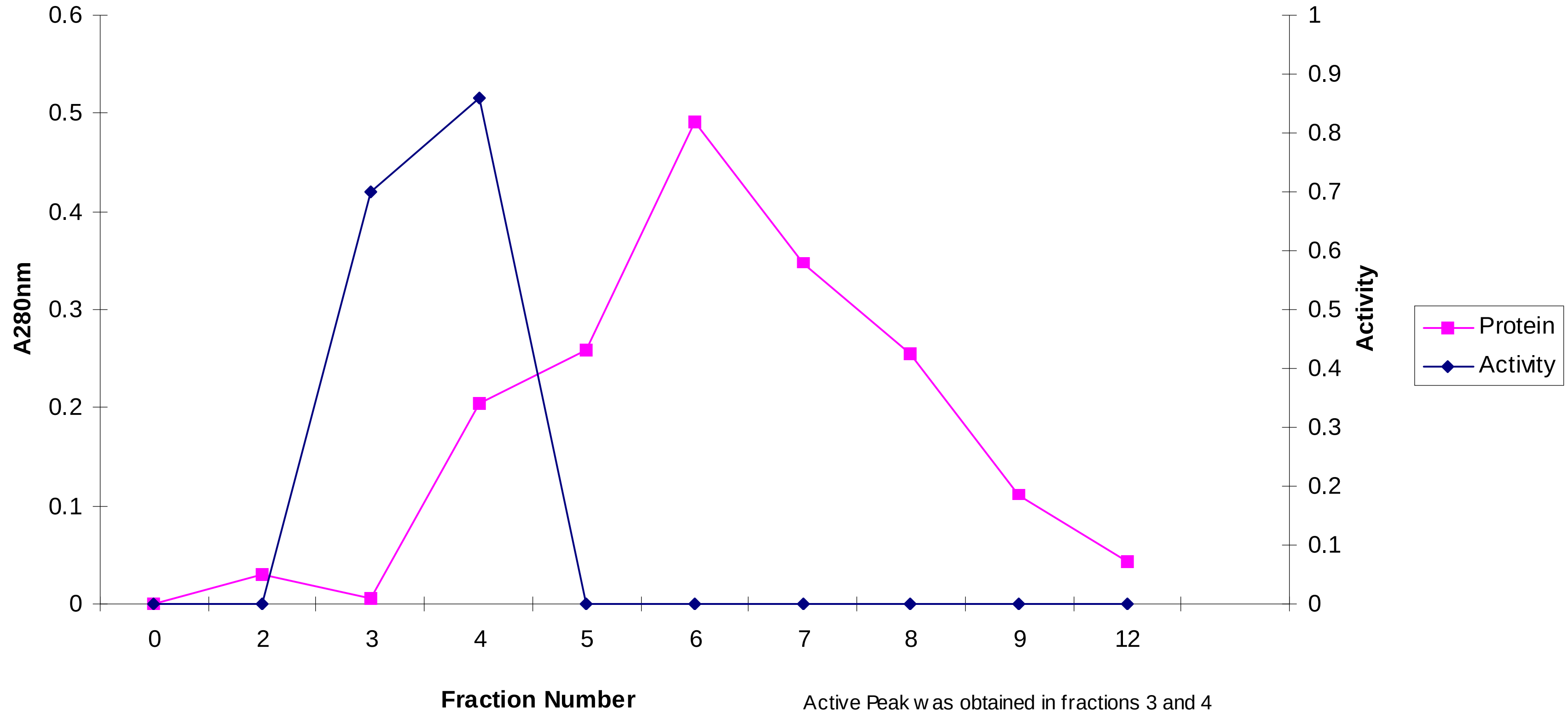


Fig 4.3 Gel Filtration Profile of Polygalacturonase

M= Standard
Marker Proteins
F2= Fraction 2
F3=Fraction 3
F4=Fraction 4
F5= Fraction 5

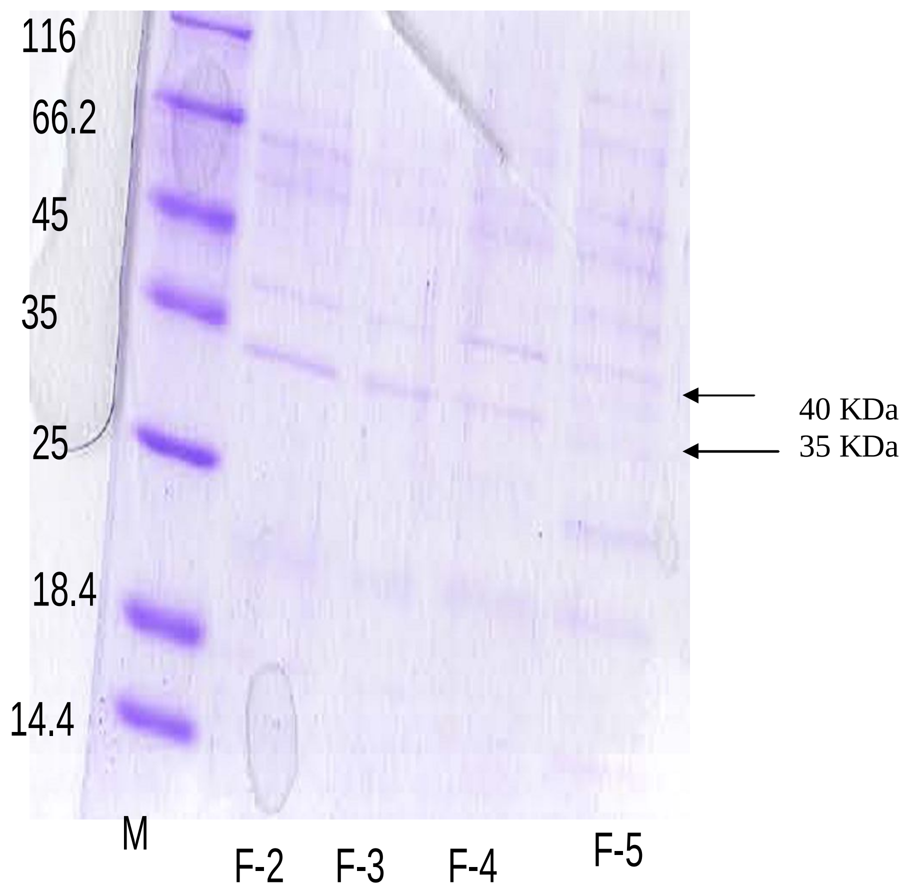
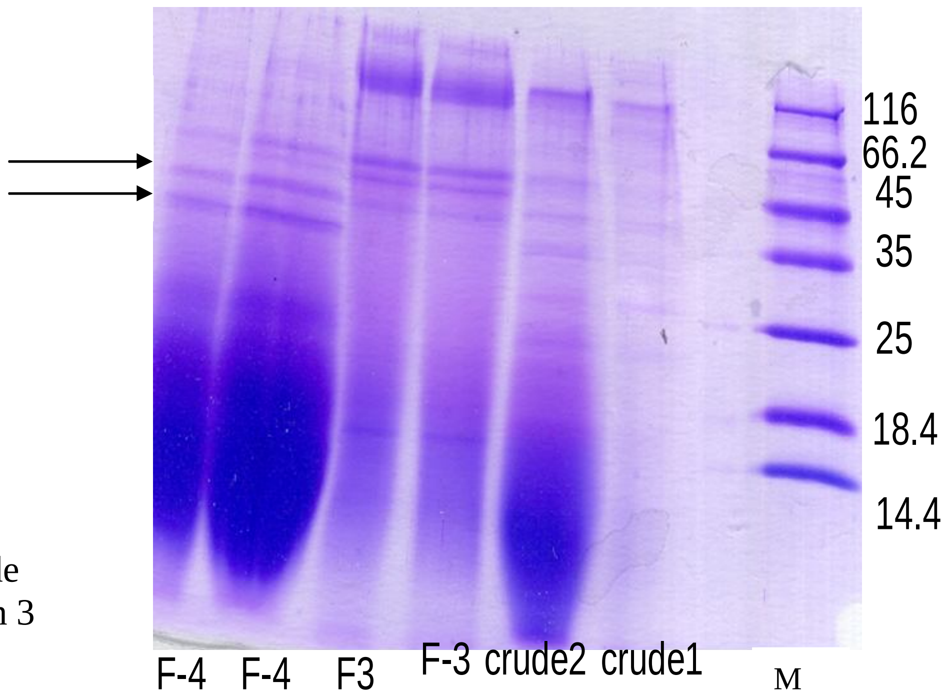


Plate . 4.5 a. Electrophoregram of Partially Purified

Polygalacturonase from Ion-Exchange Chromatography

40 KDa
35 KDa



M=Standard Marker Proteins
1 & 2= Crude
F3= Fraction 3

Plate. 4.5b.Electrophoregram of Partially Purified Polygalacturonase from Gel Filtration

4.6. REACTION PROFILE OF THE POLYGALACTURONASE.

The activity of polygalacturonase was assayed from 10 to 30 minutes at interval of 5 minutes at 40^{o}C and pH 5.0 to determine the optimum reaction time of the enzyme. The highest activity was obtained at 20 minutes of incubation and the lowest activity at 10 minutes as shown in figure 4.4. The optimum reaction time of the polygalacturonase was thus 20 minutes. The reaction profile revealed 1st order kinetics between 10- 15 minutes, mixed order between 15-20 minutes and zero order between 20- 30 minutes.

4.7. INITIAL VELOCITY STUDIES.

The effect of substrate concentration on the activity of polygalacturonase was determined at substrate concentrations of 1, 3, 5,7,13 and 15mg/ml of citrus pectin. The K_M and V_{max} of the enzyme as calculated from double reciprocal plot were 2.74 mg of citrus pectin/ml and 0.78 µmoles /min/mg respectively as shown in figure 4.5.

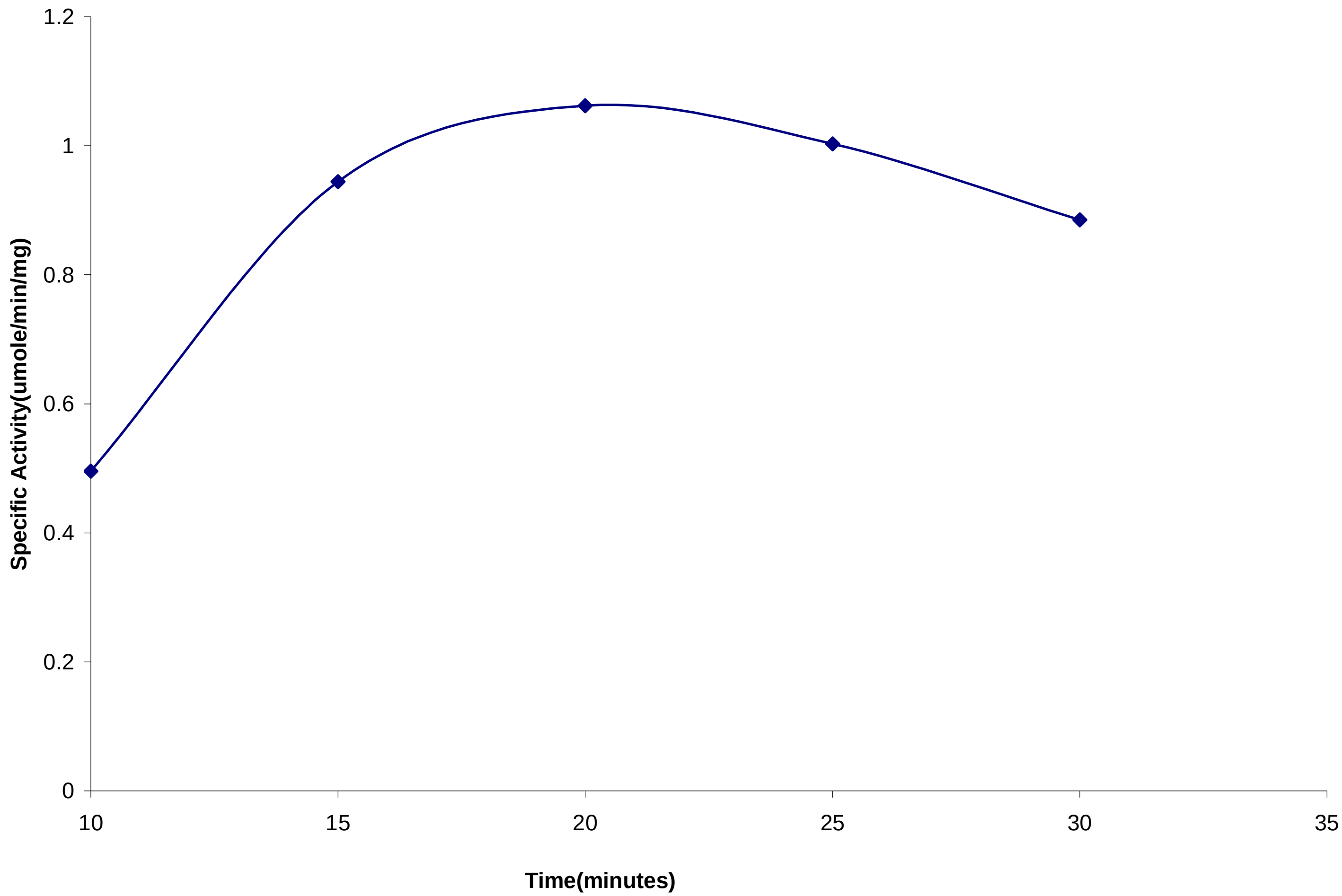


Fig. 4.4 Reaction Profile of Polygalacturonase

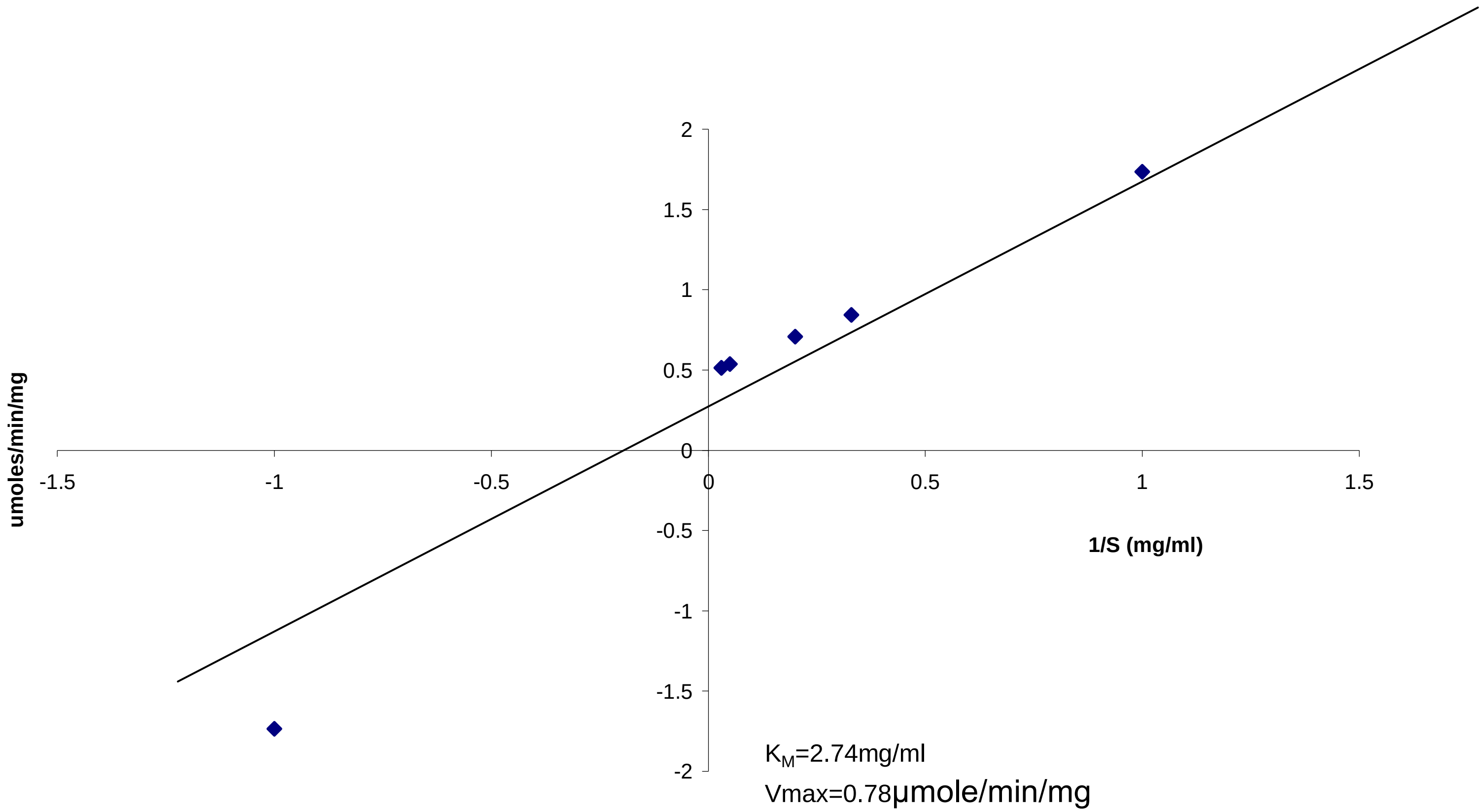


Fig.4.5 LineWeaver-Burk Plot of Polygalacturonase

4.8. EFFECT OF CHANGE OF pH ON THE ACTIVITY OF POLYGALACTURONASE.

The optimum pH of the enzyme was determined between pH 3.5 to 5.5. The highest activity of 1.42 µmoles/min/mg was obtained at pH 4.5 and the lowest activity was 0.92 µmoles/min /mg as presented in figure 4.6. Beyond pH 4.5 the activity of the enzyme dropped.

4.9. EFFECT OF TEMPERATURE CHANGE ON THE ACTIVITY OF POLYGALACTURONASE.

The optimum temperature of the enzyme was determined between 35^{o}C to 65^{o}C at an interval of 5^{o}C. The highest activity of 0.83µmoles/min/mg was obtained at 40^{o}C as shown in the figure 4.7. The lowest activity was obtained at 30^{o}C and beyond 40^{o}C the activity of the enzyme dropped. The optimum temperature of the enzyme was 40^{o}C

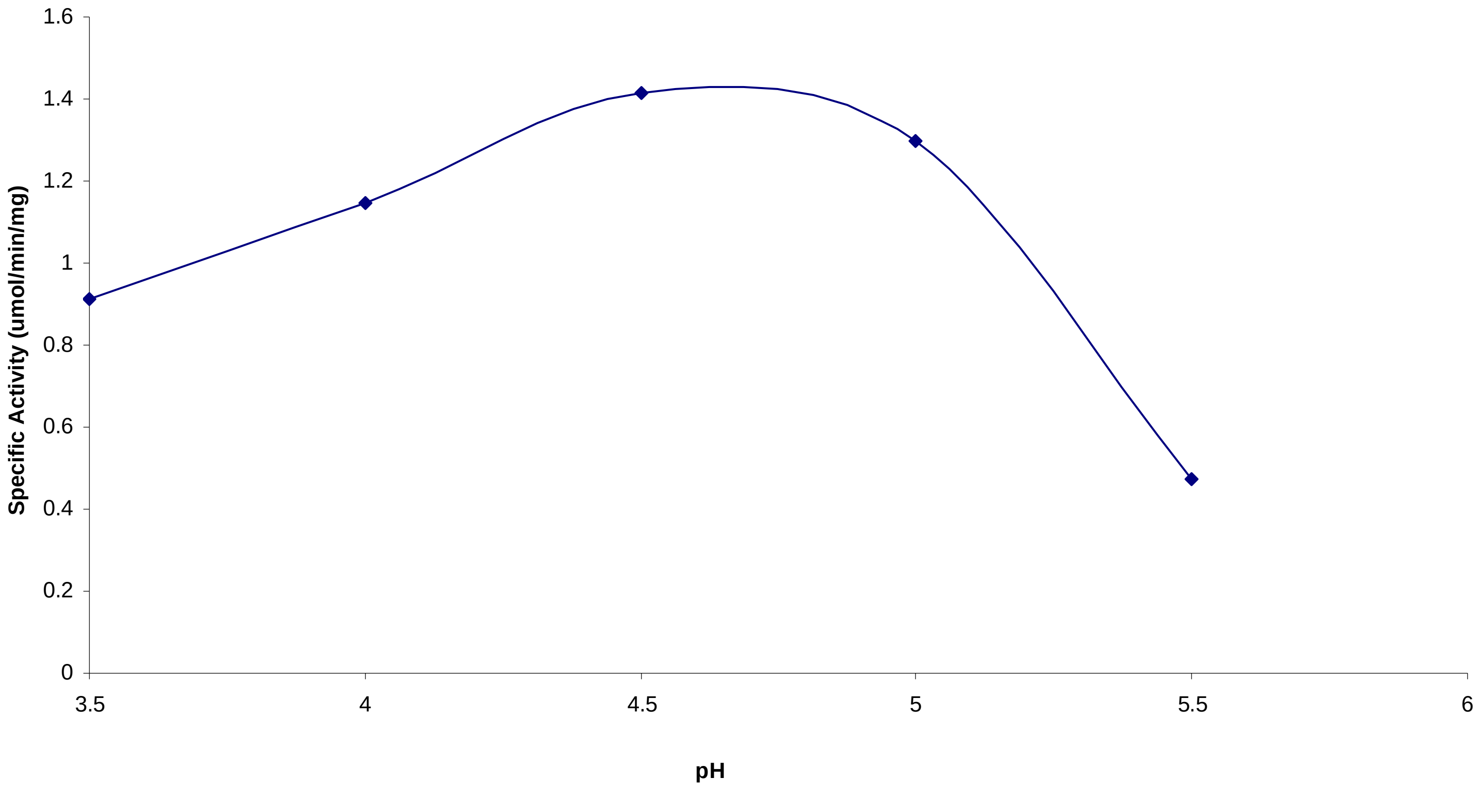


Fig. 4.6 Effect of pH Change on the Activity of Polygalacturonase

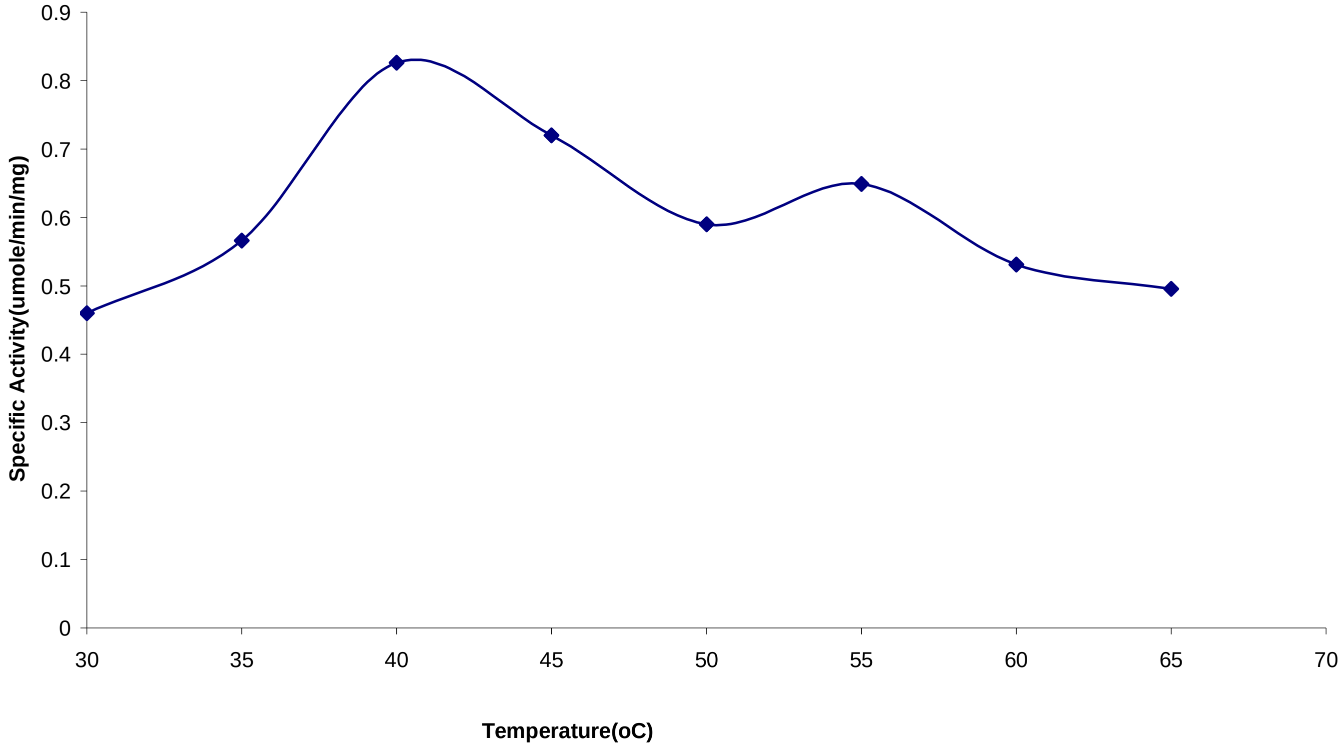


Fig. 4.7 Effect of Temperature Change on the Activity of Polygalacturonase

4.10 DETERMINATION OF pH STABILITY OF POLYGALACTURONASE.

Stability studies of the enzyme at different pH's after 24 hours of incubation at room temperature was evaluated by assaying for percentage residual activity of the enzyme at pH 3.5- 5.5 for acetate buffer; pH 6.0-7.5 for phosphate buffer and pH 7.5- 9.0 for Tris-Hcl buffer. The highest percentage residual activities obtained for the various buffers were as follows: 23.01 % at pH 4.5, in acetate, 28.32 % at pH 7.0 in phosphate, and 22.13 % at pH 7.5 in Tris-Hcl while the lowest percentage residual activitiy was obtained at pH 7.5 in Tris-Hcl buffer as presented in Appendix 6.

4.11. DETERMINATION OF TEMPERATURE STABILITY OF THE POLYGALACTURONASE.

Temperature stability of the enzyme was evaluated between 30^{o}C -70^{o}C at an interval of 10^{o}C after incubation for 1 hour. The highest residual activity of the enzyme was obtained at 40^{o}C and the lowest residual activity was recorded at 70^{o}C as presented in figure 4.8. The enzyme was more stable at 40^{o}C.

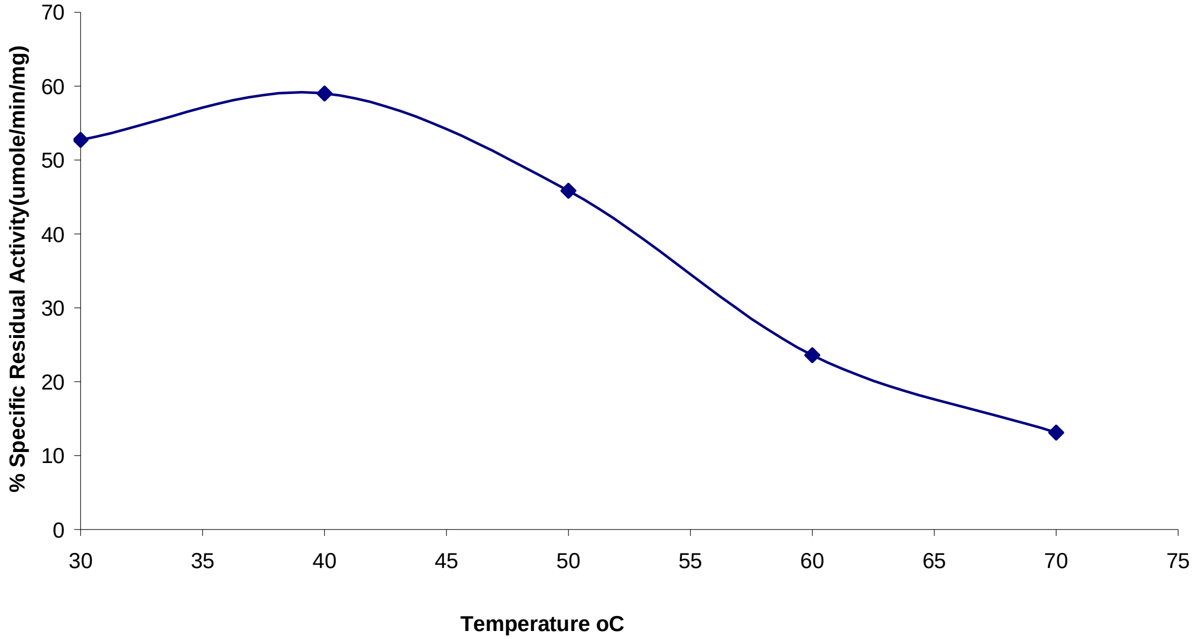


Fig. 4.8 Temperature Stability of Polygalacturonase

4.12 EFFECT OF pH ON K_M AND V_{MAX} OF POLYGALACTURONASE

The effect of pH on K_M and V_{max} of the enzyme was determined at varying substrate concentration of 1, 3, 5, 7, 9, 13 and 15 mg/ml at different pH values 3.5, 4.0, 4.5, 5.0, 5.5 using acetate buffer. The kinetic constants V_{max} and K_M were determined at each pH value using double reciprocal plots. The Dixon Plot of the enzyme showed one peak at pH 5.5 and when tangent was drawn on the curve the pKa values deduced were 4.9 and 5.6 as presented in figure 4.9.

4.13 EFFECT OF EDTA AND SOME DIVALENT CATIONS ON THE ACTIVITY OF THE POLYGALACTURONASE.

The K_M and V_{max} of the polygalacturonase in the presence of 10mM EDTA, Mg^{2+},Ca^{2+} and Mn^{2+} ions are presented in figure 4.10 together with the K_M and V_{max} values of the control.

Both the K_M and V_{max} values of the enzyme increased in the presence of EDTA, Mg^{2+},Ca^{2+} and Mn^{2+} ions as compared to the control. However, Ca^{2+} ions produced the highest increase and Mg^{2+} ions the lowest increase.

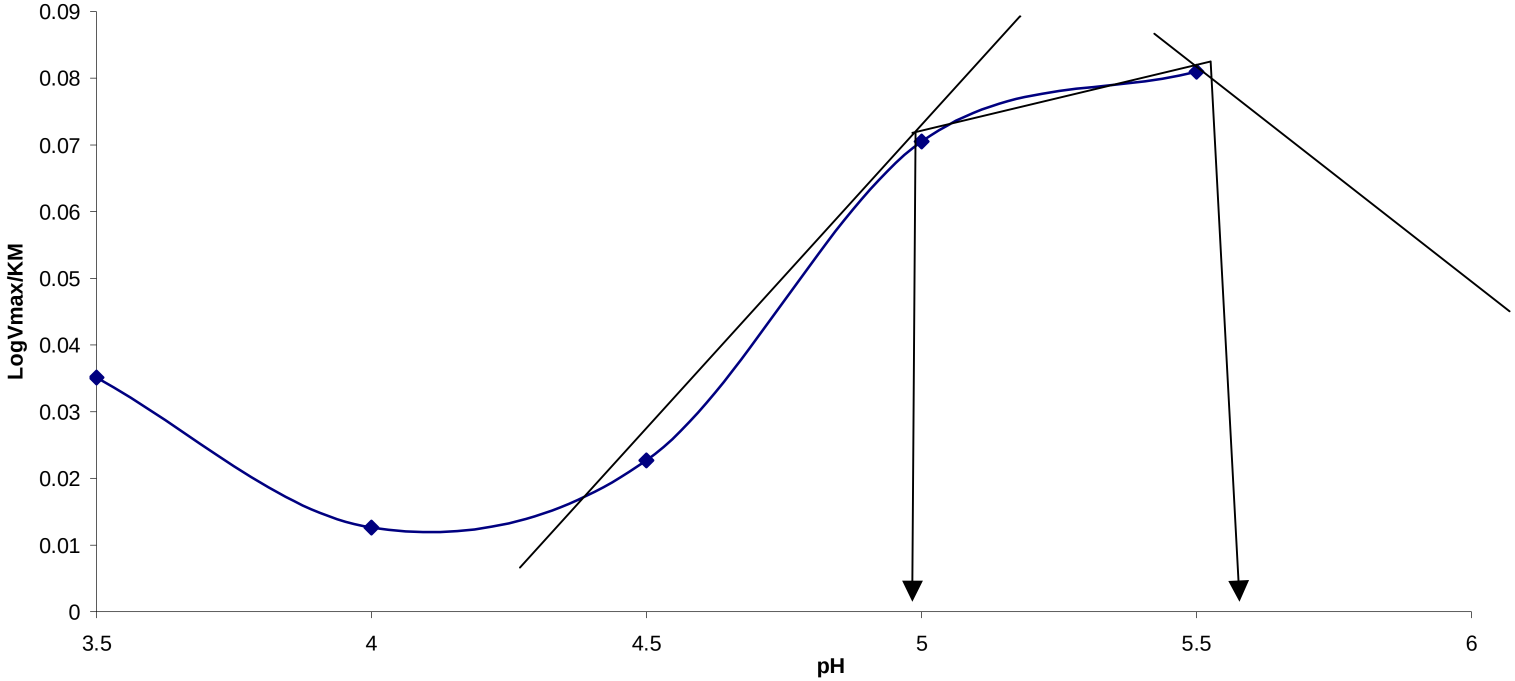


Fig. 4.9 Dixon Plot of Polygalacturonase

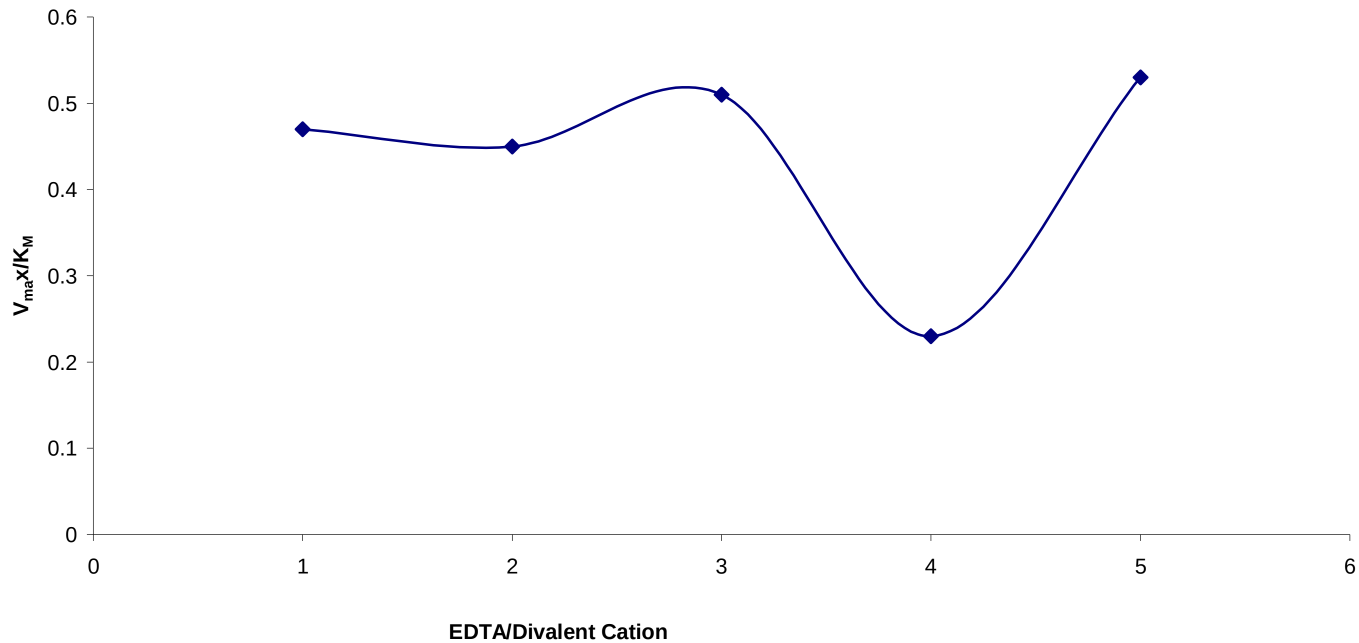


Fig. 4.10 Effect of EDTA And Divalent Cations on the Activity of Polygalacturonase

1 = Control, 2 = EDTA, 3 = Mg^{2+}, 4 = Ca^{2+}, 5 = Mn^{2+}

4.14 DETERMINATION OF ENERGY OF ACTIVATION (Ea) OF POLYGALACTURONASE

The enzyme was incubated at 30^0C, 40^0C, 50^0C, 60^0C and 70^0C in a water bath as described by Stauffer and Etson (1969). The activation energy (Ea) of the Polygalacturonase using Arrhenius plot was found to be 259.19 cal/mole as presented in figure 4.11.

4.15 INITIAL VELOCITY STUDIES OF THE IMMOBILIZED POLYGALACTURONASE.

The effect of various substrate concentrations (1, 3, 5, 7,9,13 and 15mg/ml) on the activity of the immobilized polygalacturonase was determined by assaying the activity expressed per gram of enzyme bead at each substrate concentration. The diameter and the volume of the alginate beads were 2.0 mm and 4.18 cm^3 respectively. The apparent K_M and V_{max} values were 11.1mg/ml of citrus pectin and 1.65 μmole/mg/g of enzyme bead respectively as presented in figure 4.12.

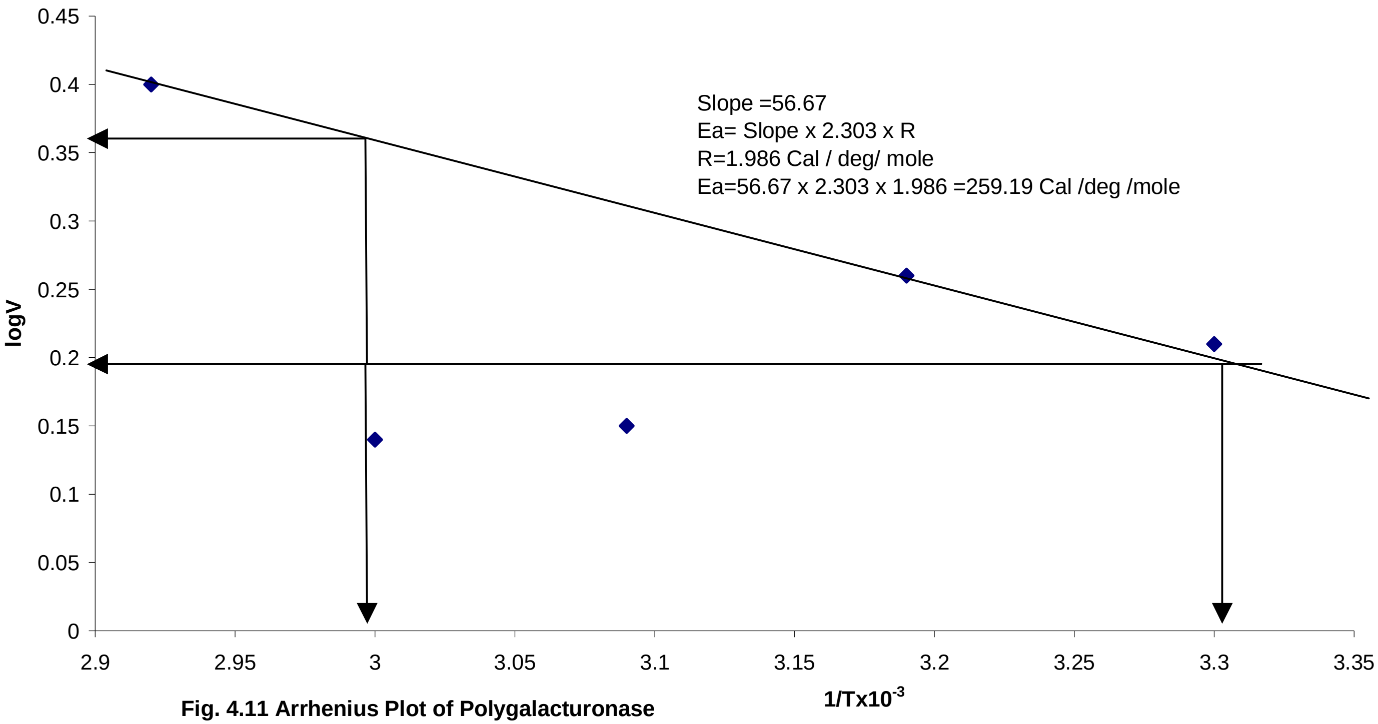


Fig. 4.11 Arrhenius Plot of Polygalacturonase

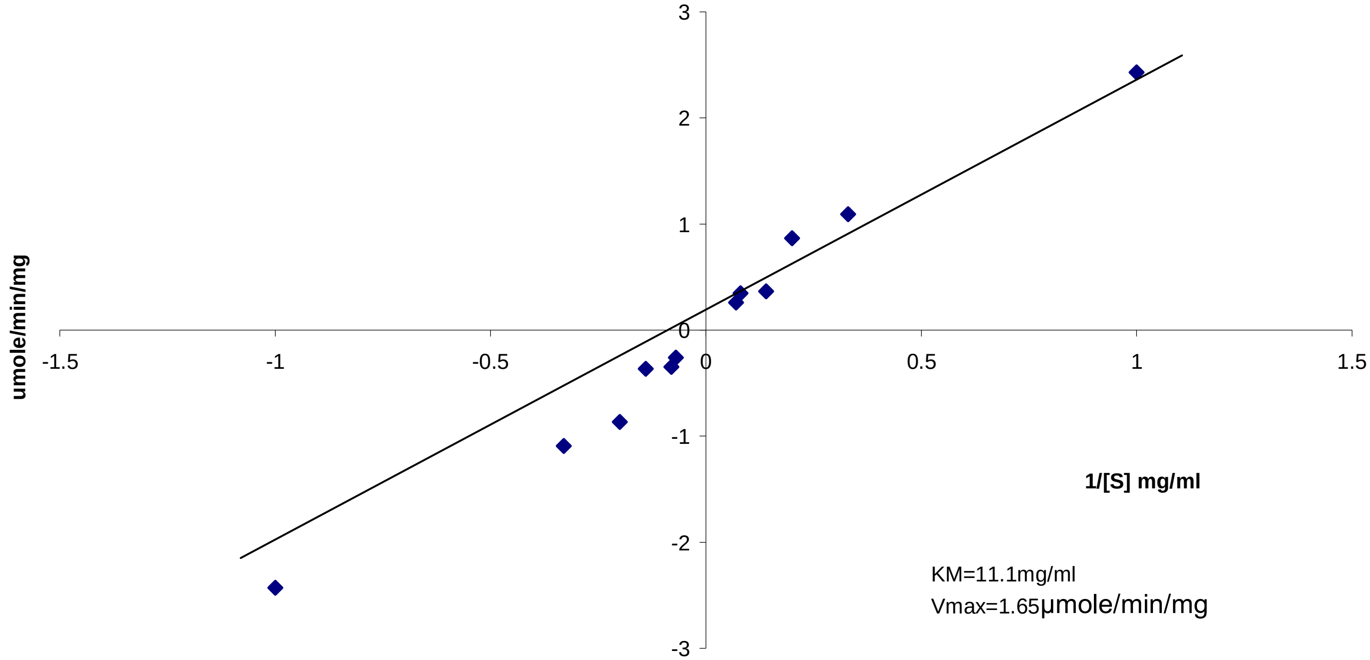


Fig. 4 .12 LineWeaver-Burk Plot of Immobilized Polygalacturonase

4.16 EFFECT OF pH CHANGE ON THE ACTIVITY OF IMMOBILIZED POLYGALACTURONASE.

The optimum pH of the immobilized enzyme was determined by assaying the activity of the immobilized polygalacturonase at pH values of 3.5-5.5 at an interval of 0.5. The highest activity of the enzyme was obtained at pH 4.5 as presented in figure 4.13. The activity of the enzyme dropped beyond pH 4.5

4.17 EFFECT OF TEMPERATURE CHANGE ON THE ACTIVITY IMMOBILIZED POLYGALACTURONASE

The optimum temperature of the enzyme was determined between 30^{o}C- 55^{o}C with an interval of 5^{o}C. The optimum temperature of the immobilized Polygalacturonase was 40^{o}C as presented in figure 4.14. The activity of the enzyme was lowest at 30^{o}C and the activity increased until it reached the peak at 40^{o}C. Beyond 40^{o}C the activity of the enzyme dropped.

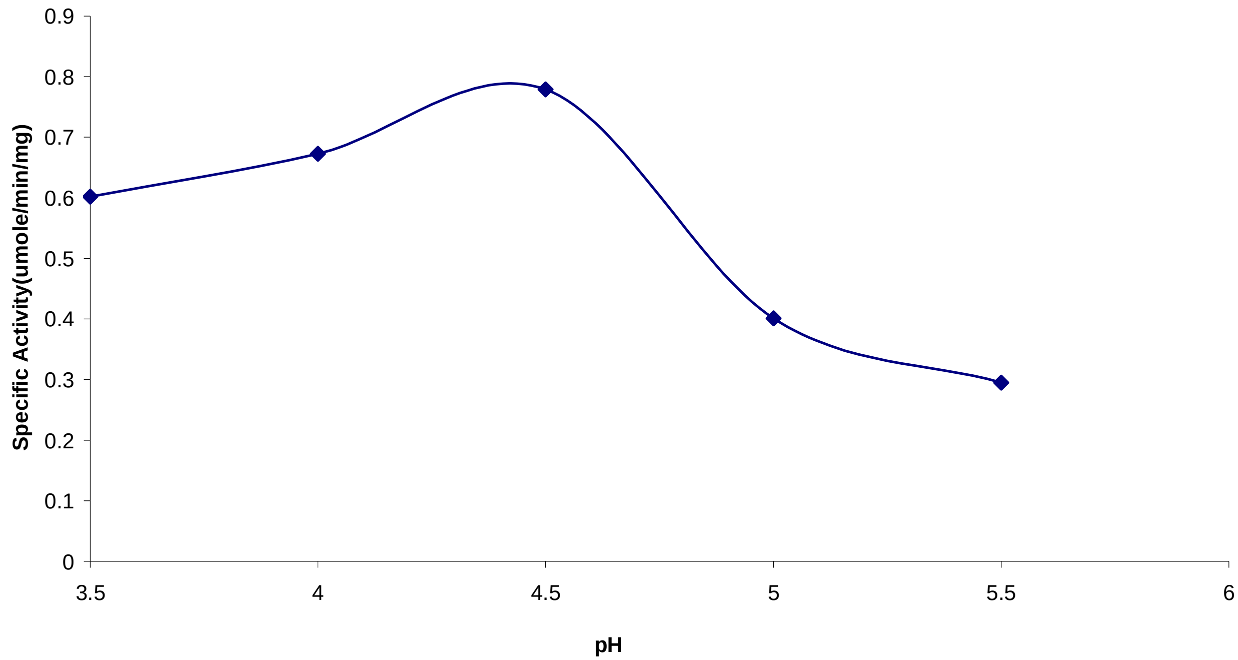


Fig. 4.13 Effect of pH Change on the Activity of immobilized Polygalacturonase

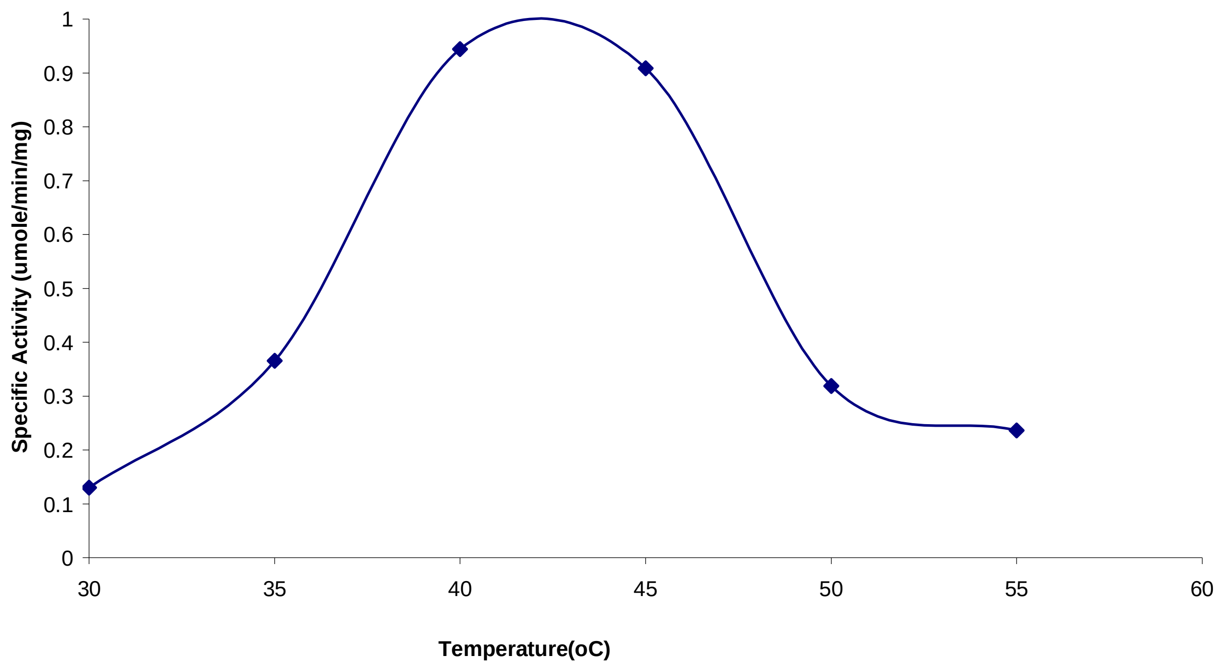


Fig. 4.14 Effect of Temperature Change on the Activity of Immobilized Polygalacturonase

4.18 DETERMINATION OF pH STABILITY OF THE IMMOBILIZED POLYGALACTURONASE.

As presented in figure 4.15 the immobilized polygalacturonase was stable at pH 4.0 and pH 5.0. The enzyme was however, more stable at pH 5.0. The lowest activity was recorded at pH 3.0 and above pH 5.0 the activity of the enzyme dropped sharply.

4.19. DETERMINATION OF TEMPERATURE STABILITY OF THE IMMOBILIZED POLYGALACTURONASE.

The temperature stability of the immobilized enzyme was determined after 1 hour incubation from 30°C -70°C at interval of 10°C. The enzyme was stable at 50°C and least stable at 70°C (figure 4.16)

4.20 EVALUATION OF CATALYTIC ACTIVITY OF THE IMMOBILIZED POLYGALACTURONASE PER CYCLE.

The immobilized polygalacturonase was used in three catalytic cycles at an interval of 30 minutes. The percentage residual activity in relation to the first activity was evaluated to get the catalytic efficiency of the enzyme. As presented in figure 4.17, the activity dropped from 100 % to 34.56 % and 14.81 % in the second and third catalytic cycles respectively.

The half life of the enzyme was found to be 10 minutes at 40°C

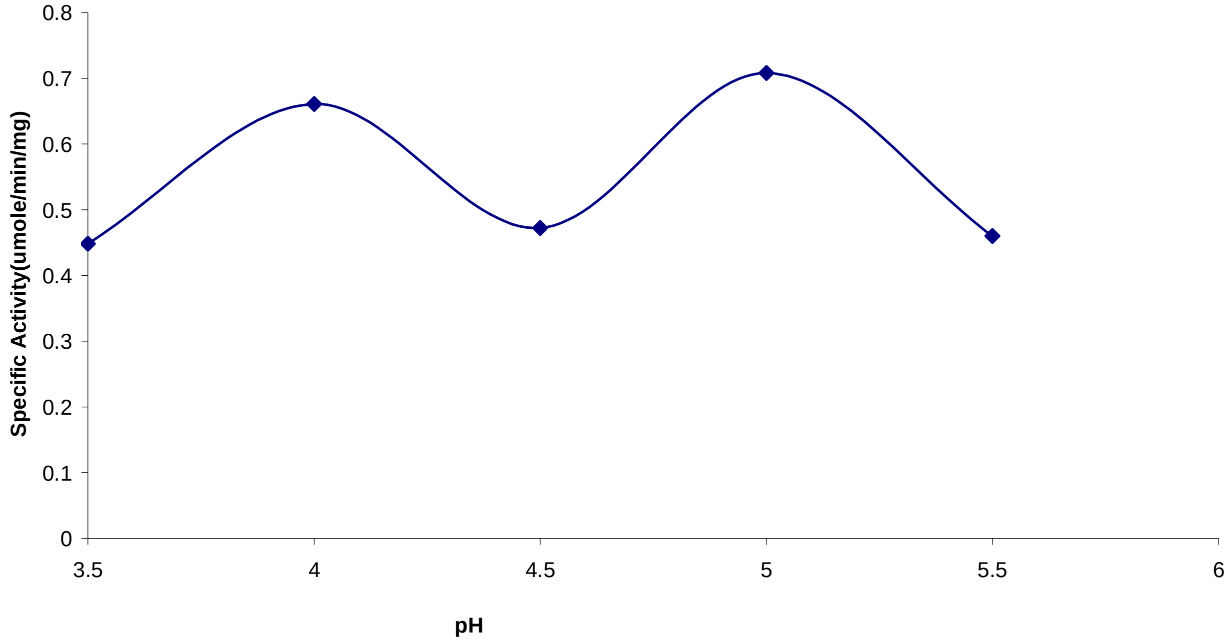


Fig. 4.15 pH Stability of Immobilized Polygalacturonase

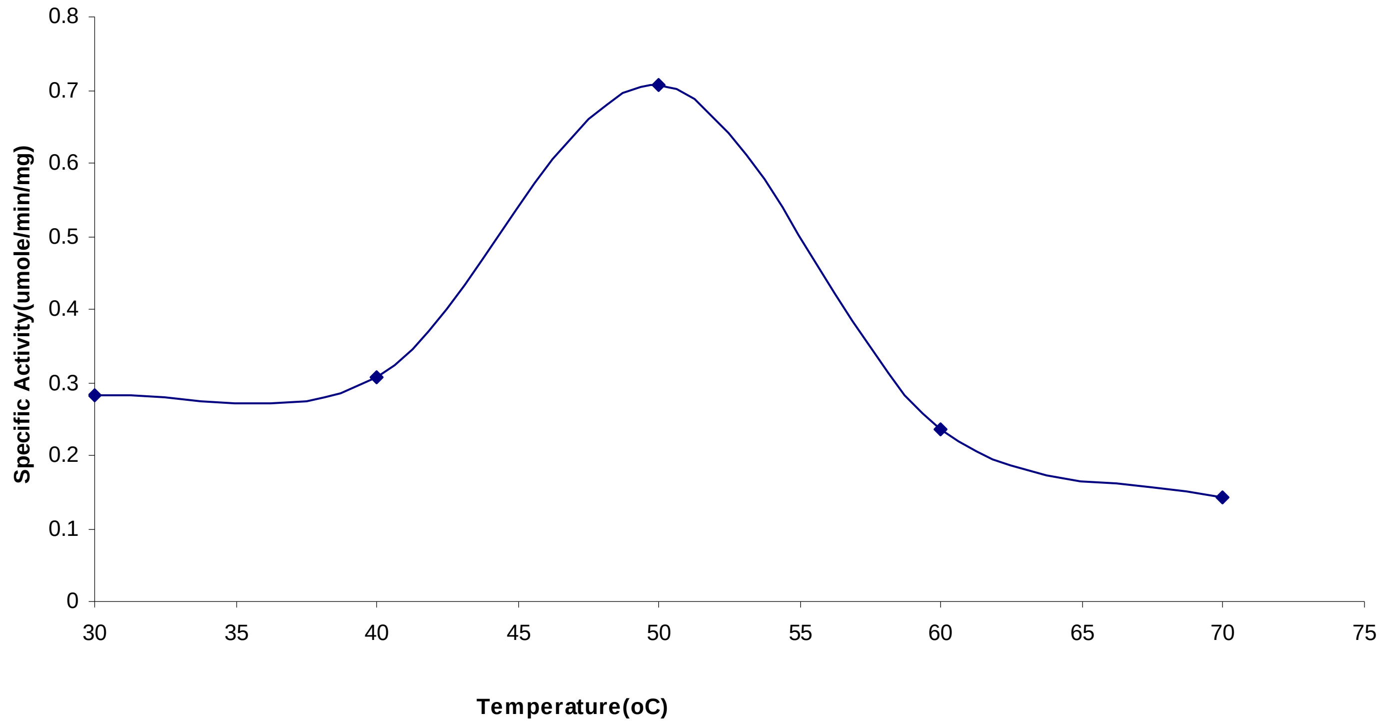


Fig. 4.16 Temperature Stability of Immobilized Polygalacturonase

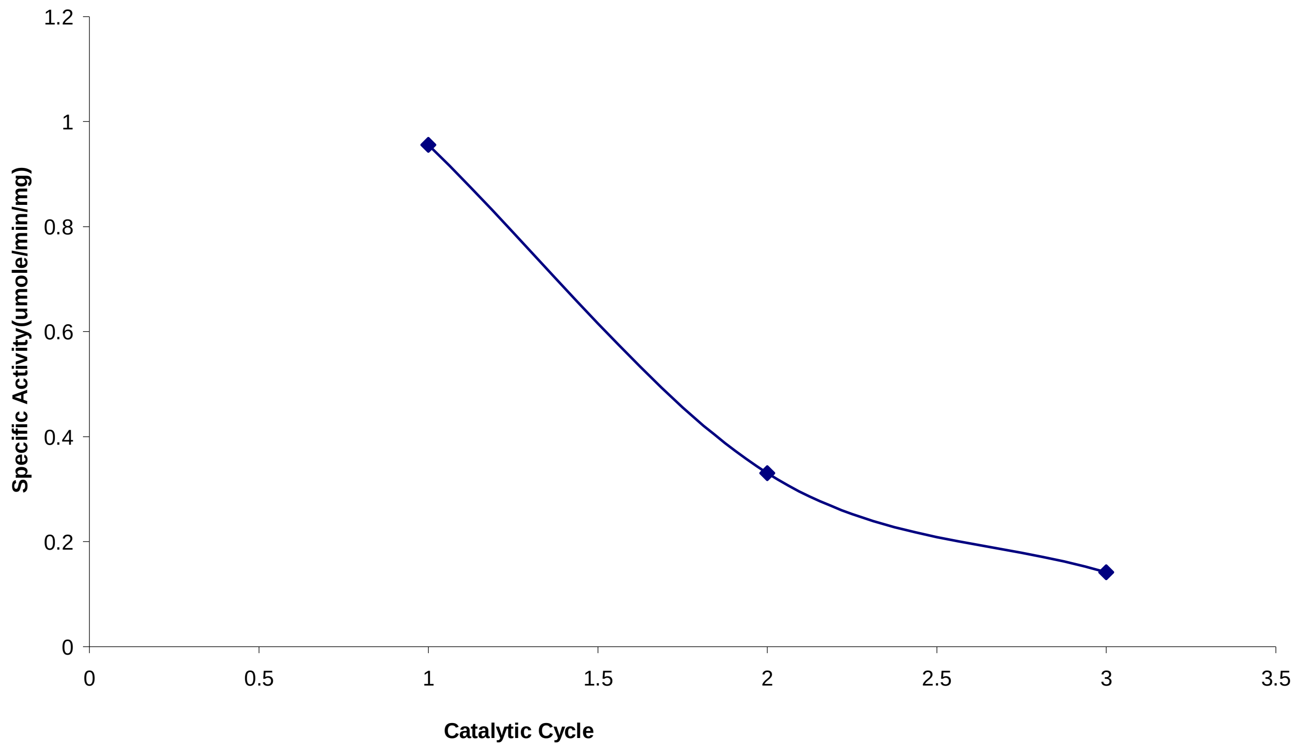


Fig. 4.17 Activity of Immobilized PG per cycle

4.21 KINETIC PARAMETERS OF NATIVE VERSUS IMMOBILIZED POLYGALACTURONASE

Table 4.21 presents the kinetic parameters of native Polygalacturonase as compared with those of the immobilized enzyme. The optimum pH and optimum temperature of both the native and immobilized Polygalacturonase was 4.5 and 40^{o}C respectively. Both the K_M and V_{max} of the native enzyme changed after immobilization. The immobilized enzyme is more stable to pH and temperature changes.

Table 4.2 Kinetic Parameters of Native Versus Immobilized Polygalacturonase

	Native Polygalacturonase	Immobilized Polygalacturonase
K_M(mg/ml) Pectin	2.74	11.1
V_{max}(µmoles/mg/min)	0.78	1.65
Optimum pH	4.5	4.5
Optimum Temp.(°C)	40	40
pH Stability	Low stability in the pH range of 3.5-9.0	Stable at pH 4 and 5
Temp. Stability(°C)	40	50

CHAPTER FIVE

5.0 DISCUSSION

The polygalacturonase was precipitated at 40, 50 ,60, 70 and 80 % ammonium sulphate saturation with the highest activity of 0.78µmole/mg/min obtained at 70 % saturation. The solubility of proteins depends on, among others, the salt concentration in the solution. At low salt concentrations, the presence of the salt stabilized the various charged groups on the enzyme protein, thus attracting the protein into solution (salting-in). However, as the salt concentration increased, the point of maximum solubility was reached and further increase of salt resulted in the protein being precipitated out of solution (salting-out).

Studies have shown that polygalacturonase can be precipitated with Ammonium Sulphate between 0-90 % depending on the source of the enzyme (Berger *et. al.,* 2000, Clausen and Green 1996, Mill and Tuttobello 1961).

Ion-exchange chromatography of the enzyme on DEAE-sepharose gave one active peak corresponding to fractions 2 and 3 which contained protein and activity with the highest activity of 1.8μmole/min/ml (1.1 μmole/min/mg) obtained in fraction 3. The enzyme was eluted in the flow through. This could be attributed to the fact that at pH 5 the charge on the enzyme and that on the DEAE- sepharose column were the same, hence the protein did not bind the column and the enzyme was therefore eluted. Proteins bind to column if the column and the protein carry opposite charges. Subsequent elution at higher salt concentration or pH gradient would elute the protein.

The gel filtration profile of the ion-exchange fraction revealed one active peak corresponding to fractions 3 and 4. Both fractions contained protein and activity with the highest activity of 0.9μmole/min/ml (0.51 μmole/min/mg)in fraction 4. The SDS-PAGE of the active fractions showed two bands that moved distinctly but closely on the gel with apparent molecular weights of 35 KDa and 40 KDa (Plate 4.5a and 4.5b) which suggests the existence of a dimeric protein consisting of both 'endo and exo' polygalacturonase activities. Kester *et. a.,l* (1996) reported a polygalacturonase with a molecular mass of 20 KDa while DeVries and Visser (2001) reported a range of 35 KDa- 80 KDa. This range is in agreement with the apparent molecular masses of the polygalacturonase isolated in this study. Previous studies have shown that some fungal polygalacturonases exists in iso forms (Clausen and Green 1996, Renata *et. al.,* 2001)

The Michaelis Menten constant (K_M) and the maximum velocity (V_{max}) of the polygalacturonase were 2.74mg/ml and 0.78 µmole/mg/min respectively as calculated from the Lineweaver-Burk plot. Studies have shown that, although Polygalacturonases share high degree of sequence homology, their rate of pectin hydrolysis, hence their kinetic constants can differ. (Lucie, 2000; De Vries and Visser 2001; Heinrichova and Rexova-Benkova 1976; Rexova-Benkova , 1973)

Suryakant (2001) reported a K_M of 0.12mg/ml and V_{max} of 111.1µM/min/mg for polygalacturonase from *Fusarium moniliforme* NCIM 1276. Lucie, 2000 also reported K_M values of <0.15 – 5.0mg/ml and specific activities of 8.8 – 7000U/mg for some fungal polygalacturonases. The K_M value of the polygalacturonase isolated in this study is comparable to those reported by previous workers.

The polygalacturonase showed optimum activity at pH 4.5.

The Dixon plot of $\log V_{max}/K_M$ versus pH revealed two ionizable groups (pKa 4.9 and 5.6) associated with catalysis of the enzyme. These pKa values appear to implicate carboxylate group of Glutamic acid and imidazole goup of Histidine in the active site of the enzyme. Previous studies have implicated a protonated Histidine and a carboxylate group in the active site of fungal Polygalacturonases(Rexova-Benkova and Siezarik 1968; Rexova-Benkova and Mrackova 1978; Rexova- Benkova 1990; Suryakant *et. al.,* 2001) Fungal polygalacturonases are known to operate in mild acidic environment. Sebastian (1996) and De Vries and Visser (1990) reported a range of optimum pH (3.8- 9.0) for *Aspergillus niger* polygalacturonases. Lucie (2000) reported pH optima for 23 polygalacturonases isolated from various species with a range of pH 3.8-6.5. The pH optima for 30 fungal polygalacturonases reported by Suryakant ranged 2.5- 6.0. Natalia *et. al.,(2004)* also reported an optimum pH of 4.5 for polygalacturonase isolated from *Monilla sp*. SB9. The optimum pH and possible amino acids in the active site of polygalacturonase is in agreement with those reported by previous workers.

In the pH stability studies, results obtained showed that, the enzyme retained 23.01 % of its initial activity at pH 4.5 after 24 hours incubation with citrus pectin at ambient temperature in acetate buffer, 28.32 % at pH 7.0 in phosphate buffer and 22.13 % at pH 7.5 in Tris-Hcl buffer. The results indicate that, though the enzyme exhibits some level of stability in different pH environments, the stability is low. The low stability could be attributed to marked changes in the charge distribution on the enzyme after 24 hours incubation leading to possible denaturation and eventual reduction of activity.

Temperature studies of Polygalacturonase revealed optimum activity at 40^{o}C and activation energy calculated from Arrhenius plot of 259.19Cal/degree/mole. According to collision theory, reactants must collide to form products after absorbing sufficient energy. Deeviet *et. al.,* (2004) reported activation energies of 4.39 and 6.83 Kcal/mole for free and immobilized commercial pectinase respectively. In a similar study Sartoglu *et. al.,*2001 reported values of 9.424 and 11.98 Kcal/mole for free and immobilized commercial pectinase. These values are comparable to the activation energy of the native Polygalacturonase in this study. The optimum temperature of the polygalacturonase is also comparable to the optimum temperature reported for many polygalacturonases isolated from several fungi, yeast plant and bacteria with a range of 37°C- 55°C(Blanco *et. al.,* 1999; Serrat *et. al.,* 2002; Frederici 1985; Polizeli *et. a.,* 1991; Semenova et al 2003 and Natalia *et. al*., 2004)

The polygalacturonase is stable at 40°C and it retained 77.7% of its optimum activity at 50°C. This stability is a good attribute in fruit processing where certain unit operations may involve changes in temperature and increased residence time. The good stability could be due to slow denaturation of the enzyme. Verlent *et. al.*, 2004 reported a polygalacturonase that is stable at 60°C. A polygalacturonase with a half-life of 18 minutes at 80°C was reported by Nirpjit and hoondal 1996. Karbasi and Vaughn (1980) reported a polygalacturonase from *Bacillus stearothermophillus* that was active at 70°C

The effect of substrate concentration on the activity of the polygalacturonase in the presence of EDTA, Mg^{2+},Ca^{2+} and Mn^{2+} was used to calculate the K_M and V_{max} of the enzyme which was compared to the K_M and V_{max} of the enzyme in the absence of EDTA and the divalent cations.

Both the K_M and V_{max} of the enzyme were increased by incubation with 10mM EDTA, Mg^{2+}, Ca^{2+} and Mn^{2+} ions. Calcium had the highest stimulatory effect while magnesium had the lowest. It is not clear how these divalent cations and EDTA exert their effects on the activity of the enzyme. It is possible that the enzyme is more stable in the presence of these ions and EDTA and they possibly do not interact with the active site residues of the enzyme, hence the increase in the K_M and V_{Max} of the enzyme. Nirpjit and Hoondal (1996) reported the existence of a thermo stable extra cellular pectinase that was stimulated by Mg2+, Ca^{2+} and Mn^{2+}. The enzyme (P1) was stimulated by 10mM Mg^{2+} which increased its activity by 115 % and the enzyme (P111) had its activity increased by 120 %

Kelly and Fogarty (1978) reported that 1 mM of Ca^{2+} causes 290 % increase in polygalacturonase lyase activity. Also, Mill (1966) reported that addition of 0.1% EDTA (disodium salt) did not alter the rate of the reaction of pectic enzymes isolated from *Aspergillus niger*.

The K_M and V_{max} of immobilized polygalacturonase calculated from Lineweaver- Burk plot were 11.1mg/ml and 1.65 µmole/min/mg/g of enzyme bead respectively when compared with the K_M and V_{max} of the native polygalacturonase there was increase in both K_M and V_{max} after immobilization of the enzyme. The increase in K_M of the immobilized Polygalacturonase may be due to inaccessibility of the substrate to the enzyme due to limitation in diffusion of substrate into the enzyme bead which is affected by the bead size, shape, pore size and enzyme loading per bead. The size and volume of the bead used in this study were 2.0mm and 4.18cm^3 respectively. It was reported that the mass transfer limitation is manifested in an increase in the Michaelis Menten constant of the immobilized enzyme as compared with the K_M of the native enzyme. Thus, the value of apparent K_M is a good indicator of the extent of mass transfer resistance. (Pier *et.*

al., 2004; Sartoglu *et. al.,* 2001; Weibel *et. al.,* 2004) The increase in the V_{max} as compared with that of the native enzyme may be attributed to increased stability of the enzyme after immobilization. Pier *et. al.,* (2004) reported an immobilized polygalacturonase with a considerably high catalytic activity and higher apparent K_M value when compared with those of the native enzyme. Sartoglu *et. al.,* 2001 also reported a commercial immobilized pectinase with an increase in both K_M and V_{max} values after immobilization. This is consistent with the findings of this study. The optimum temperature of the immobilized enzyme remained 40°C. The immobilized enzyme is stable between pH 4 to pH 5.0 and more stable at 50°C when compared with the native enzyme which was more stable at 40°C. The broad pH stability which remained in acidic region could be an advantage in fruit processing because the natural environment of the enzyme is mildly acidic. The stability of the enzyme up to 50°C makes it less susceptible to thermal inactivation during fruit processing. The half-life of the enzyme at 40°C and the activity lost per minute on thermal storage were 10 minutes and 0.0213 µmole of D-galacturonic acid.

The increase in pH stability could be due to presence of positive and negative charges in the entrapped enzyme matrix. The increase temperature stability could be due to restriction of the enzyme and mass transfer resistance .It is generally recognized that kinetic parameters of immobilized enzymes are not true constants but apparent values and could change due to restriction of enzyme and substrate flow in and out the bead.

In order to evaluate the efficiency of the immobilized polygalacturonase, it was subjected to three catalytic cycles. The second and third cycles reduced its catalytic activity to 34.56 % and 14.81 % respectively. The reduction of activity may be due to heat inactivation and gradual loss of activity due to enzyme leakage. Entrapped enzymes loose activity after several catalytic cycles due to leakage of the enzyme into the surrounding medium. (http//www.glue.umd.ench.edu/-nsw/ench485/lab13.htm. Busto *et. al., (*2005) reported a pectin lyase that could be re-used through 4 cycles while Narsimha Rao *et. al.,* reported an endopolygalacturonase from *Aspergillus ustus* that retained 28 % of its activity when immobilized and could be used through 10 cycles.

5.0 CONCLUSION AND SUGGESTIONS

The findings from this research have shown that *Aspergillus niger* (SA6) excretes Polygalacturonase. This study is a novel one therefore, the data presented in this research would help in designing immobilized Polygalacturonase systems for use in fruit processing.

The enzyme has been partially purified, characterized and immobilized using sodium alginate. The research has demonstrated that both native and immobilized Polygalacturonase showed optimum activity at pH 4.5 and 40^{o}C respectively. The electrophoregram of the enzyme has revealed that Polygalacturonase exists as a dimer (endo and exo forms). Through this research, the immobilized Polygalacturonase from *Aspergillus niger* (SA6) has been found to be stable over pH 4-5 (more stable at pH 5.0) and 50^{o}C. The pH and temperature stability is an important feature of the enzyme that could be useful in fruit processing. From the results of this work histidine and

Glutamic acid are present in the active site of the enzyme. This research has also demonstrated that EDTA, Ca^{2+}, Mg^{2+} and Mn^{2+} ions have no inhibitory effects on the Polygalacturonase. The research has demonstrated that Polygalacturonase can be used through at least three catalytic cycles with a half-life of ten minutes at $40^{o}C$.

In order to over produce the enzyme for use in fruit processing, further work should be focused on gene cloning and over expression. It is also suggested that more work should be done to evaluate the efficiency of the enzyme using different fruits.

REFERENCES

Acuna-Arguelles, M.E; Gutierrez-Rojas, M; Viniegra-Gonzalez. G. Favela-Torres, E. (1995) Production and Properties of Three Pectinolytic Activities Produced by *Aspergillus niger* in Sub-merged and Solid Sate Fermentation. *Appl. Microbiol. Biotechnol* 43, (5) 808-814.

Ahmed, R. Ahmad, S. Ighal J.; Baizy, M.A (1997) Shake Flask Studies for Preliminaries by *Aspergillus foetidus*. *Park J. Sci. Res*. 49:36-39.

Akin, D.E; Foulk, J.A; Dodd, R.B and Mc Alister, D.D. (2001)Enzyme Retting of Flax and Characterization of Processed Fibers. *J. Biotechnol* 89 (2-3) 193-203.

Akinola, S.O. and Olatunji, O.O. (2000). Pilot Production of Pectinase from Corn Pomace and Production of Clarified FruitJuices from Pulpy Fruits. Book of Abstracts. International Conference on Biotechnology, Sheraton Hotel and Towers, Abuja, October 24-26.

Alkorta, I; Gartrisu, C; Ijama, M.I and Serra I.L. (1998) Industrial Applications of Pectic Enzymes: a review. Process Biochem 33, 21 – 28.

Bahulekar, R; Ayyangar, N.R and Ponrathanam, S. (1991) Polyethyleneimine in Immobilization of Biocatalysts *Enzyme Microb. Technol* – 13, 858-868

Baker, R.A and Bruemmer J.H (1972) Pectinase Stabilization of Orange Juice Cloude. *Agric .Food Chem.*20, 1169-1173

Baracat, M.C; Vanetti, M.C.D; Araujo, E.F and Silva, D.O (1991) Growth Conditions of a Pectinolytic *Aspergillus fumigatus* for Degumming of Natural Fibers. *Biotechnol Lett.* 13, 693-696.

Bauman, J.W. (1981) Application of Enzymes in FruitJuice Technology In: Enzymes and Food Processing. G. birch, N. Blake brough and K.J. Parker (Eds) London Applied Science Publishers Ltd. PP 129-147.

Bello R.A (2000) Industrial Enzyme use in Nigeria and Investment Opportunities for Production. Book of Abstracts. International Conference on Biotechnology, Sheraton Hotel and Towers, Abuja, October 24 – 26 PP 5.

Benen, J.A.E; Kester, H.C.M and Visser J. (1999) Kinetic Characterization of *Aspergillus niger* N400 Endopolygalacturonases I, II and C. *Eur. J. Biochem*, 159, 577-585.

Bennett, J.W. (1985 a) Moulds, Manufacturing and Molecular Genetics of Filamentous Fungi. Alan R. Liss Inc. N.Y.

Berger, D.K, Delopse, M.S; Arendse, E; Plessis Du, E and Dubery, I.A. (2000) Bean Polygalacturonase Inhibitor Protein (PGIP) Inhibits Polygalacturonases from *Stenocarpella maydis*. Physiological and Molecular plant pathology. 57, 5-14.

Bickerstaff, G.F (1987) Enzymes in Industry and Medicine. Edward Arnold Publishers Ltd. 41, Bedford Square, London WClB 3DQ, PP10

Blanco P; Sierro C. Reboredo, N.M. and Villa, T.G. (1998) Cloning, Molecular Characterization and Expression of an Endopolygalacturonase Encoding Gene from *Saccharomyces Cerevisiae* IMI-8b. *FEMS Microbiol. Lett.* 164, 249-255.

Blanco, P. Siero, C and Villa, T.G (1999) Production and Extraction of Pectinases Obtained by Solid – State Fermentation of Agro – Industrial Residues with *Aspergillus niger*. *Bioresource Technol*. 71, 45-50.

Blanco, P; Sieroc, Villa, T.G (1999) Production of Pectic Enzymes in Yeasts. *FEMS Microbiol. Lett*. 175 (1): 1-9

Bradford, M.M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Biochemistry.* 72:248-254

Bussink H.J.D, Brouwer, K.B., de Graaff, L.tt., Kester, H.M.C and Visser J. (1991.) Identification and Characterization of a SecondPolygalacturonase Gene of *Aspergillus Niger*, *Curr. Genet*. 20, 301 – 307.

Bussink, H.J.D., Buxton, F.P Fraaye, B.A de Graaff, L.H. and Visser J. (1992). The Polygalacturonases of Aspergillus Niger are Encoded by a Family of Diverged Genes. *Eur. J. Biochem*. 208, 83-90.

Bussink, H.J.D, Kester, H.M.C and Visser J. (1990) Molecular Cloning, Nucleotide Sequence and Expression of the Gene Encoding Prepropolygalacturonase II of *Aspergillus niger*, *FEBS Lett*. 273, 127 – 130.

Busto, M.D; Garcia-Tramontin, K.E; Ortega, N and Perez-Mateos, M (2005). Preparation and Properties of an Immobilized Pectin Lyase for the Treatment of Fruit Juices. *Bioresource Technology* 97 1477-1483

Canal-LLauberes, R.M. (1993) Enzymes in Wine Making. In: Wine Microbiology and Biotechnology. G.H Fleet (Ed). Harwood Academic Publishers, PhiladelPhia, PP 477-506.

Cannel, E. and Moo-Young M. (1980) Solid State Fermentation Systems. *Process Biochem*. 15, 2-7

Carasik, W. and Carrol, J.O (1983) Development of Immobilized Enzymes for Production of High Fructose Corn Syrup. *Food Technol* 37, 85 – 91.

Carpita, N.C and Gibeaut, D.M (1993) Structural Models of Primary Cell Walls in Flowering plants: Consistency of Molecular Structure with the Physical Properties of the Walls During Growth *plant J*. 3, 1-30

Castilho, L.R., Modronho, R.A, and Alves T.L.M (2000) Production and Extraction of Pectinases obtained by Solid State

Fermentation of Agro-industrial Residues with *Aspergillus niger*. *Bioresource Technol*. 71, 45-50.

Cavalito, S.F; Arcas, J.A and Hours, R.A (1996) Pectinase Production Profile of *Aspergillus foetidus* in Solid State Cultures at Different Acidities. *Biotech. Lett*. 18, 251-256.

Chibata, I (1980) In. Food process Engineering (Eds Linko, P. and larinkari, J.) Applied Science Publishers London 2, PP 1-39.

Clausen, C.A and Green III, F (1996) Characterization of Polygalacturonase from the Brown-rot Fungus Postia Placenta. *App. Microbiol. Biotechnol* 45: 750-754

Costa, J.A.V; Alegre, R.M. and Hassan, S.D.M. (1998) Packing Density and Thermal Conductivity Determination of Rice Bran Solid State Fermentation. *Biotechnol. Tech*. 12, 747 – 750.

Csiszar, E; Losoncz; A. Szakacs, G, Rusznak, I. Bezur, L; and Reicher J. (2001) Enzymes and Chelating Agent in Cotton Pre-treatment *J. Biotechnol* 89 (2-3) 271-279.

Dixon, M. (1953). The Determination of Enzyme Inhibitor Constants. *Biochem J*. 55: 170 – 171.

Dixon, M and Webb, E.C (1964) Enzymes 2nd Edition Longmans,

New York PP 116-166

D'souza, S.F (1983) Cosmotic Stabilization of Mitochondria Using Chemical Cross-linkers. *Biotechnol. Bioeng* 25, 1661 – 1664.

D'Souza, S.F (1989) Immobilized Cell Techniques and Applications. *Indian J. of Microbiol* 29, 83 – 117

D'Souza, S. F. and Melo, J.S (1991) A Method for the Preparation of Coimmobilizates by Adhesion Using Polyethyleneimine. *Enzyme Microb. Technol*. 13, 508-511

D'Souza, S.F and Nadkarni, G.B (1981) Hen Egg White: A Novel Support for the Immobilization of Enzymes. *Biotechnol Bioeng*. 23, 431 – 436.

D'Souza, S.F and Nadkarni, G.B (1987) Immobilization and Stabilization ofBiomaterials for Biosensor. *J. Biosci*-11, 137-144.

D'souza, S.F; Kaul, R. and Nadkarni, G.B (1985) Ocipium Basillicum Seeds as a Pellicular Support for Immobilized Enzymes. *Biotechnol Left*. 7, 589-592.

D'urso, E.M and Fortier, G. (1996) Poly(Ethylene Glycol)- Bovine Serum Albumin Hydrogel as a Matrix for Enzyme Immobilization. *Enzyme Microb. Technol*, 18,482 – 488.

De Vries, R.P and Visser J. (2001) *Aspergillus* Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. *Microbiol. Mol Biol Rev*. 65 (4) 497 – 522.

Deshpande, A and D'souza, S.F (1988) Simultaneous Purification and Reversible Immobilization of D-amino Acid Oxidase from *Trigonopsis Variabilis* on a Hydrophobic Support *Indian J. Microbiol*. 28, 26 – 33.

Deviet, Demiral; Ahmet, R; Ozdural and Mehmet Mutiu (2004) Performance of Immobilized Pectinex Ultra SP-L on Magnetic Duolite- Polystyrene Composite Particles Part 1: a Batch Reactor Study 64(4) 417-421

Enzyme Technical Association (1999) Enzymes used in Food Processing – Washington. *NOSB Materials database*. Pp1-11

Federici, F (1985) Production, Purification and Partial Characterization of an Endopolygalacturonase from *Cryptococcus albidus Var. albidus*. Antonie Van Leeuwenhoek. 51(2): 139-150

Fraissinet – Tachet, L. Reymond-Cotton, P. And Fevre, M. (1995) Characterization of a Multigene Family Encoding an Endo polygalacturonase in *Sclerotinia sclerotiorum*. *Curr. Genet*. 29, 96-99.

Galiotou-panayotou, M. Rodis, P. And Kapantai, M. (1993) Enhanced Polygalacturonase Production by *Aspergillus niger* NRRL-362 Grown on Supplemented Citrus Pectin. *Lett. Appl*. *Microbiol* 17, 145-148.

Galiotou-panayotou, M; Kapantai, M; and Kakantzi, O (1997) Growth Conditions of Aspergillus SP. ATHUM – 3482, for Polygalacturonase Production. *Appl. Microbiol. Biotechnol*. 47, 425-429.

Gill, I; Lopez-Fandino, R; Jorba, X and Vultson. E.N (1996) Biologically Active Peptides and Enzymatic Approaches to their Production. *Enzyme Microb. Technol*. 18, 162-183

Godbole, S.S., Kubal, B.S and D'souza, S.F (1990) Hydrolysis of Concentrated Sucrose Syrups by Invertase Immobilized on Anion Exchanger Waste Cotton Thread. *Enzyme Microb. Technol* 12, 214-217.

Gramp P; E; and Urlaub, R.A (1984) The Application of Enzymes in Grape and Wine Processing. The International Symposium on Cool Climate Uiticulture and Enology. Oregon State University.

Gupta, M.N Guoqing, D and Mattiasson, B. (1993)Pectin Lyase Production by *Penecillium griseoroseum* ; Effect of Tea Extract, caffeine, Yeast Extract and Pectin. *Biotechnol*. *Appl. Biochem*. 18, 321 – 327

Haight K.G and Gump, B.H. (1995) Red and White Grape Juice Concentrate Component Ranges *J. Food Composition and Analysis 8, 71-77.*

Hartmier, W. (1988). Immobilized Biocatalysts. An Introduction. Mattiasson, B (Ed), Immobilized cells and organelles, CRC, Press. Springer Verlag, Berlin. Boca Raton 1-2

He, S.Y and Collmer, A (1990) Molecular Cloning, Nucleotide Sequence and Marker Exchange Mutagenesis of the Exo-poly-alpha D-galacturonosidase Encoding Pehx Gene *of Erwinia chrysanthemi. EC16. J. Bacteriol 172, 4988-4995.*

Heinrichova, K. and Rexova-benkova, L (1976) Purification and Characterization of another D-galacturonase from *Aspergillus niger* collection Czechoslov-chem. Commun. 42, 3569 – 3576.

Hicke, H. G; Bohme P., Becker, M. Schulze, H and Ulbricht, J. (1996) Immobilization of Enzymes onto modified Polyacrylonitrile Membranes- Application of the Acyl Azide *J. Appl. Polym Sci*. 60, 1147-1161.

Hong, S.B. and Tucker, M.L. (1998) Genomic Organisation ofix Tomato Polygalacturonases and 5' upstream Sequence Identify with tap I and win 2 genes. *Mol. Gen. Genet*. 258, 479-487.

http//.www.ench.umd.edu/-nsw/ench485/lab7b-htm

http://afmb.cnrs-mrs.fr/rpedro/CA27/ghf 28.htm.

http://www.herc.com/foodgums/pectin

http://www.glue.umd.ench.edu/nsw/ench485/lab13.htm

http://www.epa.gov/biotech-rule/pubs/fra/fra006.htm.

http: en.wikibooks.org/Potato Dextrose Media Preparation

http://www.ippa.info/applications-for-pectin.htm.

http//www.ippa.info/safety.htm

http://www.ippa.info/history-of-pectin.htm

http://www.ippa.infor/types-of-pectin.htm

Huang, J. and Schell, M.A. (1990) DNA Sequence Analysis of PgA and Mechanism of Export of its Polygalacturonase Product from *Pseudemonase solanacearum*. *J. Bacteriol* 172, 3879-3887.

Hussain, S. and Jafri, F. (1995) Covalent Immobilization of Invertase and Horseradish Peroxidase on ConcanavalinA – seralose via Carbohydrate Moieties. *Biochem Mol. Biol. Int* 36, 669 – 677.

Iguchi, K. Hirano, H. Kishida, M., Kawasaki, H and Sakai, T. (1997) Cloning of a Protopectinase Gene of *Trichosporon penicillatum* and its Expression *in Saccharomyces cerevisiae*. *Microbiol*. 143, 1657-1664.

Ishida, Y; Kakibuchi, K; Hirao, Y and Izumori, K (1997) Cloning and Characterization of a Polygalacturonase from *Penicillium Janthinellum*. *J. Ferment. Bioeng*. 84: 257-260

Jong, S.C and Grantt, M.J. (Eds) (1987) Catalogue of Fungi and Yeasts. 17th edition. American Type Culture Collection, Rockville, M.D. ISBN 0-9300918-5

Kamath, N. Melo, J.S and D'souza, S.F (1988) Simultaneous Filtration and Immobilization of Cells from a Flowing Suspension Using a Bioreactor Containing Polyethyleneimine Coated Cotton Threads. Applications in the Continuous Inversion of Concentrated Sucrose Syrups. *Appl. Biochem Biotechnol* 19, 251-258.

Karbasi and Vaughn (1980) Production of Constitutive Thermostable, Hyper Active Exo-Pectinase from Bacillus GK-8. *Biotechnology Letters.* 18:12, 1435-1438.

Kaul, R, D'souza, S.F and Nadkarni, G.B (1984)A Method for the Preparation of Hen Egg White Beads Containing Biocatalysts. *Biotechnol Bio eng*. 26, 901 – 904.

Kelly, C.T and Fogarty,W.M. (1978) Production and Properties of Polygalacturonase Lyase by an Alkalophilic MicroOrganism *Bacillus sp*. RKa *Can. J. Microbiol*. 24, 1164- 1172.

Kester, H.C.M and Visser, J. (1990) Purification and Characterization of Polygalacturonases Produced by the Hyphal Fungus *Aspergillus niger*. *Biotechnol. Appl. Biochem* 12, 150 – 160.

Kester, H.CM; kusters-Van Someren, M.A; Muller,Y.; Visser, J. (1996) Primary Structure and Characterization of Exopolygalacturonase from *Aspergillus tubingensis*. *Eur. J. Biochem* 240: 738-746

Kilara A; (1982) Enzymes and their Uses in the Processed Apple Industry. A review *Process Biochem* 17, 4, 35-41

Kolot, F.B. (1981) Microbial Carriers- Strategy for Selection. *Process Biochem* 16*,* 5, 2-9.

Komiyama, N. Sone, T. Shimizu, K; Morikubo, K and Kino, K (1994) cDNA Cloning and Expression of cry I II the Second Major Allergen of Japanese Cedar Pollen. *Biochem. Biophys. Res. Commun*. 201, - 1021 – 1028.

Kozowski, M. and Stepien, P.P. (1982) Restriction Analysis of Mitochondrial DNA of Members of the Genus *Aspergillus* as an Aid in Taxonomy. *J. Gen. Microbiol*. 128, 471476 (1).

Kubicek, C and Rohr, M. (1986) Citric Acid Fermentation.CRC Critical Reviews in Biotechnology 3. (4): 331373.

Kumar, S.D; Kukarni, A.V; Dhaneshwar, R.G and D'Souza, S.F (1992). *Bioelectrochem Bioenergetics*. 27, 153-160

Laemmli, U.K (1970) Cleavage of Structural Proteins During theAssembly of the Head of Bacteriophage T4. *Nature* 227, 680-685.

Lineweaver, H and Burk, D (1934). The Determination of Enzymes Dissociation Constants. *J. AM. Chem. Soc*. 56, 658.

Long, C. and Looman, A.C. (1995). Efficient Expression and Secretion of Aspergillus niger RH5344 Polygalacturonase in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol*. 44, 147 – 156.

Lucie P. (2000) Pectinase of Aspergillus niger: A Molecular and Biochemical Characterization. *Thesis*, Wageningen University. Netherlands. ISBN 90 – 5808-203-2.

Maldonado, M.C and Strasser De Saad, A.M. (1998) Production of Pectin Esterase and Polygalcaturonase by Aspergillus Niger in Sub-merged and Solid State Systems. *J. Ind. Microbiol-Biotechno*l. 20, 34-38.

Marolia, K.Z and D'souza, S.F (1993) A Simple Technique for the Immobilization of Lysozyme by Croos-Linking of Hen Egg White Foam. *J. Biochem Biophys*. *Methods* 76, 143 – 147.

Mattiasson, B. (1982)mmobilization of Biomaterials for Biosensor Applications. *Appl. Biochem, Biotechnol* 7, 121 – 125.

McGraw H. (1987) Encyclopedia of Science and Technology 6th ed. 13 PAC-PLAN. Pp 175-176

McMillan, J.D. and Sheiman, M.I (1974) Pectic Enzymes In: Food Related Enzymes J.R Whitaker (ed) Adv. In Chemistry Series *Amer. Chem. Soc*. Washington D.C PP 101-130.

Melo, J.S and D'Souza, S.F (1992) Immobilization of Invertase via Carbohydrate Moeity on Ocimum Basilicum Seed. *Appl. Biochem Biotechnol* 32, 159 – 170.

Meyrath, J. and Volavsek G. (1975) Production of Microbial Enzymes In: Enzymes in Food Processing (2nd Ed) G. Reed Ed New York Academic Press PP 225-300.

Micheli, P.A (1729) Nova Plantarum Genera. Illus. Florentiae – PP 1-234.

Mill, P.J (1966). The Pectic Enzymes of Aspergillus niger: A Second Exopolygalacturonase . Biochem. J. 99-562.

Mill, P.J and Tuttobello, R (1961). The Pectic Enzymes of *Aspergillus niger* 2. Endopolygalacturonase. *Biochem. J*. 79, 57

Miller, G.L. (1959) The Use of Dinitrosalicylic Acid Reagent for Determination of Reducing sugar – *Anal- Chem*. 31, 426 – 428.

Mosbach, K (1974) Separation of Iso-enzymes. *Methods in Enzymol*. Academic Press, London.34B:595-597

Nakajima, M: Nishizawa, K and Nabetani, H.A (1993) Forced Flow Membrane Enzyme Reactor for Sucrose Inversion Using Molasses. Bioproc. Eng. 9, 31 – 38.

Narsimha, Rao, M; Kembhavi A.A and Pant, A (2000) Immobilization of Endopolygalacturonase from *Aspergillus* ustus on Silica gel. *Biotechnology Letters* 22 (19) 1557-1559

Natalla, M; Simone, R.D.S; Roberto, D.S and Eleni, G. (2004) Pectinase Production by Fungai Strains in Solid State Fermentation Using Agro-industrial Bio-product. *Brazillian Archives of Biology and Technology* 47, (5) 5.

Nelson, J.M and Griffin, E.G (1916) *J. AM. Chem. Soc*. 38, 1109 – 1115.

Nelson, P.H (1991) Enzyme Applications (Industrial) In: Kirk othmer Encyclopedia of Chemical Technology 4th ed. 9:567-620.

National Bureau of Statistics (2001)Nigeria Foreign Trade Summary Abuja, Nigeria PP 41.

Nirpjit Singh Dosanji and Hoodal, G.S (1996) Production of Constitutive, Thermostable, Hyperactive Exopectinase from *Bacillus CK-8*. *Biotechnology Letters* 18 (12) 1435-1438

Pasculli, R. Geraeds, C., Voragen, F. And Pilnik, W. (1991) Characterization of Polygalacturonases from Yeast and Fungi *Lebensm. Wiss. Technol*. 24, 63-70.

Pastore, M. and Morisi, F. (1976) Lactose Reduction of Milk by Fibre-Entrapped Beta-galactosidase : Pilot Plant Experiments. Methods Enzymol 44, 822 – 844.

Pikersgill, R; Smitch, D. Worboys K, and Jenkins J. (1998). Crystal Structure of Polygalacturonase from *Erwinia carotova . J. BioChem* 273 (38) 24660-24664.

Pier Giorgio, P; Maurilio, T and Alberto, M (2004) Immobilization of Endopolygalacturonase from Aspergillus niger on Various Types of Macromolecular Supports. *Biotechnology and Bioengineering* 33(10) 1258-1266

Pilnik, W and Voragen, A.G.J (1993) Pectic Enzymes in Fruit and Vegetable Juice Manufacture in: Nagoda withama, T and Reed, G (Eds). Enzymes in Food Processing, New York: Academic Press. PP. 363-369.

Polizeli Mde, L, Jorge, J.A; Terenzi, H.F (1991) Pectinase production by Neurospora Crassa: Purification and Biochemical Characterization of Extracellular Polygalacturonase. *J.Gen Microbiol*. 137(8): 1815- 1823

Poulsen, P.B. (1981) in : Immobilized Enzymes in Bioprocess. *Enzyme Microb. Technol* 3, 271 – 273.

Raper, K.B and Fennel, D.I (1965) the genus *Aspergillus*. Williams and Wilkins Company, Baltimore, MD. Pp 1-686

Reed, G. (1975) Enzymes in Food Processing. New York Academic Press. 2nd Edition Pp 48-50

Reichlin, M.E. (1980) in: Immobilized Enzymes in Bioprocess. Methods Enzymol.

70, 159 – 165.

Renata,I; Barense, Maria; Angelica dos, S.C , Chellegatti; Maria Jose V. Fonseca; Surain Said (2001). Partial Purification and Characterization of Exopolygalacturonase II and III of *Penicilium frequentans. Brazilian J. of Microbiology* 32 (4)

Rexova – Benkova, I. (1973). The Size of the Substrate Binding Site of an *Aspergillus niger* Extra Cellular Polygalacturonase. *Eur. J. Biochem*. 39, 109-115.

Rexova-Benkova, I and Slezarik, A (1968) On the Role of Histidine in the Active Center of the Endopolygalacturonase of *Aspergillus niger*. *Collection*. *Czechoslov Chem. Commun*, 35, 1253-1260

Rexova-Benkova, I and Mrackova, M, A (1978) Active Groups of Extracellular Endo-D-galacturonase of *Aspergillus niger* Derived from pH Effect on Kinetic Data. *Biochem. Biophy. Acta* 523, 162-169

Rexova-Benkova, I (1990) Evidence for the Role of Carboxyl Groups in the Activity of Endopolygalacturonase of *Aspergillus niger*, Chemical Modification by Carbodiimide Reagent. *Collection. Czechoslov Chem. Commun*, 55, 389-1395

Rombouts, F.M and Pilnik W. (1978) Enzymes in Fruits and Vegetable Juice Technology. *Process Biochem*.

13, (8) 9-13.

Ronald, P.C, Carl, W.B and Melvin, S.F (1997) Isolation and Characterization of an Endopolygalacturonase from *Cochliobolus stativus* and a Cytological Study of Fungal Penetration of Barley,. *Phytophatology* 87, 1148-1159.

Rong, L. Karcher, S.J. and Gelvin, S.B. (1991) Genetic and Molecular Analyses of Pic A, a Plant – Inducible Locus on the *Agrobacterium tumefaciens* chromosome. *J. Bacteriol* 173, 5110 – 5120.

Sada, T. Kato, H.S Terashima, M. and Kikichi, Y (1987) *Biotechnol. Bioeng* 30, 117 – 122.

Sartoglu, Kamal; Demir, Nilay; Acar, Jale; Mutlu, Mehmet (2001) The Use of Commercial Pectinase in the Fruit Industry, part 2: Determination of Kinetic Behaviour of Immobilized Commercial Pectinase. *Journal of Food Engineering* 47, 4, 271-274

Samson, R.A (1994) Current Systematics of the Genus *Aspergillus*. In the Genus *Aspergillus*; from Taxonomy and Genetics to Industrial Applications (Eds Powell, K.A, Ren wick, A and Peberdy, J.F) Plenum press London – PP 261 – 276.

Samejima, H; Kimura, K and Ado, Y (1980)Recent Development and Future Directions of Enzyme Technology in Japan. *Biochemie* 62 299-315

Sankaran, K, Godbol; S.S and D'souza S.F (1989) Simultaneous Purification and Reversible Immobilization of D-amino Acid Oxidase from Trignopsis Variabilis on a Hydrophobic Support. *Enzyme Micro*. *Technol*. 11, 617-619.

Sanzo, A.V; Hassan, S.D.M, Costa, J.A.V and Bertolin, T.E. (2001) Enhanced Glucoamylase Production in Semi-continous Solid State Cultivation of *Aspergillus niger* NRRL 3122 *Ceincia and Engehare* 10, 59-62.

Sarkanen, S. (1991).Enzymatic lignin degradation an extra cellular view in: Leathem, G.F and Himmel, M.E (eds) Enzymes in Biomass Conversion, ACS Symp. Series 460, *American Chem.. Soc.* PP. 247 – 269.

Sawada, K. and Ueda, M. (2001) Enzyme Processing in Textiles in Reverse Micellar Solution *J. Biotechnol*. 89(2-3) 263-269.

Sebastian, F. Cavalitto-Jorge A, Arcas and Roque-A, Hours (1996) Pectinase Production Profile of *Aspergillus foetidus* in Solid State Cultures at Different Acidities. *Biotechnology letters* 18, 3, 251 – 256.

Semenova, M.V; Grishutin, S.G; Gusakov, A.V, Okunev, O.N. Sinitsyn, A.P (2003) Isolation and Properties of Pectinases from the Fungus *AspergillusjJaponicus. Biochemistry(Moscow)* 68:5, 686-697

Serrat, M; Bermudez, R.C; Villa, T.G (2002) Production, and Charadterization of a Polygalacturonase from a New Strain of *Kluyveromyces marxianus* Isolated from Coffee Wet- processing Wastewater. *Appl. Biochem. Biotechnol*. 97(3) 193-208

Sharma, B.P and Messing, R.A (1980) Pitcher, W.H. (ed) In: Immobilized Enzymes for Food Processing CRC press, Boca Raton, Florida PP185 – 209.

Shevchik, V.E; Kester, H.C.M; Benen, J.A.E; Visser, J; Robert-Baudouy, J. and Hugouvieux-Cotte-pattat, N. (1999) Characterization of the Exopolyglacturonase Lyase pelx of *Erwinia chrysanthemi* 3937, J. Bacteriol 181, 1652-1663.

Siekstele, R. BartKeviciute, D and Sasnauskas, K. (1999) Cloning Targeted Disruption and Hetrerogenous Expression of the *Kluyeromyces marxianus* Endo Polygalacturonase gene (EPGI) *Yeast* 15, 311 – 322.

Soares, M.M.C.N; Silva, R and Gomes, E (1999) Screening of Bacterial Strains for Pectinolytic Activity Characterization of the pgase Produced by *Bacillus species*. *Rev. Microbiol*, 30, 229 – 303.

Sohel D, Aparna Sharma and Munishwar Narth Gupta(2007) A Multipurpose Immobilized Biocatalyst with Pectinase, Xylanase and Cellulase activities 1: 16

Solis, S; Flores, M.E. and Huitron, C. (1997) Imporvement of Pectinase production by Interspecific Hydrides of *Aspergillus* strains *Lett. Appl. Microbiol*. 24. 77 – 81.

Solis-pereira, S. Favela-Torres, E. ; Gutierrez-Rojas, M. Roussos, S; Sauccedo-castafieda, G. And Vinegra-Gonzales, G. (1996) Production of Pectinases by *Aspergillus niger* in

Solid State Fermentation at High Initial Glucose Concentration. *World J. of Microbiol Biotechnol* 12, 257-260.

Stauffer, C.R. and Etson, D. (1969) Enzyme Assay and Kinetic Characterization. *J. Biol. Chem*. 244:5333-5338

Stutzenberger, F (1992) Pectinase production In: Encyclopedia of Microbiology (Lederberg J. ed-in-chief) Academy Press, New York, 3, 327-337.

Suryakant K. Niture, Aditi pant and Ameeta R. Kumar (2001) Active Site Characterization of the Sngle Endo-Polygalacturonase Produced by *Fussarium monilf*orme NCIM 1276. *Eur... J. Biochem.* 268, 832-840.

Tampion, J. and Tampion M.D (1987) Immobilized Cells. Principles and Applications. Cambridge University Press. (United Kingdom) 264PP

Thakur, B.R; Singh, R.K and Handa, A.K. (1997) Chemistry and Uses of Pectin. A review, Critical Reviews in Food Science and Nutrition. 37, 47-73.

Thorm. C. and Church, M.B. (1926) The *Aspergilli:* Baltimore, MD, Williams and Wilkins, PP. 1-272.

Turner, A.P.F; Karube, I and Wilson, G (1987). Biosensors: Fundamentals and Applications. Oxford Univ. Press. Oxford, NewYork. Pp291-314

Tuttobello, R and Mill P.J. (1961) Pectic Enzymes of *Aspergillus niger. Biochem J.* 79, 57 – 64.

Verlent, I. : Van loey, C.A ; Smout, C. : Duvetter, T and Hendriclox, M.E (2004) Purified Tomato Polygalacturonase Activity During Thermal and High Pressure Treatment. *Biotechnology and Bioengineering* 86 (1) 63-71.

Viera, F.B, Barragan, B.B and Busto, B.L (1988) Reversible Immobilization of Soyabean b-Amylase on Phenyl Borronate Agarose. *Biotechnol. Bioeng*. 31, 711 – 713

Weetall, H.H. (1985) Enzyme Immobilized on Inorganic Supports. *Trends Biotechnol* 3, 276 – 280.

Weibel, M.K; Roberto, Barrios; Delotto,R; Humphery, .A. E(2004) Immobilized Enzymes , Pectin Esterase Covalently Coupled to Porous Glass Particles. *Biotechnology and Bioengineering.* 17, 1 *85-98*

Whitaker, J.R (1991) Microbial Pectolytic Enzymes in: Microbial Enzymes and Biotechnology (eds) Forgarty, W.M. and Kelly, C.T.) Elsevier Applied Science, London and New York PP. 133 – 175.

Wubben, J.P; Mulder, W. T; Have, A. Vankan J.A.L. and Visser,J. (1999) Cloning and Partial Characterization of Endopolygalacturonase Genes from *Botrytis cinerea. APP.Environ Microbiol*. 65, 1596 – 1602.

Yu, Y.T and Chang, T.M.S (1982) in: Immobilized Enzymes in Bioprocess. *Enzyme Microb. Technol*

4, 327 – 331.

APPENDIX

Appendix 1 PREPARATION OF COLUMN FOR GEL FILTRATION

Ten grams of Sephadex G-75 was dissolved in distilled water and left to swell for about 3 hours. Part of the slurry has poured into the 15.5cm XK 16/20 column until it reached the appropriate height. The packing was done without air bubbles. The column was then washed several times with acetate buffer 0.05M pH 5.0 to equilibrate the column at pH 5 and achieve proper packing using a flow rate pump. The fraction collector was set to collect 56 fractions (5ml) per fraction at a flow rate of 0.5ml per minute.

Appendix 2 Preparation of Column for Ion-Exchange Chromatography

The Fast Performance Liquid Chromatography (FPLC) equipment was washed with vacuum filtered distilled water, 0.05M acetate buffer and NaCl in acetate buffer. A slurry of DEAE –Sepharose was poured into the 15.5cm XK 16/20 column until it reached the appropriate height. The packing was done without air bubbles. The column was equilibrated with 0.05 acetate buffer pH 5.0 untill proper packing was achieved using a flow rate pump adjusted to 3ml/ minute. Fifty-six fractions were collected at 5ml per fraction

Appendix 3 Preparation of Dinitrosalicylic Acid Reagent

Exactly 10g of DNS were dissolved in 200ml of 2M NaOH with warming. It was heated to 90^{o}C for 5-15 minutes for the red-brown color to develop. Exactly 200g of Potassium Sodium Tartarate (40%) were dissolved in 500ml of distilled water. The two solutions were mixed with constant stirring and diluted to 1 liter with distilled water.

Appendix 4 SDS –PAGE Buffers and Stains

SAMPLE BUFFER

(i) 125mM Tris-Hcl pH 6.8

(ii) 4 % SDS

(iii) 10 %(v/v) Glycerin

(iv) 10 % (v/v) β-Mercaptorethanol

(v) 0.25 % Bromophenolblue

(vi)

ELECTROPHORESIS BUFFER

(i) 25mM Tris-Base

(ii) 192mM Glycin

(iii) 0.1 % SDS

STAINING SOLUTION

(i) 0.1 % Coomassie brilliant blue R250

(ii) 7% (v/v) Acetic acid

(iii) 14% (v/v) Methanol

DE-STAINING SOLUTION

(i) 50ml Ethanol

(i) 71ml Acetic acid

Appendix 5 SDS-PAGE (Method of Laemli 1970)

Both the standard marker proteins and the enzyme sample were first denatured at 95^{o}C for 5 minutes. The gel plates were than cleaned with methylated spirit dried and joined together to form the cassette, which was clamped in a vertical position. The following separating gel was mixed in a beaker to give (12.5%) gel.

Tris –Hcl buffer pH 8.8 1.95ml

Distilled H_20	0.275ml
Acrylamide + Bis 30%	2.405ml
SDS 10 %	54.5µl
TEMED 5 %	54.5µl
APS 10 %	78µl

The solution was mixed carefully and then using a micropipette it was applied on the gel cassette carefully down one edge between the glass plates. The solution was added until it reaches to the top about 1cm before the loading wells. This was done fairly quickly to avoid the setting of the gel before loading. The excess solution was discarded in the appropriate waste container not in the sink because of its toxicity.

To ensure the gel sets with a smooth surface distilled water was run very carefully down one edge into the cassette using a micro pipet until a layer of about 2 cm exist on top of the gel. The gel was allowed to stand for 15 minutes to harden before applying the stacking gel. While the gel was setting the following stacking gel was prepared.

Tris-Hcl buffer PH 6.8	235µl
Distilled $H_2 0$	1.27ml
Acrylamide + Bis 30 %	0.475ml
SDS 10 %	20µl
TEMED 5 %	20µl
APS 10 %	40µl

The water on top of the separating gel was poured off after it has set. The stacking gel was carefully added on the separating gel until the solution reaches the cut away edge of the gel plate. The well forming comb was then placed into the solution and allowed to set in 20 minutes. Refractive index changes around the comb indicated that the gel has set. The position of the bottoms of the wells was then marked with a marker pen to facilitate loading of sample.

The comb from the stacking gel was removed carefully and rinsed to remove any non-polymerized acryl amide solution from the wells using electrophoresis buffer. The top reservoir of the apparatus was filled with electrophoresis buffer checking for possible leaks. 20µl samples and standard markers were loaded onto the gel wells by placing the syringe needle through the buffer and locating it just above the bottom of the well. The samples loaded were recorded and the power pack was connected to deliver initially 80 volts and after 5 minutes 140-150 volts for about 1 1/2 hour. When the bromophenol blue reached the bottom of the gel it was switched off. And the gel apparatus dismantled opened and the gel was stained with shaking and heating in the oven for 30 seconds. Then destained with heating in oven and shaking. The cycle continued until clear bands were seen.

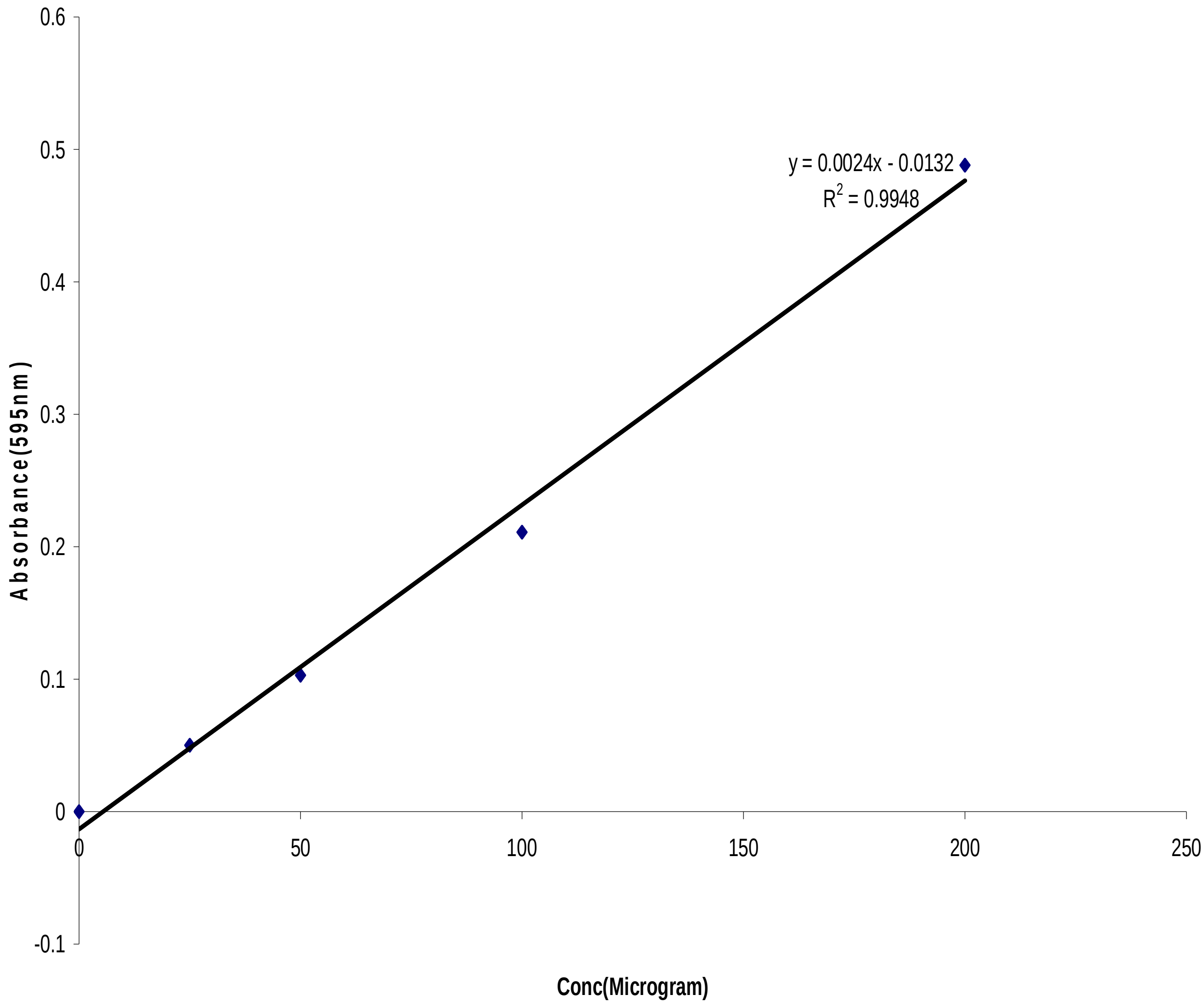
Appendix 6 pH Stability of Polygalacturonase

pH	Specific Activity (umole/min/mg) (Initial)	%Residual Activity(umole/min/mg) (Final
3.5	0.1239	9.44
4.0	0.1416	11.21
4.5	0.295	23.01
5.0	0.0649	5.31
5.5	0.1711	13.57
6.0	0.2655	20.65
6.5	0.236	18.29
7.0	0.3599	28.32
7.5a	0.00531	0.413
7.5b	0.2832	22.125
8.0	0.0708	5.5342
8.5	0.2006	15.6704
9.0	0.177	13.8296

Appendix 7 Standard Curve for Bovine Serum Albumin (BSA)

A stock solution of 1mg/ml (BSA) was serially diluted to obtain concentrations of 0.025, 0.05, 0.1 and 0.2mg/ml. 20μl of appropriate dilution of the BSA was mixed with 1ml of Bio-rad dye reagent and vortexed. The mixture was incubated at room temperature for 5 minutes. Absorbance was read at 595nm using spectrophotometer. Absorbance values were plotted against protein concentration to get a standard curve (Bradford, 1976)

Appendix 7 Standard Curve for Bovine Serum Albumin (BSA)



Appendix 8 Standard Curve for D-galacturonic Acid

Exactly 1mg/ml of D-galacturonic acid was serially diluted to obtain 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9mg/ml. 1ml of D-galacturonic acid standard was mixed with 1 ml of Dinitrosalicylic Acid (DNS) reagent and the mixture incubated at 90^0C for 5 minutes. After cooling in running tap water, absorbance was read at 575nm using a spectrophotometer. A blank of 1ml of DNS and 1ml of buffer was used to zero the spectrophotometer(Miller,1959).

TABLE. 8
TEMPERA
TUE
STABILIT
Y OF

Appendix 8 Standard curve for D-Galacutronic acid