PHYSIOLOGICAL STUDIES ON *BACILLUS* SPECIES ISOLATED FROM FERMENTED AFRICAN LOCUST BEAN [*PARKIA BIGLOBOSA* (JACQ.) BENTH.]

By

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A B S T R A C T

The physiological activities of Bacillus species that ferment African locust bean (Parkia biglobosa (Jacq.) Benth.) to produce 'iru' were investigated. The strains studied belong to the B. subtillis group and were designated BS1, BS2, BS3, BL1, BL2, BL4 and BP2. These seven strains showed significant differences (at $\alpha = 0.05$) in growth and extracellular proteinases production. The following (descending) order was obtained for the growth of the organisms in liquid medium:

BS3 > BS1 > BL4 > BL2 > BS2 > BL1 > BP2

The order of proteolytic activity (in descending order) of the strains in nutrient broth medium containing African locust beans was:

BL2 > BP2 > BS2 > BL4 > BS3 > BL1 > BS1

The best three strains (on the basis of proteinase production) BL2, BP2 and BS2 showed further variation in the production of other extracellular enzymes. The three strains produced amylase and polygalacturonase constitutively and varied amounts of sucrase and galactanase. Phytase activity was not detected in culture broth of strain BS2. None of the strains BS2, BL2 and BP2 produced pectinmethylesterase in nutrient broth medium with or without African locust bean. The three strains were lipolytic on tributyrate agar plates and produced trace amounts of lipase in broth medium

containing African locust bean. In most cases, presence of African locust bean in culture medium enhanced production of extracellular enzymes significantly in the three strains.

Agitation was found to be necessary for optimal production of extracellular proteinases by the strains BS2 and BL2. Among the carbon sources used, fructose and glucose repressed proteinase production significantly (at $\alpha = 0.05$) in strain BS2 while raffinose and starch favoured proteinase production. Among the carbohydrates which favoured the production of proteinases are, in order

raffinose > starch > arabinose > galactose > sucrose

The effects of different nitrogen sources on proteinase production by strain BS2 were also investigated. Casein enchanced production but the effect was not significant (at $\alpha = 0.05$) while other nitrogen sources repressed proteinase production significantly. The nitrogen sources repressed proteinase production in the order:

Leucine > Urea > KNO_3 > NH_4NO_3 > Aspartic acid > Glutamic acid > Alanine

The presence of African locust bean in culture medium enhanced proteinase production in the three strains, but the effect was not significant (at $\alpha = 0.05$).

The crude proteinases of strain BS2 had optimum activity at pH 7.5. Optimum temperature for activity of the proteinases was 35°C, and the proteinases were relatively stable at 60°C, but were

quickly denatured at temperatures $\geq 70^{\circ}C$. The apparent Km of the crude proteinases of the strains BS2, BL2 and BP2 were approximately 39.14mg/ml, 33.29mg/ml and 44.1mg/ml respectively. Multiple proteinase bands were obtained after electrophoretic separation of the crude enzymes in all strains.

During purification, the proteinases were precipitated out between 55 - 70% of ammonium sulphate saturation levels. There was a substantial loss in proteolytic activity during the salting out process. Three proteolytic activity peaks were obtained during ion-exchange chromatography of crude enzymes of strains BS2 and BL2. The peaks I, II and III were identified to be serine proteinase, neutral proteinase and an esterase (with low proteolytic activity) respectively. The serine proteinases of the two strains BS2 and BL2 showed hydrophobic properties. The molecular weights of the serine, neutral and esterase proteinases for strain BS2 were 29800, 24000 - 27400 and 33900 - 38400 respectively while those of strain BL2 were 18200 - 19700, 22600 and 33500 respectively.

The purified neutral proteinase had higher specific activity than the serine proteinase while the esterase was characterized by low specific activity. The esterase was responsible for the multiple proteinase bands pattern observed in the electrophoresed crude enzymes. The possibility of selecting strains capable of producing wider variety and higher yields of extracellular enzymes to bring about more digestible fermented product is discussed.

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CERTIFICATION

I hereby certify that this work was carried out by Mrs. E.Y. Aderibigbe under my supervision in the Department of Botany and Microbiology, University of Ibadan, Nigeria.

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D E D I C A T I O N

This work is dedicated to

my Lord Jesus Christ,

my dear husband Felix,

and our darling children.

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ABBREVIATIONS

APS	:	Ammonium peroxide sulphate
BSA	:	Bovine Serum Albumen
BTEE	:	Benzoyl-L-tyrosine ethyl ester
CTA	:	Chymotrypsinogen A
CYT.C	:	Cytochrome C
DFP	:	Diisopropyl fluorophosphate
DNSA	:	Dinitrosalicylic acid reagent
EDTA	:	Ethylenediaminetetraacetic acid
HIC	:	Hydrophobic Interaction chromatography
IEC	:	Ion-Exchange Chromatography
KD	:	Kilodaltons
NB	:	Nutrient broth
PAGE	:	Polyacrylamide gel electrophoresis
PMSF	:	Phenylmethanesulphonylfluoride
TAME	:	p-Tosyl-L-arginine methyl ester
TCA	:	Trichloroacetic acid
TEMED	:	N,N,N',N' - tetramethylenediamine

CHAPTER 1

INTRODUCTION

Food fermentation is the process by which microorganisms effect desirable changes in the food substrate. These changes are due to the activity of extracellular enzymes excreted by the fermentative organisms. Through fermentation, variety of foods are available all over the world. The nutritive value of the food is increased in many cases. Certain vitamins (thiamine and riboflavin) are synthesized during fermentation of African locust bean to produce 'iru' (Eka, 1980). Roelofsen and Talens (1964) found about threefold increase in riboflavin and niacin during 'tempeh' fermentation.

Apparently in all cases, fermentation results in improved digestibility. Complex components such as carbohydrates, proteins and lipids are broken down to simple sugars, amino acids and fatty acids which are easily assimilated in the gut (Padhye & Salunkhe, 1979; Anosike & Egwuatu, 1980). In some cases, fermentation enhances desirable flavour. During fermentation, development of characteristic flavour is achieved in production of 'ogi' (Banigo & Muller, 1972) and 'tempeh' (Wood, 1985). Toxic substances and anti-nutritional factors naturally present in some food substances

are eliminated or reduced during fermentation. The cyanide in fresh cassava and phytic acid in corn are eliminated during fermentation of these food substances (Akinrele, 1964; Oke, 1967).

Many fermented foods have been part of the diet in Southeast Asia, the Near East, Central and West Africa for centuries. Table 1 shows a list of some of the plant protein-rich fermented foods peculiar to Africa and some closely-related ones produced in the Orient. In West and Central Africa, the most popular leguminous fermented product is from African locust bean. It is known by different names in different parts of West Africa. These include 'iru' (among the Yoruba ethnic groups), 'dawadawa' (among the Hausa ethnic groups) 'soumbala' (in French-speaking countries), 'kpalagu' (in Ghana), 'kinda' (in Sierra Leone) and 'netetou' (in Gambia) (Odunfa, 1986).

The African locust bean (Parkia biglobosa) tree is a perennial tropical plant that grows in the grassland regions of West Africa. The fruits develop as elongated pods and turn brown when matured. The seeds are embedded in sweet edible yellow pulp. Harvested seeds are washed in handwoven raffia baskets and sun-dried. In this dried form, the seeds can keep for several months till they are processed to make 'iru'.

The production of 'iru' is a traditional family art, which involves the use of local utensils. The seeds are boiled in pots for 12 hours during which the seeds become soft and swollen. They

Table 1: Some of plant protein-rich fermented foods in Africa (West & Central) and the Orient

Name	Substrate	Microorganisms involved	Use of food	Area of production
Iru	Locust bean (*Parkia biglobosa*)	*Bacillus subtilis* *B. licheniformis* *B. pumilus* and *Staph. saprophyticus*	Soup flavouring condiment	West & Central Africa
Ogiri	Melon seeds (*Citrullus vulgaris*)	*Bacillus* spp. *Escherichia; Proteus* *Pediococcus*	"	South -western Nigeria
Ogiri-igbo	Castor oil seed (*Ricinus communis*)	*Bacillus* spp. *B. subtilis* *B. megaterium* & *B. firmus*	"	South -eastern Nigeria
Ogiri-saro	Sesame seed (*Sesamum indicum*)	*Bacillus* spp.	"	Sierra Leone
Natto	Soybean	*Bacillus natto*	Meat substitute	Northern Japan
Thua-nao	Soybean	*Bacillus subtilis*	Flavouring condiment	Thailand
Tempeh	Soybean	*Rhizopus oligosporus* *Aspergillus oryzae*	Cake used as snack	Indonesia

Source: Odunfa, 1985a; Reddy *et al.*, 1986.

are dehulled in mortar by pressing with the feet. The dehulling process is aided by adding a little quantity of sand as an abrasive agent. Loose testa are washed off. Cleaned cotyledons are steamed for about an hour after which they are drained and the hot beans are spread in calabash trays (layers of about 10 cm deep). Jute sacks are used to wrap the tray to provide warmth and humid atmosphere. The beans are left for 36 hours to ferment. The greyish fermented product has a sticky film covering the beans.

Among the rural dwellers, 'iru' is used as a relish in preparing soups, though in urban cities, it is becoming less popular. Some of the reasons for non-popularity among urban dwellers are the crude method of its preparation and unattractive presentation. Other reasons are the 'off-flavour' of the product and competition with imported boullion cubes.

Efforts have been made to study the fermentation process with the aim of improving the production process. A mixed bacterial community comprising of Bacillus spp., Staphylococcus and Lactobacillus spp, were found to be associated with the fermentation (Odunfa, 1981). When fermented 'iru' samples were obtained from different localities in Nigeria, strains of B. subtilis, B. licheniformis and B. pumilus were consistently isolated and found to be predominant (Odunfa & Oyewole, 1986).

Chemical composition of the fermented and unfermented beans

have been compared (Leung et al., 1968). Locust bean contains 24.6% digestible protein (Oyenuga, 1968), it is therefore a valuable source of protein supplement in foods. This value is comparable to the protein content of the more widely consumed cowpea. The flatus-producing oligosaccharides in the beans have been shown to be degraded and to disappear during fermentation (Odunfa, 1983). Proteolysis is one of the major biochemical changes during 'iru' fermentation. The total amount of soluble amino acids increased throughout fermentation (Odunfa, 1985b). There was a steady increase in proteolytic activity till it reached a peak at 36th of fermentation.

Studies on the optimization of process conditions for the fermentation have also been conducted. Optimum temperature and time required for fermentation were found to be 35°C for 36 hours respectively (Odunfa & Adewuyi, 1985a). Strains of Bacillus subtilis isolated from 'iru' were better than B. natto (a strain of B. subtilis) isolated from 'natto' in bringing about a desirable product (Odunfa & Adewuyi, 1985b).

The ability of the species of B. subtilis group to ferment African locust bean or other legumes is attributed to their capability to produce a variety of extracellular enzymes. The organisms grow in close association with the food substrate and elaborate extracellular enzymes during fermentation. The food substrate-related extracellular enzymes of the group include carbohydrases,

pectinases, lipases and proteinases. Among the carbohydrases are amylases, cellulases, hemicellulases, xylanase, levansucrase and β-glucanases (Fogarty, et al. 1974a; Priest, 1977). Of the total amylases produced by most strains α-amylase is known to known to constitute a larger percentage than the β-amylase and amyloglucosidase. Alpha amylase hydrolyzes the α-1, 4-glucosidic bonds in starch and constituent polysaccharides (amylose & amylopectin). Two principal types of α-amylase are produced by strains of B. subtilis, the saccharifying and liquefying enzymes. The saccharifying enzyme produces about twice the yield of reducing sugars from starch than does the liquefying enzyme (Fogarty et al., 1974a).

The B. subtilis α-amylase seems to be constitutive and controlled by the size of the pool of nucleic acid precursors (Coleman, 1967); when a carbon-limited medium was used, it was shown that the presence of an inducer was not necessary for amylase production by B. licheniformis (Meers, 1972). It is also known that maximal production of α-amylase occurs after the end of the log phase and during sporulation (Markkanen & Bailey, 1974). Yields of α-amylase and protease could be improved in strains of B. licheniformis and B. subtilis by selecting mutant strains or by transformation (Ingle & Boyer, 1976). The α-amylase of many B. subtilis strains is most active in the pH range 5.0 - 7.0 and at temperatures as high as high as 75°C - 80°C. It

is resistant to alkaline pH than many other bacterial and fungal amylases. The characteristic high optimum temperature and resistance to inactivation by heat make the enzyme extremely useful in the mashing process in the brewing industry.

Medium composition and concentration influenced growth and enzyme synthesis in B. subtilis var. amyloliquefaciens 3053 (Korculanin et al., 1972). The effect of cultural conditions on production of exo-amylase and exoprotease by B. subtilis was investigated by Eka and Fogarty (1972). Highest exoamylase yields were achieved in medium at pH 6-7, temperature range 27-33°C and after 60 hours of incubation. Horvath and Inczefi (1972) found that ammonium free medium inhibited α-amylase production in B. subtilis. Catabolite repression of α-amylase synthesis by glucose has been reported in B. subtilis and B. licheniformis (Glenn, 1976). Also the use of continuous culture gave 4-5 times higher yield of α-amylase in B. subtilis as compared with batch cultivation (Fencl & Pazlarova, 1982).

Galactanases have been purified from culture broth of wild type B. subtilis and B. subtilis var. amylosacchariticus 1043 (Emi & Yamamoto, 1972; Labavitch et al., 1976). The galactanases were found to attack soybean arabinogalactan and not coffee bean arabinogalactan. An exocellular levansucrase isolated from B. subtilis Marburg strain by Pascal and Dedonder (1972) was found to be inducible. But Berthou et al. (1974) were able to isolate a constitutive exocellular levansucrase from a mutant

strain BS5C4 of B. subtilis. Bacterial pectinases (depolymerases) which split the α-1, 4-glycosidic bonds of pectin are known (Fogarty & Kelly, 1979). These pectinases are specific for pectic/ polygalacturonic acid (Priest, 1984). They are referred to as polygalacturonases (hydrolytic in action) or polygalacturonate lyases (transeliminative in action). According to Ottow (1972), Steinigeweg & Ottow (1974), the B. licheniformis - pumilus-subtilis group showed pectinolytic activity by liquefaction of pectin gel medium, but not all strains in the group showed positive activity.

Phospholipases/lecithinases attack phospholipid lecithins which are lipids with a fatty acid unit substituted by a phosphate group. A strain of Bacillus subtilis BS-72 was found to produce lipase which was still active on butter oil at -18°C, pH 8.3 (Singh et al., 1976). Kennedy and Lennarz (1979) reported the production of extracellular lipase by B. subtilis (wild type cells) and mutant CMK_{33}. The strains of B. subtilis 100 and 207 were found to form both exolipase and endolipase (Lobyreva & Marchenkova, 1979). When a total of 96 Bacillus spp. (including B. licheniformis, B. pumilus, B. subtilis, B. megaterium and B. polymyxa) were isolated from smoked fish by Obi (1980), 66.7 - 100% of the tested species were strongly lecithinase positive.

The Bacillus subtilis group is known to produce extracellular proteinases (Foarty et al., 1974b; Priest, 1977). Both the serine proteinase (subtilisin) and neutral proteinase are secreted (Boyer

& Carlton, 1968; Millet, 1970; Dancer & Mandelstam, 1975; Shishkova et al., 1975) and studied extensively by Rappaport et al. (1965), Keay and Moser (1969), Fujiwara and Tsuru (1974), Feder et al. (1978). Millet (1970) and Prestidge et al. (1971) found a third extracellular enzyme which showed a high esterolytic but low proteolytic activity. It has been reported that some strains of Bacillus spp, such as the halophilic strains produce proteinases with pH optima in the alkaline range (Priest, 1977). Excretion of proteinases by the B. subtilis group has been found to be associated with the sporulation phase (Coleman, 1967; Schaeffer, 1969). El-Said and Ghali (1976), Hageman et al. (1984) found that nutrient composition of culture medium affects the time at which the cells reach sporulation and extracellular proteinase production phase.

The subtilisins differ from the neutral proteinase on the bases of sensitivity to inhibitors, specificity of action and esterolytic activity (McConn et al., 1964). Mutant strains of B. subtilis capable of producing 16-37 fold amount of proteolytic activity over the wild strain have been isolated by Higerd et al. (1972). The subtilisins are strongly inhibited by sulfhydryl compounds such as Diisopropyl fluorophosphate (DFP) and phenyl-methanesulphonyl fluoride (PMSF) (Rappaport et al., 1965; Boyer & Carlton, 1968).

The DFP and PMSF inhibit by reacting with seryl residue 221 in the polypeptide chain. The subtilisins are stable between pH 5-10 (optimum pH: 7-8.4), but rapidly denatured at pH 1-2 and inactivated at 65°C (Perlmann & Lorand, 1970). The composition of culture medium was found to have an influence on alkaline proteinase production (Emtseva, 1975; Daguerre et al., 1975).

Generally two types of subtilisins have been identified which are subtilisin Novo or BPN[1] and subtilisin Carlsberg. Bacillus licheniformis and B. pumilus produce mainly the subtilisin Carlsberg/ subtilopeptidese A, while B. subtilis NRRL B3411 and B. subtilis var. amylosacchariticus produce the BPN[1]/Novo/subtilopeptidase B (Keay et al., 1970). Though the two subtilisins are similar in physico-chemical properties, they are quite distinct in immunological cross-reactions ratio of esterase to proteinase activity, amino acid sequence and composition. The subtilisin Carlsberg is characterized by a higher esterase to proteinase ratio (Perlmann & Lorand, 1970).

Detailed study of the serine proteinases from B. subtilis by Palubinskas et al. (1976) by adsorption and subsequent gel filtration on Sephadex G-75 showed three factions having different physico-chemical properties. The first fraction had a molecular weight of 23,000-24,000 and optimum pH 6.5, while the second fraction had a molecular weight of 29,000 and optimum pH 11.0. The third fraction was a mixture of proteinases with average molecular weight of 26,000 and optimum pH 7.0, 8.5 and 11.0. Alikhanin et al. (1974) reported a method of detecting B. subtilis alkaline proteinase band in polyaccrylamide gel.

The method involved the use of chromogenic substrate, carbobenzoxy-glycyl-L-leucine p-nitroanilide. A light yellow band indicated the location of subtilisin in the gel. Farmer and Hageman (1975) also described a method of detecting thiobenzyl ester in polyaccrylamide disc gels. The gel is incubated in substrate solution in the presence of nitro-blue tetrazolium. The subtilisins have high commercial values because they are used in making detergents and leather tanning. The subtilisins are

(i) produced at low cost;

(ii) have good stability characteristics at different pH, temperature and in the presence of detergent components; and

(iii) effective in stain removal at low concentration (0.1 - 1%).

These characteristics of subtilisins meet neceesary requirements of a detergent enzyme. The leather industry also utilize them in initial soaking, dehairing and removal of unwanted interfibriliar material (Fogarty et al., 1974b).

The neutral proteinase is a zinc-containing metallo-enzyme with one atom of zinc per molecule. It has optimum pH between pH 6.5 - 7.5 and active in the range pH 5.5 - 10 (McConn et al., 1964; Feder, 1967). The enzyme is stable up to 50°C for 15 minutes in the presence of calcium ions. It is prone to autolysis and irreversible loss of activity in the absence of Ca^{2+} ions. The enzyme is inhibited by metal chelating agents especially EDTA which removes the zinc atom, but insensitive to PMSF and DFP (Tsuru et al., 1967).

Neutral proteinase is devoid of esterolytic activity and least stable (McConn et al., 1964). The enzyme has a molecular weight of 44,700 ± 800 (Yasunobu & McConn, 1970; Fogarty et al., 1974b). The aminopeptidase isolated from B. licheniformis by Rodriquez-Absi and Prescott (1978) was found to be a metalloenzyme which yielded a molecular weight of 43,270 by sedimentation equilibrium and 37,500 and 36,000 by two polyacrylamide electrophoretic procedures.

The composition of culture medium has been shown to affect the synthesis of metalloproteinase (Kalunyants et al., 1979). Neutral proteinases have been used extensively in food processing especially baking, fish and brewing industries to remove unwanted proteinaceous materials (Yasunobu & McConn, 1970; Fogarty et al., 1974b).

The esterase has been difficult to classify with certainty. It has been found to hydrolyse benzoyl-L-tyrosineethyl ester (BTEE) more readily than p-Tosyl-L-arginine methyl ester (TAME) (Millet, 1970). The enzyme was reported to be inhibited by DFP and PMSF just like serine proteinases (Prestidge et al., 1971). It has been described as a serine enzyme with high esterolytic activity but low proteolytic activity (Mamas & Millet, 1975; Priest, 1977).

Studies have confirmed that the Bacilius subtilis group are predominant and are responsible for the fermentation of African locust bean to produce 'iru' (Odunfa, 1981; Odunfa & Adewuyi, 1985b; Odunfa & Oyewole, 1986). Thus detailed studies of the strains involved in the fermentation is necessary. Various aspects of

physiological studies have been done on strains of Bacillus species from other sources. The strains vary in their capabilities to produce extracellular enzymes (Fogarty 1974 et al. a & b; Priest, 1977 & 1984). Up to date there is no information on the physiology of strains of Bacillus subtilis group involved in the fermention of African locust bean. Knowledge of their physiological and biochemical activities is a very important step towards optimization of the fermentation process. Though the proteinases play the most prominent role during fermentation (Odunfa, 1983); it is important to know other food hydrolytic enzymes that are excreted by attested strains during fermentation. Such attested strains that are capable of producing a variety of desirable enzymes will be better choice for optimizing the fermentation process.

The chemical constituents of food substrate affects the amount and types of extracellular enzymes elaborated by the organisms during fermentation. Some sugars such as sucrose, glucose, fructose and galactose are known to be present in fermenting African locust bean (Odunfa, 1983). These sugars are likely carbon sources for the bacteria during fermentation of the beans. Thus it is necessary to investigate the effect of these carbon sources and some nitrogen sources on the production of extracellular proteinases.

By the traditional method of processing, the beans are layered into shallow calabashes forming about 10cm deep layers of beans. In such a case, the amount of air reaching the lower layers may be

reduced, thereby having lower oxygen tension at such depths. The effect of agitation on production of extracellular proteinases by the fermenting organisms needs to be confirmed. Thus other complementary optimal conditions necessary for proteolytic fermentation of the beans will be known. This information can be harnessed such that products with higher percentage of free amino acids and nutritive value are made.

There are variations in the biochemical properties of strains isolated from 'iru' (Odunfa & Oyewole, 1986). These variability might extend to the capability of the organisms to produce extracellular proteinases. Strains that are proficient in producing large amounts of proteinases can be developed as starter cultures for large scale production of 'iru'. Strains of B. subtilis have been developed as starter cultures and used in the fermentation of soybean to produce 'thua-nao' in Northern Thailand (Sundhagul et al., 1970; Sundhagul et al., 1973) and 'natto' (Sakurai, 1960; Hesseltine & Wang, 1979).

A detailed study of the types of proteinases produced by the strains of B. subtilis involved in fermentation of African locust bean is also necessary. Such information will help to compare these strains with other known strains of Bacillus species. Characterization of the proteinases might lead to the discovery of new proteinases in the Bacillus subtilis from 'iru'.

The main objective of this study is to know the physiological characteristics of the Bacilius species involved in the fermentation of African locust bean in order to enhance the fermentation process. This main objective is to be realised through the following specific objectives:

1. To study the growth of strains of B. subtilis isolated from 'iru';
2. To assay for extracellular enzymes produced by strains of B. subtilis group isolated from 'iru':
3. To obtain the cultural conditions necessary for optimal production of the extracellular proteinases;
4. To compare proteinase production by strains of B. subtilis group isolates from 'iru';
5. To purify and characterize the proteinases excreted by representative strains of Bacilius species from 'iru'.

CHAPTER 2

MATERIALS AND METHODS

(a) Organisms

The organisms used in this study are strains of *Bacillus subtilis* group and were part of the culture collections in the Department of Botany and Microbiology, University of Ibadan, Ibadan. They were previously isolated from 'iru' samples obtained from different towns in Southwestern Nigeria (Odunfa & Oyewole, 1986). Seven strains that were more efficient in degrading casein on agar plates (Harrigan & McCance, 1976) were selected and subcultured onto nutrient agar slants and plates. These strains are BS1, BS2, BS3 (strains of *Bacillus subtilis*), BL1, BL2, BL4 (strains of *B. licheniformis*), and BP2 (strain of *B. pumilus*).

(b) Inocula

Inocula for experiments were prepared by growing each strain in 0.1% peptone at 37°C on a rotary shaker for 12 hours.

(c) Growth and pH measurements

Growth in media was assessed by measuring the optical densities at 540nm using a Spectronic 21 spectrophotometer (Milton Roy Company). Uninoculated media served as controls. The pH of media was measured using Du Pont Instruments pH meter equipped with glass electrode.

(d) Production and assay of extracellular enzymes

(i) Proteinase activity

Proteolytic activity was assayed by modifying the casein digestion method of Kunitz (1947). A 1% casein (Sigma) solution was prepared in 0.05M potassium phosphate buffer pH 7.0 and heat denatured by heating at 100°C in a water bath for 15 minutes. The solution was allowed to equilibrate at 35°C before use. To one millilitre of casein solution in a tube was added 0.5ml of enzyme preparation, mixed and incubated at 35°C for one hour in a water bath. The reaction was terminated by adding 3 ml of cold 10% TCA. The control tube contained only casein solution during incubation at 35°C, after which TCA was added before the addition of enzyme preparation. The undigested proteins were allowed to precipitate by standing the tubes at 4°C for one hour. The tubes were centrifuged at 11,000 rpm (4°C) for 15 minutes.

Supernatants were analysed for unprecipitated protein either by reading the absorbance at 280nm with a spectrophotometer (Hitachi Model 100-40) or by adding Folin-Ciocalteu's phenol reagent (Lowry et al., 1951). All assays were done in triplicates.
Using the direct spectrophotometric reading method, one unit of proteolytic activity (UC) was arbitrarily defined as the amount of enzyme causing an increase of 0.01 per hour in the absorbance at 280nm (Olutiola & Nwaogwugwu, 1982). When Lowry et al. (1951) method was used, one unit of proteolytic activity (UP) was the amount of

enzyme that will yeild 10 µmol of non-precipitable (TCA) protein. Tyrosine was used for standard curve.

(ii) Esterase activity

Esterolytic activity was assayed using the method of Hummel (1959). A 10^{-3} molar solution of benzoyl-L-tyrosine ethyl ester (BTEE) was used as substrate (Millet, 1970). Measurements were made with quartz cells with 1 cm light paths. One millilitre of substrate was placed in each cuvette. The control cuvette received 50 µl of buffer (Eppendorf pipette), while to the second cuvette was added 50 µl of buffered enzyme solution. After each addition, the contents of each cuvette was mixed for 5-10 seconds by stoppering them and inverting twice. A Hitachi spectrophotometer fitted with a heating system maintained at 30°C was used to measure absorbance changes at 256nm and 1 minute intervals.

One unit of esterolytic activity (UE) was defined as the amount of enzyme causing an increase of 0.001 per minute in the absorbance at 256nm (Boyer & Carlton, 1968).

In subsequent enzymes assay, basal medium contained (per litre) KCl, 1g; $MgSO_4.7H_2O$, 0.125g; $CaCl_2$, 10^{-3}M;Nutrient broth, 13g. Other flasks contained in addition to the above, 4% African locust beans, 0.2% pectin, 0.2% sodium polypectate, 5% rice flour and 0.2% sodium phytate (w/v) respectively. The total volume of media per 100ml - Erlenmeyer flask were 50ml. The media were sterilized by autoclaving at 121°C (15lb) for 15 min. and inoculated with 0.5ml of inoculum.

Flasks were incubated at 35°C on a rotary shaker. Samples taken were centrifuged to remove bacterial cells and supernatants assayed for pectinase, amylase, lipase and phytase activities.

(iii) Pectinase activity

Polygalacturonase (PG) activity was assayed by measuring the increase in free reducing sugars (Miller, 1959) produced by enzyme action on sodium polypectate (NaPP). Sodium polypectate solution (0.5%) was prepared in citrate phosphate buffer pH 5.0. To 1ml of NaPP solution was added 0.5ml of enzyme solution. The reaction mixture was incubated for 3 hours at 30°C after which it was terminated by adding 3ml of DNSA reagent solution (prepared freshly). To the control tube, DNSA reagent solution was added to the substrate before adding the enzyme solution. The mixture was heated in boiling water-bath for 10 minutes, then cooled rapidly. Ten millilitres of distilled water was added to each tube. Undigested NaPP was removed by centrifugation. Absorbance of supernatants was read at 575nm using a Pye Unicam SP6 250 spectrophotometer. Each assay was in triplicates.

The amount of reducing sugar formed was calculated from a standard curve prepared with known concentrations of polygalacturonic acid. One unit of polygalacturonase activity (UPG) was defined as the amount of enzyme that will liberate 10µg of polygalacturonic acid per hour at 30°C.

$$UPG = \frac{\mu g \text{ of polygalacturonic acid}}{3 \times 10}$$

Pectinmethylesterase was assayed by titrimetric method (Bateman, 1963). A 1.2% pectin solution was prepared in distilled water and used as substrate. The pH of the enzyme solutions was adjusted to pH 7.0. The reaction mixture, 20ml of pectin solution, 2ml of 1M NaCl and 3ml of enzyme solution were added into a beaker. The reaction mixture was incubated at 30°C for 3 hours. The reaction was terminated by titration with 0.1N NaOH to pH 7.0. The titre volume of NaOH required to adjust the pH to 7.0 was used to calculate the methoxy units released.

One unit of pectinmethylesterase (UPME) was defined as the amount of enzyme which under assay conditions requires the addition of a miliequivalent of 0.1N NaOH per minute to maintain the reaction at pH 7.0.

$$UPME = \frac{\text{titre volume (ml.)}}{60 \times 3} \times 10^3$$

(iv) Amylase activity

Amylase activity was assayed by the method of Sumner and Howell (1935) and Bernfeld (1955). A 1% starch solution was made in 0.02M phosphate buffer pH 6.5. To 1ml of the starch solution

was added 1ml of enzyme solution, and the reaction mixture was incubated at 40°C for 1 hour. The amount of reducing sugars released in the reaction mixture was estimated by adding 2ml of DNSA reagent (Miller, 1959) and heated in a boiling water bath for 5 minutes. Five millilitres of distilled water was added and the absorbance at 540nm wad read using a Pye Unicam SP6-250 spectrophotometer. The amount of readucing sugars produced was read from a standard curve prepared with known concentrations of maltose. One unit of enzyme activity (UA) was expressed as the amount of reducing sugars (mg maltose) produced per hour at 40°C.

(v) Lipase activity

Lipase activity was assayed by modifying the method of Yong and Wood (1977). Glycerol tributyrate was used as substrate. The substrate mixture was prepared by mixing 20ml glycerol tributyrate, 0.08g sodium taurocholate, 0.44g $CaCl_2$ in 20ml water, 2g gum acacia (to stabilize emulsion), 120ml 0.1M acetate buffer pH 5.0. The mixture was blended into fine emulsion using a Waring Blender. To 1ml of substrate emulsion was added 0.5ml of enzyme solution and the mixture was incubated at 40°C for 1 hour. The reaction was terminated by adding 8ml of absolute ethanol. The resulting mixture was titrated with 0.02M NaOH using phenolpthalein (0.1g in 50ml absolute ethanol and 5ml water) as an indicator. Blanks were prepared by adding 8ml absolute alcohol before adding enzyme solution. The difference in titre values of blank

and reaction mixtures gave the amount of alkali required to neutralize the liberated fatty acids.

(vi) Phytase activity

Phytase activity was assayed using sodium phytate (Sigma) as substrate (Wang et al., 1980). To 1ml of 1.6mM sodium phytate in 0.5M acetate buffer pH 4.5 was added 1ml of crude enzyme solution. The mixture was incubated at 37°C for 1 hour, after which it was terminated by adding 2ml of 10% TCA. The inorganic orthophosphate (Pi) liberated was determined by the method of Taussky and Shorr (1953). One unit of enzyme activity is the amount of enzyme required to liberate 1 µmole of Pi per hour under the assay conditions.

(vii) Sucrase activity

The enzymatic hydrolyses of raffinose and arabinogalactan were also quantified by measuring the amount of reducing sugars released. In raffinose hydrolysis, a 0.5% substrate solution of raffinose in buffer pH 7.0 was prepared. To 0.5ml of enzyme solution was added 1ml of substrate solution. The enzyme reaction mixture was incubated at 35°C for 2h and terminated by adding 2ml of DNSA reagent. The mixture was heated at 100°C in boiling water bath for 15 minutes and cooled immediately. Five millilitres of water was added and absorbance was read at 575nm. The amount of reducing sugars formed was read from a standard curve. One unit of sucrase activity was defined as the amount of enzyme activity

that will liberate 10 µg of reducing sugars at 35°C per hour.

(viii) Galactanase activity

For hydrolysis of arabinogalactan (galactanase), a 1% substrate solution was made in distilled water. To 0.5ml of enzyme solution was added 1ml of substrate solution. The reaction mixture was incubated at 35°C for 2h, and the reducing sugars formed were also estimated using the DNSA reagent. One unit of galactanase activity was defined as the amount of enzyme that will liberate 10 µg of reducing sugars at 35°C per hour.

(e) Extraction and analysis of arabinogalactan or galactomannan from African locust bean (Parkia biglobosa)

Fifty grammes of African locust bean cotyledons was ground into a fine mesh using a mortar and pestle. The water-soluble sugars were extracted by adding hot distilled water to the mesh. The resulting slurry was placed in a boiling water bath for 30 minutes. The slurry was filtered first with a muslin cloth, then with Whatman No. 1 filter paper. The supernatant was allowed to cool. Absolute ethanol was slowly added to 250ml of the aqueous extract with rapid stirring till the alcohol concentration was 60% by volume (Adams, 1965; Whistler & Marx, 1965). The sugars were allowed to precipitate out overnight at 4°C. The resulting precipitate was removed by

centrifugation and oven dried at 70°C for 48 hours.

The dried precipitate was redissolved in 1N HCl and hydrolysed by heating at 100°C for 4 hours in a water bath (Smith, 1943). Qualitative analysis of the sugars present was done by paper chromatography (using Whatman No. 1). The sample (50 µl) was spotted along with standard sugars galactose, mannose and arabinose. The paper was developed for 30 hours by descending chromatography using N-Butanol-acetic acid - water (4:1:1 v/v) as running solvent. Chromatogram was allowed to dry in the air and the sugar spots were developed by using silver nitrate (Trevelyan et al., 1950). Excess silver nitrate in background was removed by washing through 20% sodium thio-sulphate.

(f) Paper chromatography

The enzymatic hydrolysis products of rafinose were determined qualitatively. To the crude enzyme solutions (from medium containing African locust bean) of the three strains were added crystals of raffinose to make 1% (w/v) each. A few drops of toluene were added to each medium and the mixtures were incubated at 35°C for 24 hours. The products of hydrolysis were determined by paper chromatography (Whatman No. 1). The paper was developed for 30 hours by descending chromatography using N-butanol-acetic acid - water (4:1:1v/v) as running solvent. Sugar spots on chromatograms were developed using silver nitrate

(Trevelyan et al., 1950).

(g) Culture conditions for proteinase production

Media used were a modification of that used by Millet (1970) and contained the following (per litre): KCl, 1g; $MgSO_4.7H_2O$; 0.125g; $MnCl_2.4H_2O$, 2mg; $CaCl_2$, 0.01g; $FeSO_4$, 0.278mg potassium phosphate buffer pH 7.0, 20mmol; trace elements solution SL10 (appendix 1), 1ml; vitamins solution (appendix 2), 0.5ml. In addition, the flasks contained varying concentrations (0.8% - 0.02%) of nutrient broth (Merck). All the components were autoclaved except the trace elements and vitamins solution which were filter-sterilized. Each flask was inoculated with 0.1ml of BS2 inoculum. Samples taken at intervals were assessed for growth and pH.

The effect of different carbohydrates on proteinase production was investigated. Fermenting locust bean is known to contain glucose, fructose, galactose, sucrose, raffinose (Odunfa, 1983). The effect of these sugars, arabinose and starch as sole carbon sources on proteinase production by one of the strains BS2 was studied. The basal medium contained the following (in g/l): KCl, 1g; $MgSO_4.7H_2O$,0.125g; $CaCl_2$,0.01g; Tryptone (Oxoid)5g. The flasks contained in addition 2g/l of the sugars glucose, fructose, galactose, sucrose, arabinose, raffinose and starch respectively. Glucose and fructose were filter-sterilized using Millipore filter (0.45u), the other components were

autoclaved. The total volume of each medium was 50ml per 100ml - Erlenmeyer flask. Sterilized media were inoculated with 0.5ml of BS2 inoculum and flasks were incubated at 35°C on a rotary shaker (New Brunswick, New Jersey) at 125rpm for 36 hours. Bacterial cells were removed by centrifugation at 6,000 rpm for 15 minutes using an MSE refrigerated centrifuge. Supernatants were assayed for proteolytic activity.

The effects of some inorganic and organic nitrogen sources, and African locust bean were also investigated. Media contained the above-mentioned basal salts and starch (0.2%). In addition, flasks contained 0.5% Urea, KNO_3, NH_4NO_3, DL-Alanine, L-Aspartic acid, DL-Glutamic acid, DL-leucine, peptone and casein respectively. Sterilized media were also inoculated with 0.5ml of BS2 inoculum and the flasks were incubated at 35°C on a rotary shaker at 125 rpm for 36 hours. Bacterial cells were removed by centrifugation and supernatants were assayed for proteolytic activity.

The effect of agitation on proteinase production was also studied. The medium contained the following per litre; KCl, 1g; $MgSO_4.7H_2O$, 0.125g; $CaCl_2$, 10^{-3}M; $MnCl_2.4H_2O$, 10^{-5}M; $FeSO_4$, 10^{-6}M; potassium phosphate buffer pH 7.0, 20mM; nutrient broth, 1.6g; African locust bean cotyledons, 8g. In 250ml Erlenmeyer flasks, 100ml of medium was prepared and autoclaved. The flasks were inoculated with 0.2ml of BS2 and BL2. One flask of each inoculum was agitated on a rotary shaker at 120rpm, while the other flasks were left stationary. All flasks were incubated at 37°C.

Samples taken at regular intervals were centrifuged to remove bacterial cells and the supernatants were assayed for proteolytic activity.

(h) Characterization of the crude proteinases

(i) Effect of pH on proteinase activity and stability

Buffers with pH values of 2 to 11 were prepared (Hale, 1966). Buffers with pH 2; 3 and 4 were prepared using citrate HCl; pH 5 and 6 were citrate-NaOH; pH 7,7.5, 8 and 9 were Tris-HCl, while pH 10 and 11 were borate-NaOH. For the test of optimal pH of activity, buffers with pH 7, 7.5. 8,9,10 and 11 were used to prepare the casein substrate solution. Procedure for proteinase assay is as previously stated. In case of the test for stability at different pH values, buffers with pH 2-10 were used. Equal volumes of each buffer was mixed with crude enzyme solutions. Samples taken at 5-minute intervals were assayed for proteolytic activity.

(ii) Effect of temperature on proteinase activity and stability

Proteolytic activity was tested by incubating enzyme reaction mixture at different temperatures 25°C to 50°C for 1 hour. The thermal stability at high temperatures was assayed at 60°C, 70°C, 80°C, 90°C and 100°C. Samples were taken at 5-minute intervals and assayed for proteolytic activity.

(i) Gas chromatographic analysis of short-chain fatty acids

The supernatants of media (containing locust bean cotyledons) and some standard fatty acids were run on the gas chromatograph

(GC) (Carlo Erba Instrumentatione, Italy). The column (200x0.2cm) was packed with 60/80 mesh/carbopack C/Carbowax 20M/0.1% H_3PO_4 (made by Supelco). Carrier gas was nitrogen with flow rate of 40-50ml/min. The detector was a flame ionisation detector (FID). Temperature of injector and detector were 200°C while column temperature was 160°C.

To 20ml of deionized water in conical flasks were added 20ul each of propionic acid, butyric acid, valeric acid and caproic acid. 100µl of this mixture was taken and acidified by adding 5 µl of 10M formic acid and mixed. Two microlitres of this was injected into the GC. To 100 µl of each sample supernatant was added 5ul of 10M formic acid, and 4µl of this was injected and run in the GC. Other standard short-chain fatty acids, isobutyric acid, 2-methyl-butyric acid and isovaleric acid were prepared and also injected into the GC.

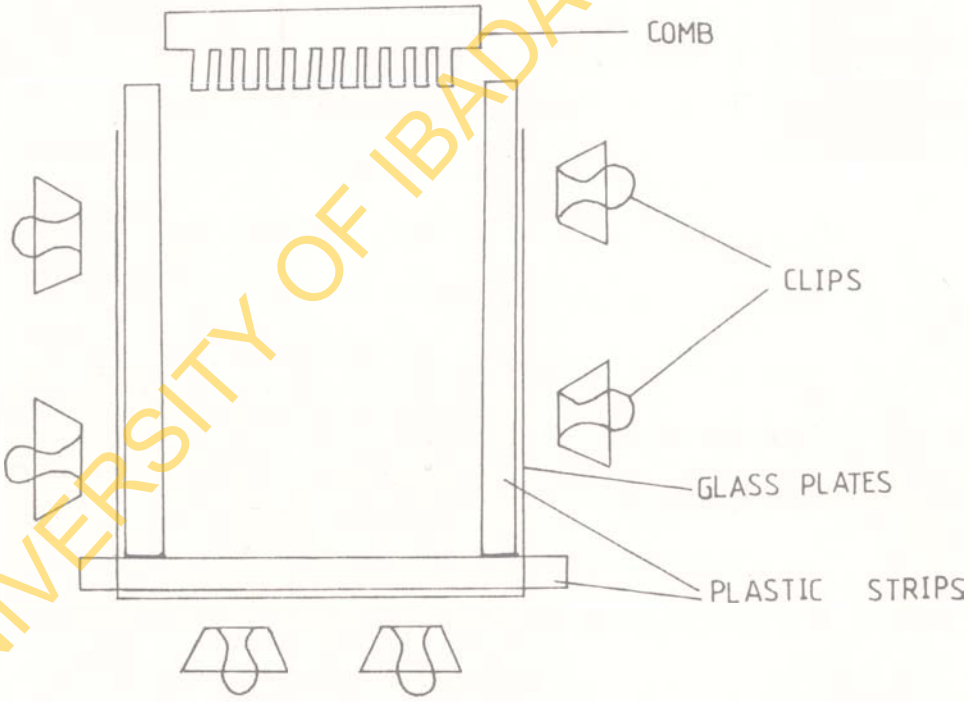
(j) Polyacrylamide gel electrophoresis (PAGE)

(i) Preparation of polyacrylamide gradient slab gel:

The method of Lorentz (1976) was used. The procedure was as follows:

Two glass plates (25 x 17cm) were clipped together with plastic strips (1mm thick) inserted between them, both at base and on the sides. The plastic strips were placed 2mm to the edges of the glass places (Fig. 1). The sides and the base were reinforced to be leakproof by sealing them (both inside and outside of the strips) with 1% agar gel. From

Fig. 1: Diagrammatic representation of apparatus for casting gradient polyacrylamide slab gel.



the measurement of the dimensions of glass plates, the volume of the gel mixture required was calculated. The glass plates were wrapped in towel and kept in the refrigerator to cool, this allows for better setting of the polyacrylamide gel mixture.

Solutions A, B, and C were prepared thus:

Solution A (50% acrylamide): into a beaker was weighed 23.4g acrylamide and 0.72g of the cross-linker N, N'-Methylenebisacrylamide. Water was added to make up total weight to 50g. A magnetic stirrer follower was added and the beaker was covered up with a parafilm. The mixture was stirred on a magnetic stirrer until the acrylamide dissolved. The solution was clarified of undissolved particles by filtering with a Whatman No. 1 filter paper. Solution B (buffer): To 35ml of 1M HCl was added 30.3g Tris salt. Water was added to make up the volume to 250ml. Solution C (APS): a solution of 0.063g/0.5ml distilled water was prepared. The APS (ammonium peroxidesulphate) initiates the polymerization reaction and it was always prepared fresh prior to use. A gradient mixer was set up on a magnetic stirrer, the outlet vessel contained a glass rod. The clipped glass plates was set upright with a clamp and the plastic comb inserted half-way.

A 5-20% polyacrylamide gradient gel was prepared by mixing the following in the gradient mixer vessels:

	Left (5% acrylamide)	Right (20% acrylamide)
Solution A:	1.6ml	6.4ml
Solution B:	6.ml	6ml
Solution C:	32 µl	16 µl
Distilled water :	8.37ml	3.57ml
TEMED :	16 µl	16 µl

TEMED which accelerates the polymerization reaction was added last. Both taps were opened immediately and the gel mixture flowed into the space between the glass plates till it was filled up. The comb was fully inserted such that there was no air-space between the gel and the comb (plastic well-former). The remaining polyacrylamide gel mixture in the vessels was washed off immediately under running tap to prevent gel setting inside. A film of water was added on the setting gel to prevent dehydration. The gel was left for at least 5 hours to set. The set gel was wrapped in a towel and kept in the refrigerator till when needed.

(ii) Electrophoretic procedures

The cooling machine was connected to the gel cooling system and switched on. The temperature was set at 5°C to effect adequate cooling during electrophoretic runs. The running buffer, Tris-Giycine was poured into both upper and lower buffer tanks. The buffer was made by dissolving 0.6g Tris and 2.88g glycine in water and making up the volume to 1 litre. The buffer had a pH of 8.3. The plastic strip at the lower end,

the comb and all clips were gently removed from the gel. It was then carefully placed in the lower tank of buffer, ensuring that no air space was trapped. The slab gel was clipped on the two sides and tightened with a glass bar to the gel cooling system. More running buffer was added to the upper tank till it overflowed onto the gel.

Samples (10 µl each) were applied into the sample wells. The samples were prepared by mixing equal volumes of enzyme preparation and 50% of glycerine (glycerine/buffer 50:50). Mixture of standard protein markers and 2 µl of bromophenol blue was applied into one of the wells. The electrodes were connected and the mains switched on. The current was at 40A and voltage at 450V, the voltage being the limiting factor. The electrophoresis proceeded for 2½-3 hours till the blue marker front approached the lower end of the gel. The power supply was switched off and the gel removed. Both plastic strips on the sides were removed and the upper glass plate was removed carefully using a flat spatula. A hypodermic needle was used to draw a line on the gel, marking the running front. The gel was removed for staining procedures.

The proteinases in the crude enzyme preparations of the 7 strains of Bacillus subtilis group were run electrophoretically on 5-20% polyacrylamide gradient slab gels. For each strain, supernatants of culture medium samples taken at 12, 24, 36 and 42 hours (in the medium without locust beans) and at 12, 18, 24, 30 and 36 hours (in the medium with locust beans) were used. Five microlitres (5 ul of each sample was added into microlitre-vial tubes. To each was added

5 µl of glycerine/0.1M Tris-HCl buffer pH 7.8 (50:50 v/v).

Standard marker proteins Thyroglobulin, Ferritin, Catalase, BSA & bromophenol blue were mixed and electrophoresed in each gel to calibrate the gel and calculate the molecular weights of the proteinase bands in the gels. Molecular weights of the standard proteins (all of analytical grade) were 655, 455, 240 & 68 kilodaltons (KD) respectively. The standard proteins and blue tracking dye mixture were applied into one sample well while the experimental samples were applied into the other sample wells using a microlitre syringe.

Electrophoresis was performed at 450V for 3 hours, by the end of which the tracking dye had moved and approached the lower end of the gel. The gel was then processed to stain for proteinase bands.

For all strains, the 42 hour samples (from medium with locust beans) were run on a gel, while the 12 hour samples (from medium without locust beans) were also run on another gel. Uninoculated media served as control runs. Both gels were stained for esterase bands. Thus esterase bands pattern in the presence and absence of locust bean could be compared.

(iii) Staining of gels

The method of Andary & Dabich (1974) was used to stain for proteinases in gels. A 1% w/v casein solution in buffer was prepared, and denatured by heating at 100°C in a water bath for 15 minutes. Two millimolar $CaCl_2$ was added to the buffer o.1M Tris-HCl pH 7.8 (to ensure stability of neutral proteinase if present). The gel was

incubated in 150ml of the casein solution at 37°C for 1 hour. The casein solution was changed to maintain the pH required for maximal activity. Gel was further incubated in buffer alone at 37°C for an hour before it was placed in a staining bath containing coomasie brilliant blue stain solution for 2-10 hours. Destaining of gel was in methanol/ acetic acid solution until the proteinase bands (clear zones) were differentiated from the background (blue-stained).

This method is based on the principle of casein digestion in the gel. Subsequent to separation of the proteinases on the basis of their electrophoretic mobilities, casein diffused into the gel and was digested at the bands where proteinases were present during incubations in casein and buffer solutions at 37°C. Thus there were clear bands where casein had been digested against a blue-stained casein background. On inspecting the gel after destaining on a light box, all proteinase bands could be detected. This proved to be a conveinient method of detecting the different proteinase bands present in a enzyme preparation, even at low concentration compared to the method of scanning in a gel scanner (Gilford) at 280nm (Andary & Dabich, 1974). Gels were stored in 1% acetic acid until they were photographed.

Esterase bands in gels were detected by staining using the method of Gomori (1953) and Davis (1964), (Majima et al., 1982: Higashi & Johnson, 1986). Eighty milligrams of α-naphthylacetate was dissolved in 10ml acetone, while 200mg of Fast-blue salt B was dissolved in 30ml deionized water. Both solutions were stirred into

200ml of 0.1M Tris-HCl buffer pH 7.8 (with 2mM $CaCl_2$). The electrophoresed gel was placed in this solution and incubated at room temperature (with occasional shaking) until the bands were fully developed (after 30 minutes). The gel was washed with several changes of water before it was stored in 1% acetic acid.

Staining for proteins in gels were done using the method of Weber and Osborn (1969). Coomasie-blue stain solution was prepared by dissolving 1.25g of Coomasie-blue R-250 in a mixture of 454ml of 50% methanol and 46ml of glacial acetic acid. Insoluble materials were removed by filtration through Whatman No. 1 filter paper. Gel was incubated in 200ml of the Coomasie-blue solution in a staining bath at room temperature for 2-10 hours. Gel was destained in several changes of the destaining solution (methanol/acetic acid/water, 50:10:40) until the protein bands were clearly differentiated from the background.

When protein bands were hardly visible by Coomasie-blue stain, the silver-stain method (Switzer et al., 1979; Merril et al., 1980) was used. This method was more sensitive and could detect protein bands present in very low concentrations. Destained gel (from Coomasie-blue stain) was placed in 200ml distilled water for 10 minutes to wash off the destaining reagents. The water was discarded. Gel was then soaked for 10 minutes in 250ml of oxidizer solution (0.0034M $K_2Cr_2O_7$ and 0.0032N nitric acid). It was washed four times for 30 seconds in 200ml of deionized water, and placed in 200ml of 0.012M silver nitrate for 30 minutes. This was followed by rapid rinsing with two 300ml protions of the image developer solution

which contained 0.28M sodium carbonate and 0.5ml of 34% formaldehyde per litre. The gel was gently agitated in a third portion of this solution until the image had reached the desired intensity. Development was stopped by discarding the developer and adding 200ml of 1% acetic acid.

Ten-fold concentrated stock solutions of oxidizer and silver nitrate were prepared and suitably diluted when needed, while the image developer solution was prepared freshly prior to use. Stock solution of oxidizer was prepared by dissolving 1g $K_2Cr_2O_7$ in 0.17ml nitric acid (65% HNO_3) and making up the volume to 100ml. The silver nitrate stock solution was 2% (w/v) in distilled water.

(iv) Determination of molecular weights

The molecular weight of each proteinase and esterase band was calculated from their relative electrophoretic mobilities using standard protein markers (Weber & Osborn, 1969; Maurer, 1971). The electrophoretic mobilities of proteins are known to be dependent both on the net charge and molecular weight of the polypeptide chains. Use of gradient gel made the gel to serve as a molecular sieve, thus separating proteins on the basis of their molecular size (Hames & Rickwood, 1983).

Each gel was mounted on a glass plate and covered with a transparent sheet. This was placed on a light box and measurement of the distances of proteinase and esterase bands migration were taken using a ruler. The distance of dye migration was also measured. The relative mobilities (R_f) of the proteinase and esterase bands

were calculated thus:

$$R_f = \frac{\text{distance of proteinase/esterase band migration}}{\text{distance of bromophenol blue migration}}$$

R_f values for the marker proteins were also calculated, and these values were plotted against the molecular weights of the proteins on logarithm-scale graph. The molecular weights of the proteinase and esterase bands were read off from the graph using their R_f values.

From the results obtained from PAGE of crude enzyme solutions above, the intensity of lower molecular weight proteinase bands increased with time of incubation, especially in the absence of locust beans (see Plate 8). Thus to confirm the source of these lower molecular weight proteinase bands, the crude enzyme preparations were further incubated sterile. Hundred millilitres (100ml) of sterile nutrient broth medium in flasks were incolulated with 1ml of BS2, BL2 and BP2 inocula. For each strain samples were taken at 12, 18, 24 and 36 hour intervals (A1, B1, C1, D1 respectively). Each sample taken was centrifuged to remove bacterial cells and then filter-sterilized into sterile flasks. The sterile crude enzyme solution samples (except sample D1) were further incubated at 37°C and samples taken at subsequent times of sampling (Table 2). Samples were lyophilized to smaller volumes and dialysed against water, then buffer for 6 hours, before running them on gel elecrophoresis. Gels were stained for proteinases.

Table 2: Layout of times of sampling of crude enzymes solution of strains BS2, BL2 and BP2.

Time (hours) of sampling	12	18	24	36
0	A1	-	-	-
6	A2	B1	-	-
12	A3	B2	C1	-
24	A4	B3	C2	D1

(k) Purification and characterization studies

(i) Ammonium Sulphate Precipitation

The salting-out process involved the use of dry ammonium sulphate ($(NH_4)_2SO_4$) and was carried out in an ice-bath on a magnetic stirrer. Initially, the level of saturation required to precipitate out the proteinases were investigated. Five millilitres of crude enzyme solution were added into empty test tubes. Calculated amounts of $(NH_4)_2SO_4$ were added to each tube to obtain 40% - 95% of saturation (Coombs, 1982). The precipitation was carried out for 6 hours and the tubes were allowed to stand overnight at 4°C before centrifugation at 11,000rpm for 20 minutes. The precipitates were each redissovlved in 5ml Tris-HCl buffer pH 7.8 (+2mM $CaCl_2$), while the supernatants were dialysed against several volumes of water, then buffer. Protelytic activity was assayed both in supernatants and redissolved precipitates.

From the results obtained above 70mls of crude enzyme solution was saturated to 50% with dry $(NH_4)_2SO_4$. The suspension was centrifuged at 11,000rpm (4°C) for 20 minutes and the precipitate was redissolved in buffer. More $(NH_4)_2SO_4$ was added to the 50% - saturated enzyme solution (volume now 80ml) till 70% saturation level. The enzyme was allowed to precipitate out overnight at 4°C and later centrifuged at 11,000rpm. The precipitate was redissolved in buffer and dialysed against several volumes of buffer. Some of the supernatant were also dialysed against buffer.

The dialysed supernatant and redissolved precipitate (at 70% saturation) were assayed for proteolytic activity and compared with proteolytic activity in crude enzyme and redissolved precipitate (at 50% saturation).

Subsequently, BS2, BL2 & BP2 were grown in medium (containing 0.2% NB, 0.8% locust beans, 20mM Tris-HCl buffer pH 7.6 + 2mM $CaCl_2$ & basal salts) for 36 hours. Each culture medium (320ml) was centrifuged at 5,000rpm for 15 minutes to remove bacterial cells. To 250ml of each crude enzyme preparation was added dry $(NH_4)_2SO_4$ till 50% saturation, then centrifuged at 11,000rpm for 20 minutes. The precipitate collected was redissolved in buffer and dialysed against several volumes of buffer. Dry $(NH_4)_2SO_4$ was further added to the supernatant till 70% saturation. This was again centrifuged and the precipitate collected was redissolved in buffer and dialysed against several volumes of buffer. Some of the supernatant (70% saturation) was dialysed also.

Protein content, protelytic activity and esterolytic activity were assayed in 50% saturation precipitate, 70% saturation precipitate and supernatant, and crude enzyme preparation. The redissolved 70% saturation precipitate were used in further purification studies.

(ii) Ion-Exchange Chromatography (IEC)

The anion exchangers DEAE Sephadex A-50 and DEAE Sephadex A-25 (Pharmacia) were used. Ten grammes of anion exchanger were both swollen and equilibrated on 0.1M Tris-HCl buffer pH 7.8 (+2mM $CaCl_2$). The slurry was deaerated by heating in water bath at 100°C for at least

2 hours. Fine suspensions were decanted after 90-95% of gel had sedimented Swollen gel was allowed to cool before packing into column (1.5 x 20cm). Packed column was allowed to equilibrate at 40°C and the bed stabilized by washing through with two volumes of buffer. The flow rate (using a peristaltic pump unit) was calibrated while the fractions-collector unit was set before the enzyme preparation was applied on the column. In all IEC runs, the $(NH_4)_2SO_4$ - precipitated crude enzymes were used. Except when stated, precipitates were redissolved in 0.1M Tris-HCl buffer pH 7.8 containing 2mM $CaCl_2$.

After applying the enzyme preparation, column was washed again with buffer before elution with buffer containing a linear gradient of NaCl (0 to 1M). Absorbance at 280nm of all fractions collected in test tubes were measured using a Hitachi (Model 100-40) spectrophotometer. Proteolytic activity and esterolytic activity were assayed in fractions. Fractions within each peak of proteolytic activity were pooled and assayed for proteolytic activity, esterolytic activiy (where necessary) and protein content. After each run on the column, the anion exchanger was unpacked and regenerated by washing thrice with 5M NaCl in buffer. The gel was washed with several changes of buffer and deaerated by heating in water bath at 100°C for some hours. The cooled slurry was repacked for another run.

When a substantial proportion of total enzyme activity were found to be eluted in the void volume of the anion exchanger, attempt was made to test the binding capacity of a cation exchanger CM 52-cellulose

with the $(NH_4)_2SO_4$ - precipitated crude enzyme of BP2. The precipitated enzyme was redissolved in 0.1M phosphate buffer pH 6.5, and dialysed against several changes of same buffer. Sample of enzyme solution was assayed for proteolytic activity, while 4ml of the enzyme was added to 1.5ml of swollen, degassed CM 52-cellulose gel in a test tube. The slurry was mixed, and the mixture allowed to stand for a while. Supernatant of the sedimented slurry was taken and assayed for proteolytic activity.

(iii) Gel Filtration

The different enzyme activity peaks were further purified by running the pooled fractions on gel filtration columns. Sephadex G-75 and Sephadex G-50 (Pharmacia) were equilibrated in buffer (0.1M Tris-HCl buffer pH 7.8 + 2mM $CaCl_2$) and packed in columns 1 x 97cm and 1 x 93cm respectively. Packed columns were allowed to equilibrate at 4°C and washed with several volumes of buffer. The flow rate (using a Pharmacia persistaltic pump) was calibrated and the fractions collector unit was set. Pooled proteinase activity peaks from the IEC column were run on the Sephadex G-75 column. The column was washed with several volumes of buffer and eluent fractions were collected. Absorbance at 280nm of fractions were measured using a spectrophotometer, and proteolytic/esterolytic activities were assayed in fractions.

The enzyme activity peaks from Sephadex G-75 were pooled, and proteolytic activity and protein content were assayed. Each of the enzyme activity peaks II & III were applied on the Sephadex G-50 column.

The column was washed with several volumes of buffer and eluent fractions were collected. Also absorbance at 280nm of fractions were measured while proteolytic/esterolytic activities were assayed in fractions.

(iv) Inhibition Tests

After gel filtration, inhibition tests were carried out on few selected fractions from enzyme activity peaks before pooling. This was done to confirm homogeneity of the peaks (Millet, 1970). Inhibition tests were carried out using EDTA and PMSF which are specific inhibitors of neutral and serine proteinases respectively. Stock solutions with different concentrations of EDTA and PMSF were prepared in buffer as follows:

E_1 = 0.004M EDTA P_1 = 0.002M PMSF

E_2 = 0.01M EDTA P_2 = 0.01M PMSF

E_3 = 0.02M EDTA

Equal volumes of inhibitor solution and enzyme solution were mixed which resulted in two-fold dilution of inhibitor and enzyme. The mixture was incubated at 4°C for 2 hours before it was assayed for proteolytic activity. Degree of inhibition was expressed as percentage inhibition compared to the enzyme solution without inhibitor.

(v) Determination of molecular weights by gel filtration

Molecular weights of the three enzyme activity peaks were determined by their elution volumes during chromatography on Sephadex G-75 and Sephadex G-50 (Cooper, 1977). Calibrations were done using Cyt.C CTA and BSA as standard proteins, while dextran blue 2000 was used to

determine the void volume. Amounts of each protein required to give an O.D. at 280nm of 0.5 were calulated and weighed. These were dissoved in 1ml of buffer. Also 2.5mg of dextran blue 2000 was added. The standard proteins and dye mixture were applied on each gel column (1 x 97cm, Sephadex G-75). Elution was with 0.1M Tris-HCl buffer pH 7.8 (+2mM $CaCl_2$). When Sephadex G-50 was used, two standard proteins CytC & CTA with dextran blue 2000 were mixed and applied on the column (1 x 93cm). The absorbance at 540nm (for dye) and 280nm (for proteins) were measured and plotted. The following parameters were measured and calculated:

V_t = total volume of gel bed

V_x = space occupied by gel beads

V_o = space occupied by solvent surrounding gel beads

= void volume

V_e = elution volume

K_{av} or K_d = the coefficient of distribution

$$= \frac{V_e - V_o}{V_x}$$

The K_d values of the enzyme activity peaks were calculated from their elution volumes and their respective molecular weights were read from a standard curve of K_d versus molecular weights of the standard proteins.

(vi) Protein Determination

The protein content of samples at the different stages of purification was assayed by the Bio-Rad protein assay method.

The microassay procedure (Anon, 1981) was used. A stock solution of 25mg/100ml of bovine serum albumin (BSA) obtained from Boehringer, Mannheim was used as protein standard.

To 0.8ml of suitably diluted sample in microlitre sample tubes was added 0.2ml of Bio-Rad Dye Reagent concentrate. Each was mixed using a Vortex mixer and allowed to stand for 30 minutes. The absorbance at 595nm of each solution versus blank (contained 0.8ml of buffer & dye reagent concentrate) was read. The protein content of samples were read from a standard curve of O.D (595nm) versus concentration (1-25 ug/ml) of the standard protein.

(vii) PAGE of enzyme activity peaks

The three proteolytic activity peaks obtained from the IEC and gel filtration columns were pooled separately and applied on polyacrylamide gradient slab gels. Standard proteins (Thyroglobulin, Ferritin, Catalase & BSA) were also run on the gel. Electrophoresis was performed at 450V for 3 hours in Tris-glycine buffer pH 8.4. Electrophoresed gel was cut into strips and stained for proteinase, esterase and protein bands.

SDS-PAGE was also performed with gel containing 0.1% SDS. The samples were prepared using the method of Webber et al., (1972). Denaturing buffer was prepared by adding SDS and β-mercaptoethanol (final concentrations of 1% and 0.1% respectively) to 0.1M Tris-HCl buffer pH 7.8. Ten microlitres each of enzyme sample and denaturing buffer were mixed in a sample tube and held in boiling water bath for 3½ minutes. Some granules of sucrose was added to increase viscosity before applying the samples on the gel for electrophoresis. The running buffer also

contained SDS (1%) and electrophoresis was performed at 250V for 4 hours.

(viii) Hydrophobic Interaction Chromatography (HIC)

The proteinase bands pattern on PAGE of peak I highly suggested hydrophobic interaction between the enzyme proteins. Thus peak I was subjected to HIC on Phenyl Sepharose CL-4B which was equilibrated in buffer containing 2M NaCl at 4°C. The binding capacity of the HIC with the enzyme was first tested. To 1ml of swollen phenyl sepharose equilibrated with buffer (with 2M NaCl) was added 1.5ml of enzyme preparation. The mixture was stirred and allowed sometime to interact before centrifuging. Some of the supernatant was removed and assayed for proteolytic activity. Triton X-100 was added to the remaining slurry in the tube to give a final concentration of 0.1% Triton. The slurry was mixed again, then centrifuged and supernatant was assayed for proteolytic activity. The binding capacity of the enzyme was expressed as a percentage of the proteolytic activity present in the enzyme preparation prior to addition of HIC medium.

The pooled peak I was applied on the packed column and washed with buffer containing 2M NaCl. Elution was performed with buffer containing a negative salt gradient 2 to 0M NaCl and positive gradient solution of 0 to 0.1% Triton X-100. Absorbance at 280nm and proteolytic activity were assayed in the collected fractions. When enzyme failed to be eluted, column was unpacked and washed with a volume of buffer containing 0.2% Triton X-100. Enzyme was recovered,

lyophilized to a small volume, then dialysed against several volumes of buffer. The gel was regenerated (Pharmacia Handbook) and repacked. After application of enzyme sample, elution was attempted with buffer containing 10% methanol, then 20% methanol. In all fractions collected, none had detectable proteolytic activity. To confirm whether enzyme was still active after 20% methanol treatment, column was upacked and the gel washed with one volume of buffer containing 0.2% Triton X-100. Proteolytic activity was assayed in the filtered solution.

CHAPTER 3

RESULTS

(a) Morphology of microorganisms

Examination by phase contrast microscope of 12h cultures of the 7 strains of Bacillus revealed variation in cell length arrangement and motility. Strains BS1 and BS3 cells were in singles or pairs and were actively motile. Strain BL4 cells were in singles, pairs or short chains while strains BS2, BL1, BL2 and BP2 cells formed long chains. Plates 1, 2 and 3 show photomicrographs of the representative samples of B. subtilis, B. licheniformis and B. pumilus respectively.

(b) Growth

Figure 2 shows the growth of the 7 strains of Bacillus subtilis group in nutrient broth medium. The strains BS2, BL1 and BP2 showed slower growth with time. Strains BL2 and BL4 were closely related in growth, while strains BS1 and BS3 had higher growth than others. In all cases, the strains started sporulating by 18h of growth. The spores were ellipsoidal in shape and placed subterminally or centrally in the cells. Growth of the seven strains between 36h-42h of incubation were significantly (at $\alpha = 0.05$) different. Using one-way anova and Duncan's multiple range test, the following (descending) order was obtained for the growth of the organisms in liquid medium: BS3 > BS1 > BL4 > BL2 > BS2 > BL1 > BP2

The effects of pH of culture medium on growth of the three strains BS2, BL2 and BP2 are shown on Table 3. Strains BS2 and BP2 had pH

Plate 1: Photomicrograph of Bacillus subtilis (BS3) x 1000.

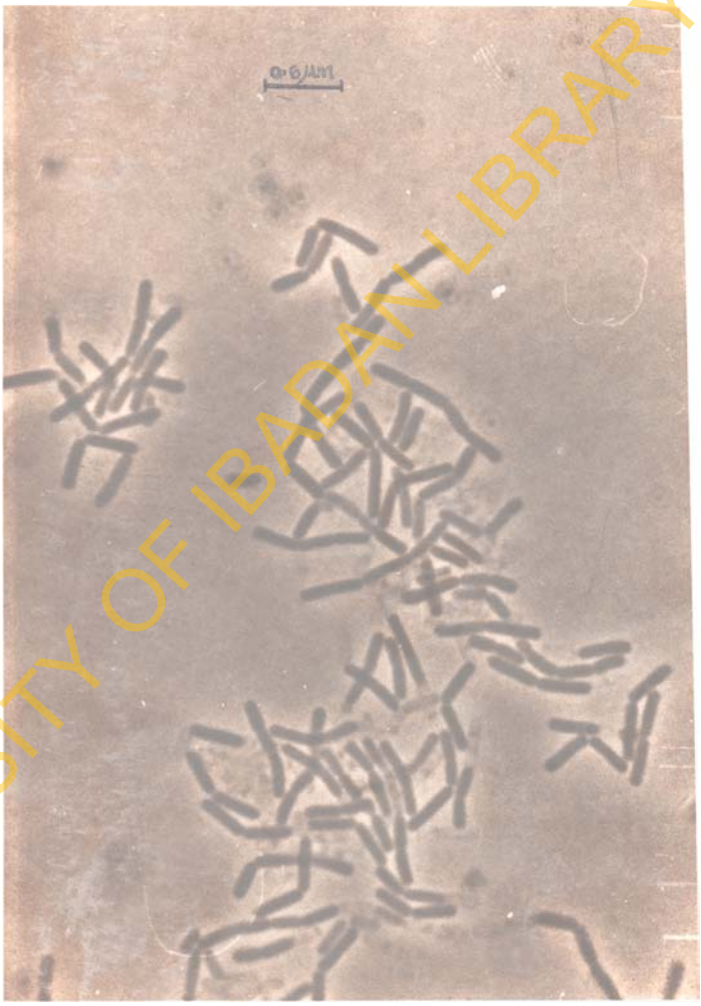


Plate 2: Photomicrograph of Bacillus licheniformis (BL4) x 1000.

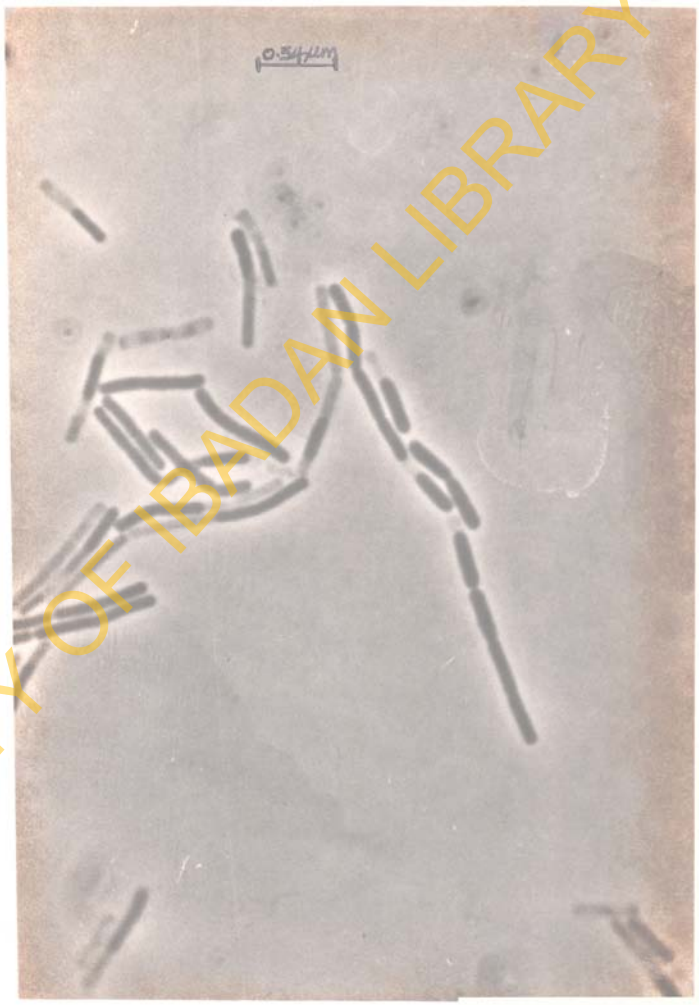


Plate 3: Photomicrograph of Bacillus pumilus (BP2) x 1000.

Fig. 2: Growth (optical density at 540nm) of the 7 strains of Bacillus species.

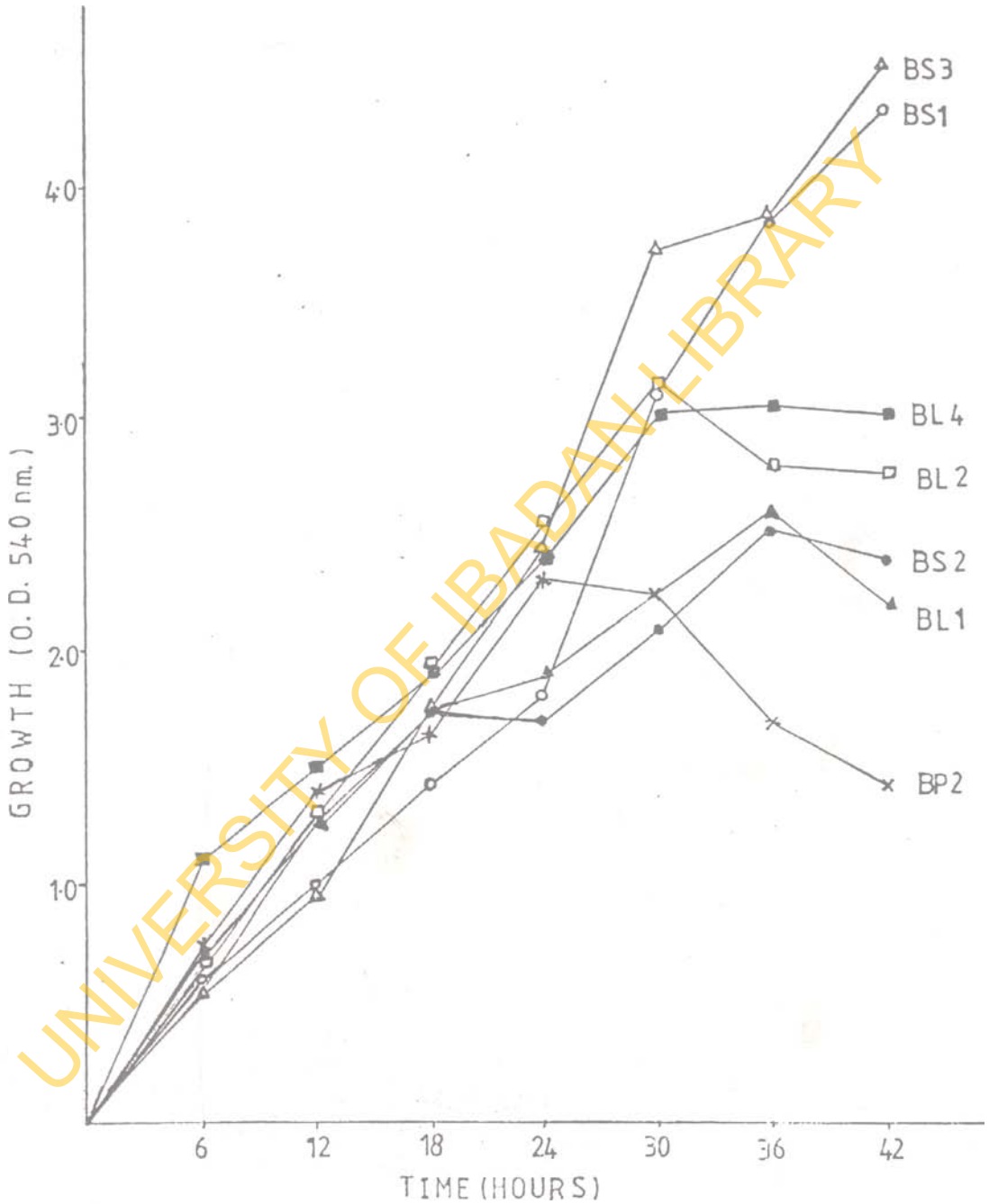


Table 3: Effect of pH of culture medium on growth of *Bacillus* species strains BS2, BL2 and BP2 (O.D. 540nm at 36h).

pH	BS2	BL2	BP2
6.5	0.035	0.02	1.22
7.0	1.61	0.01	1.28
7.4	1.1	0.75	1.56
8.0	0.865	0.525	0.81
9.0	0.845	1.04	0.02
10.0	0.11	0.005	0.0

optimum for growth in the range pH 7.0-7.4, while strain BL2 had optimum pH for growth between pH 7.4-9.0. The strain BP2 was more tolerant of acidic pH while strain BS2 tolerated alkaline pH.

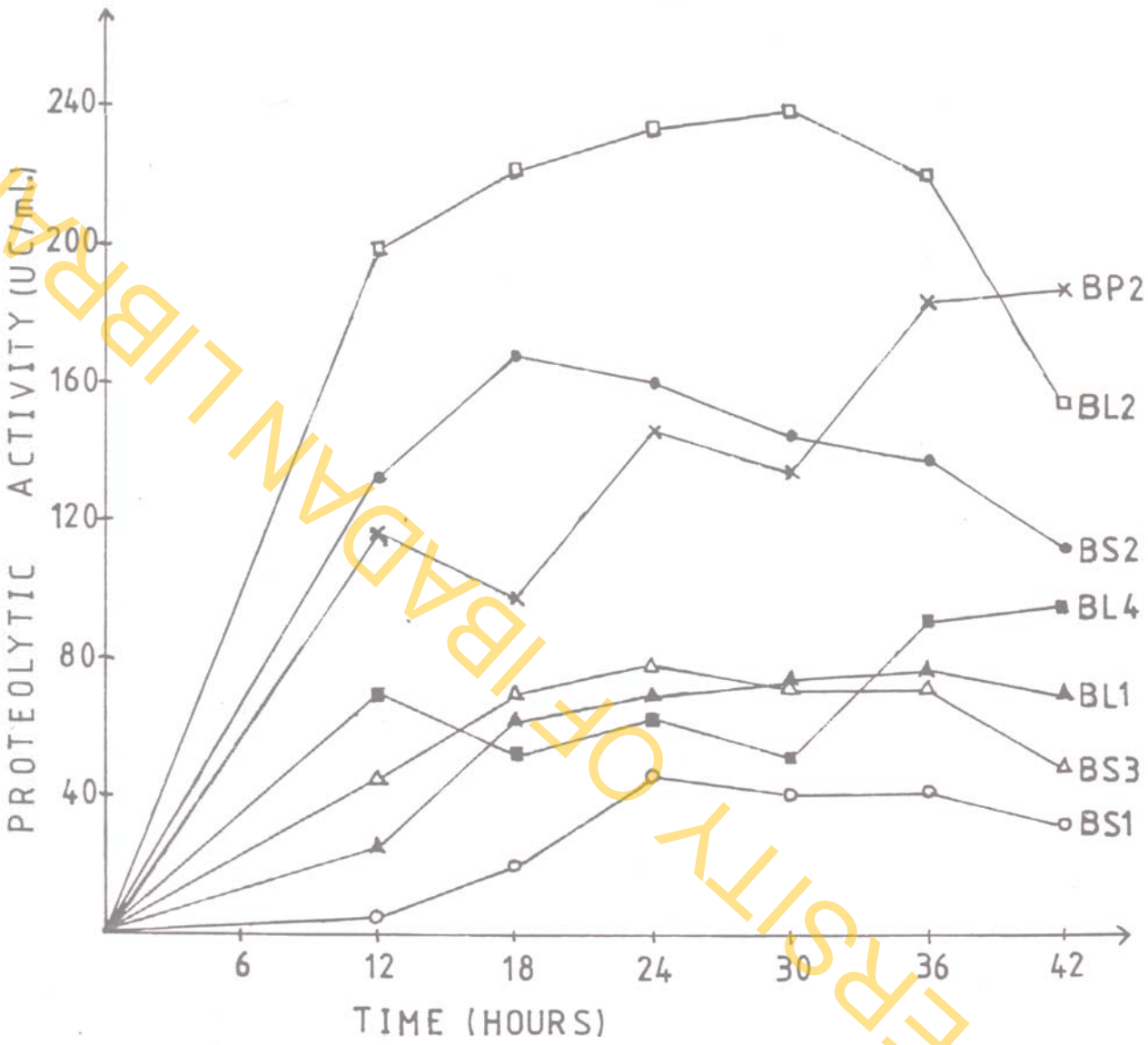
(c) Comparison of proteolytic activities of the Bacilus spp.

The proteolytic activities of the 7 strains in medium with African locust bean are shown in Figure 3. Strain BL2 showed the highest activity followed by strains BP2 and BS2, while strain BS1 showed the least activity. Significant (at $\alpha = 0.05$) differences were also recorded in the amounts of extracellular proteinases produced by the strains. Statistically, the following (descending) order was obtained when the proteolytic activities of the seven strains were compared: BL2 > BP2 > BS2 > BL4 > BS3 > BL1 > BS1 The result indicated that strains within a species group differ in the quantity of extracellular proteinases excreted.

(d) Extracellular enzymes production by strains BS2, BL2 and BP2

The three strains produced extracellular polygalacturonase constitutively. The strain BS2 produced significantly higher amounts (at $\alpha = 0.05$) of polygalacturonase than strains BL2 and BP2 at 36h of incubation, either in the presence or absence of African locust bean (Fig. 4). The presence of African locust bean in the culture medium enhanced production of polygalacturonase significantly in the three strains. African locust bean enhanced production of polygalacturonase in strain BS2 than pectin and sodium polypectate (Fig. 5). Only small amount of polygalacturonase activity was detected in medium containing nutrient broth and pectin at 24h of incubation.

Fig. 3: Proteolytic activity of the 7 strains of Bacillus species in nutrient broth containing 0.7% (w/v) African locust bean.



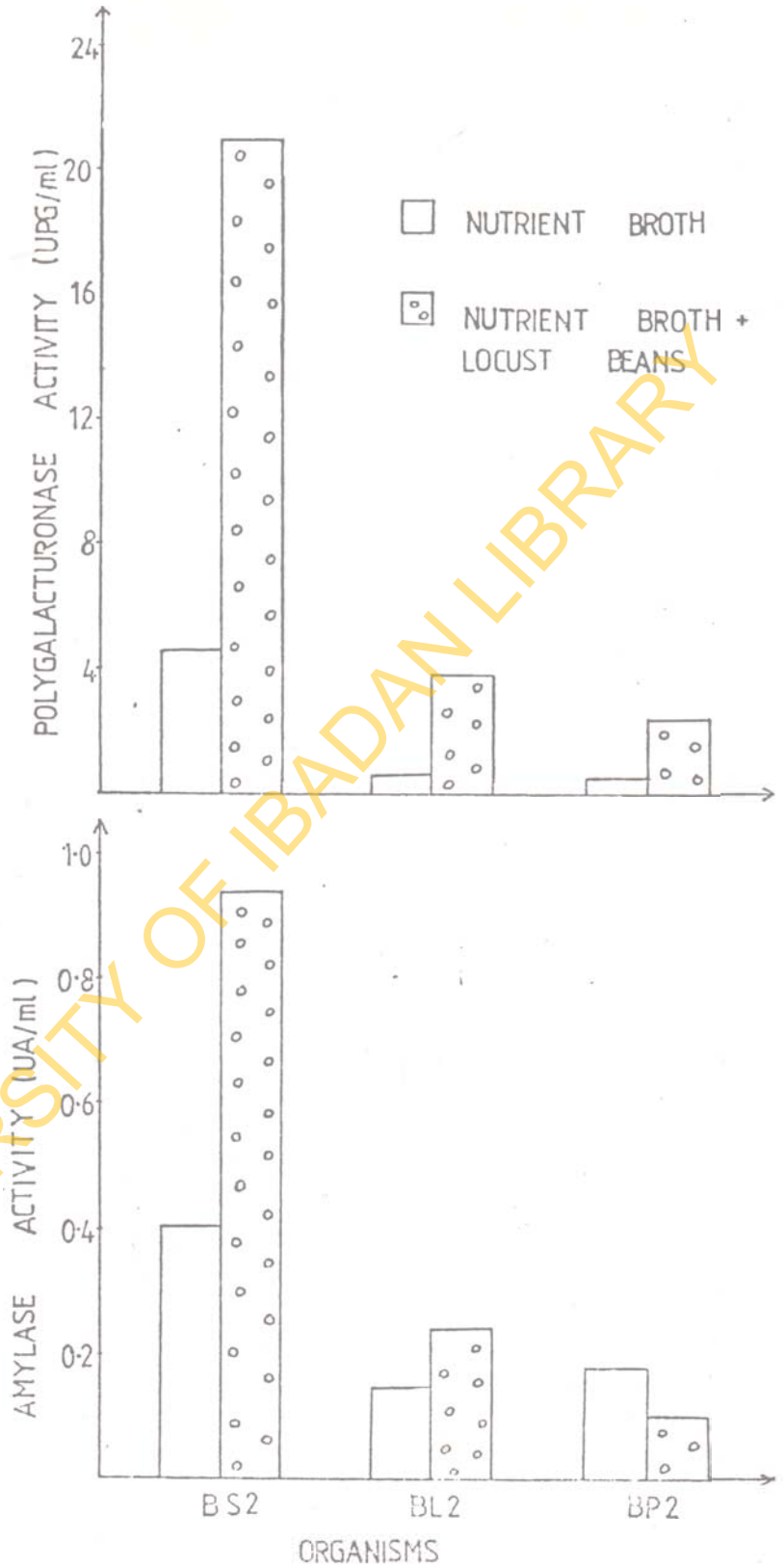


Fig. 4: Polygalacturonase and amylase activities of the strains BS2, BL2 and BP2.

Fig. 5: Polygalacturonase activity of the strain BS2 in media with time. · Δ , nutrient broth with sodium polypectate; ▲, nutrient broth with pectin; ●, nutrient broth with African locust bean.

Sodium polypectate enhanced production of the enzyme at early stages of incubation, but the production fell after 12 hours. Polygalacturonase activity increased throughout the period of incubation in medium containing African locust bean.

Pectinmethylesterase (PME) activity was not detected in crude enzyme supernatants of the three strains BS2, BL2 and BP2, (as there were no differences in the titre volumes of sodium hydroxide used for the control and the experiment). Presence of pectin in one of the culture media did not induce production of PME by strain BS2.

The three strains, BS2, BL2 and BP2 produced amylase constitutively Strain BS2 produced significantly higher amounts of amylase than strains BL2 and BP2 (Fig. 4). The presence of African locust bean in culture medium enhanced amylase production in strain BP2. African locust bean also caused an initial delay in amylase production in strain BS2 (Fig. 6). The peak of amylase production of strain BS2 in nutrient broth medium was at 36 hours.

Extracellular lipolytic activity was not detected in crude enzymes supernatants of strains BS2, BL2 and BP2 in medium containing nutrient broth only. The culture medium containing nutrient broth and African locust bean had very low lipolytic activity detectable (Table 4). The three strains were lipolytic on glycerol tributyrate agar plates.

Strain BS2 was screened for production of extracellular phytase. Phytase activity was not detected in media used which

Fig. 6: Amylase activity of strain BS2 in media with time. ○, nutrient broth only; ●, nutrient broth with African locust bean.

Table 4; Lipolytic activity (difference in tire volume of NaOH between control and experiment) of the strains BS2, BL2 and BP2 in medium containing African locust beans.

		Volume of 0.02N NaOH (ml)		
BS2		x_1	x_2	
	12h	0.15	0.1	0.125
	24h	0.05	0.05	0.05
	36h	0.05	0.05	0.05
	42h	0.0	0.0	0.0
BL2	36h	0.1	0.0	0.05
BP2	36h	0.1	0.25	0.175

contained rice flour, sodium phytate and African locust bean respectively.

The crude enzymes solutions of the three strains hydrolysed raffinose to mellibiose and fructose (Plate 4), indicating sucrase activity. Sucrase activity was confirmed as the enzymes solution of the strains also hydrolysed sucrose to glucose and fructose (Plate 5).

Spectrophotometric assay of sucrase activity by the three strains showed that strain BS2 had the highest activity, while strain BL2 had the least activity (Fig. 7). The arabinogalactan that was extracted from African locust bean was also hydrolysed by crude enzymes solution of the three strains, indicating galactanase activity. Strain BP2 had the highest galactanase activity while strain BS2 had the least activity (Fig. 7).

(e) Extraction of arabinogalactan from African locust bean

The paper charomatogram of the acid-hydrolysed extract from African locust bean revealed two major monosaccharides, arabinose and galactose (Plate 6). This indicated that arabinogalactan is one of the main carbohydrates present in African locust bean.

(f) Production of short-chain fatty acids by strains BS2 and BL2

Gas chromatographic analysis of the crude enzymes supernatants of strains BS2 and BL2 showed the presence of isobutyric acid,

Plate 4: Paper chromatogram showing hydrolytic products of raffinose by enzyme solutions of strains BS2, BL2 and BP2. Raf., raffinose; Mel., Mellibiose; Suc., sucrose; Glu., glucose; Gal., galactose; Fru., frutose.

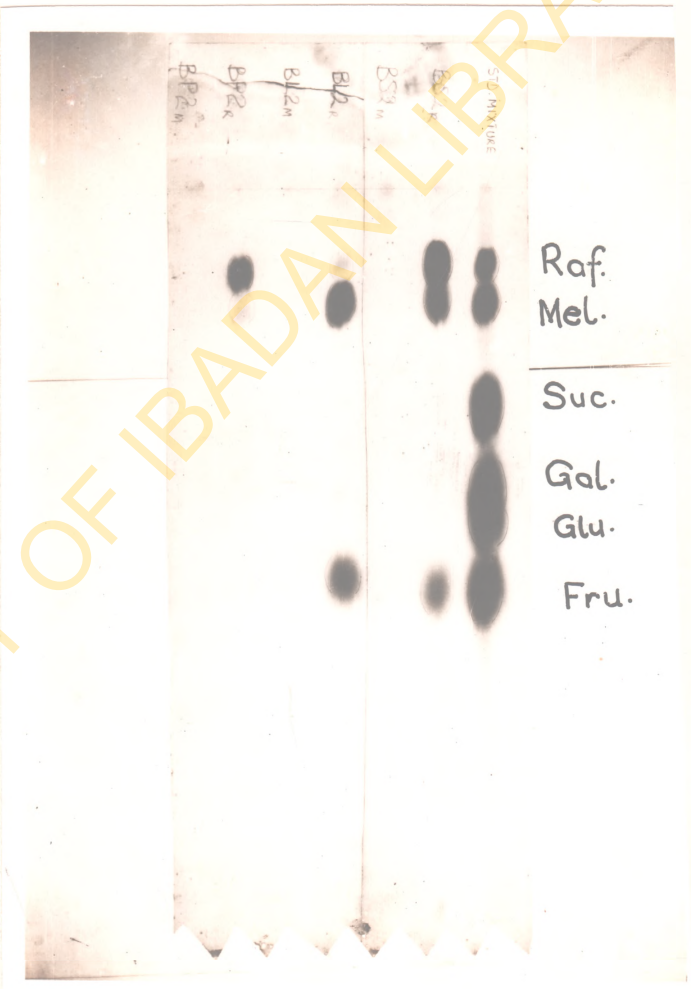


Plate 5: Paper chromatogram showing hydrolytic products of sucrose by enzyme solution of strain BS2. Suc., sucrose; Glu., glucose; Fru., fructose.

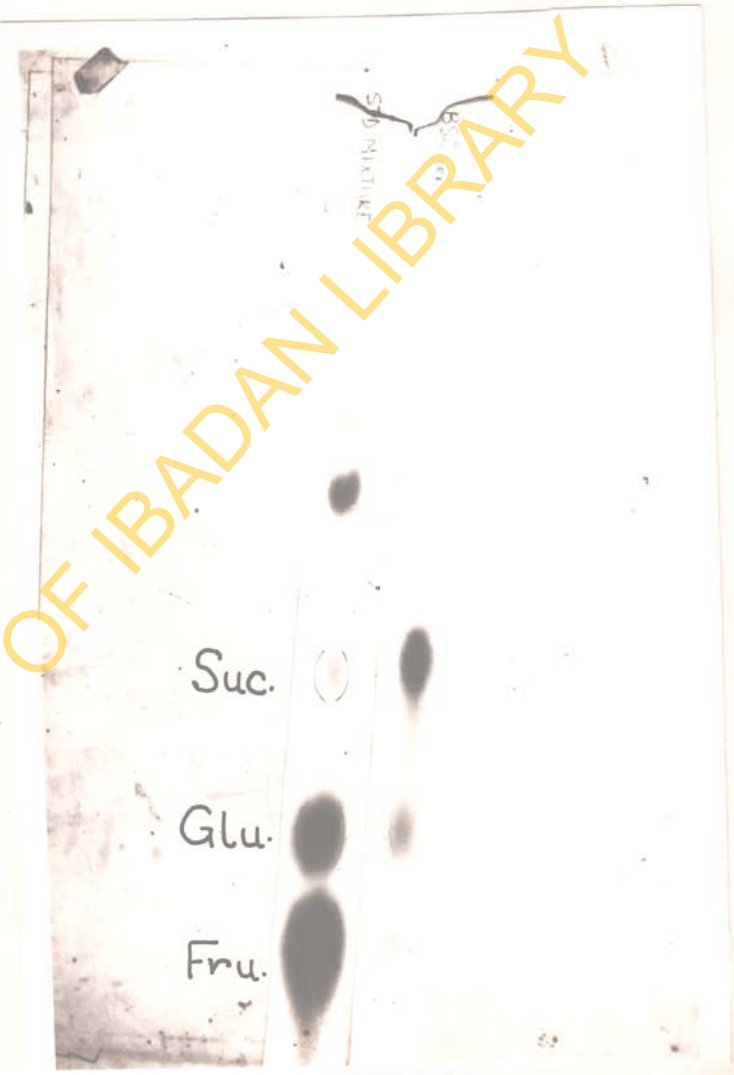
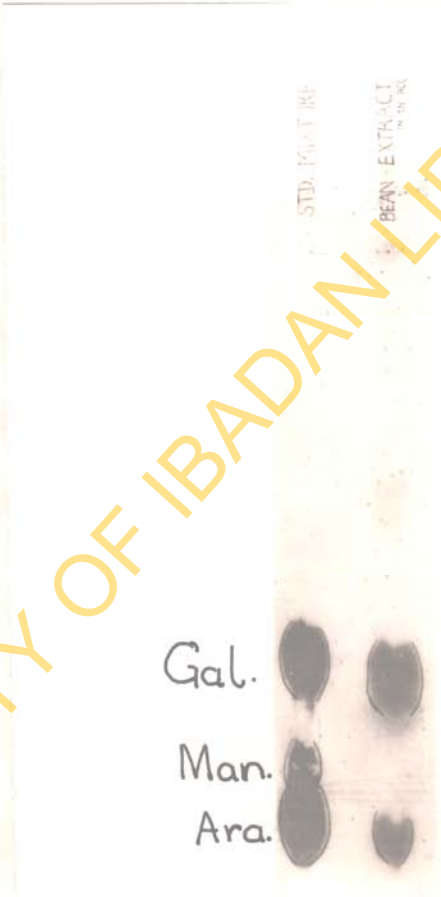


Fig. 7: Galactanase and sucrase activities of the strains BS2, BL2 and BP2.

Plate 6: Paper chromatogram of African locust bean extract acid-hydrolysate and standard sugars. Gal., galactose; Man., mannose; Ara., arabinose.



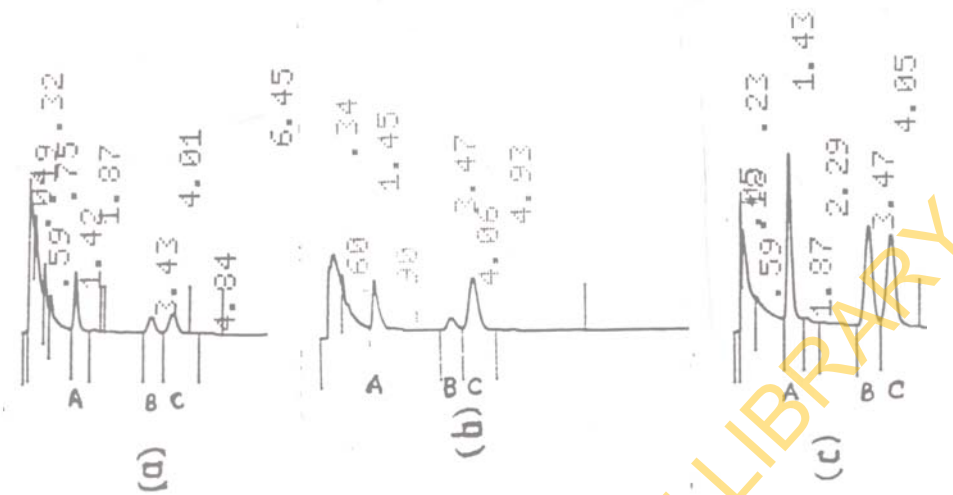
2-methylbutyric acid and isovaleric acid (Fig. 8). Propionic, butyric, valeric and caproic acids had retention times (RT) of 0.81, 1.91, 5.0 and 14.34 seconds respectively. The peaks obtained from GC run of BS2 supernatants had RT values of 1.42, 3.43 and 4.01 (Fig. 8a) while the peaks from BL2 supernatants had RT values of 1.45, 3.47 and 4.06 (Fig. 8b). When the standard fatty acids isobutyric acid, 2-methylbutyric acid and isovaleric acid were chromatrographed on the GC, the peaks with RT values of 1.43, 3.47 and 4.05 respectifely were obtained (Fig. 8c). Thus the short-chain fatty acids produced by the strains were identified as isobutyric, 2-methylbutyric and isovaleric acids.

(g) Culture conditions for optimal proteinase production

Preliminary studies showed that a concentration range of 0.1%-0.2% nutrient broth (NB) in the medium was required for minimal significant growth by the strain BS2. High (> 0.2%) NB concentrations resulted in greater changes in pH values in media. To effect minimal changes in pH of medium during fermentation, NB concentration of 0.1% - 0.2% is desirable. Thus a basal NB concentration in the range was used in subsequent experiments except where stated.

There was reeuction in the amount of extracellular proteinases produced by strain BS2 when flask was incubated without agitation (Fig. 9). By 36h when the highest level of production was reached in agitated medium, only 23% of this activity was detectable in flask not agitated. When the non-agitated medium was incubated for longer period (till 96h), there was continued steady increase

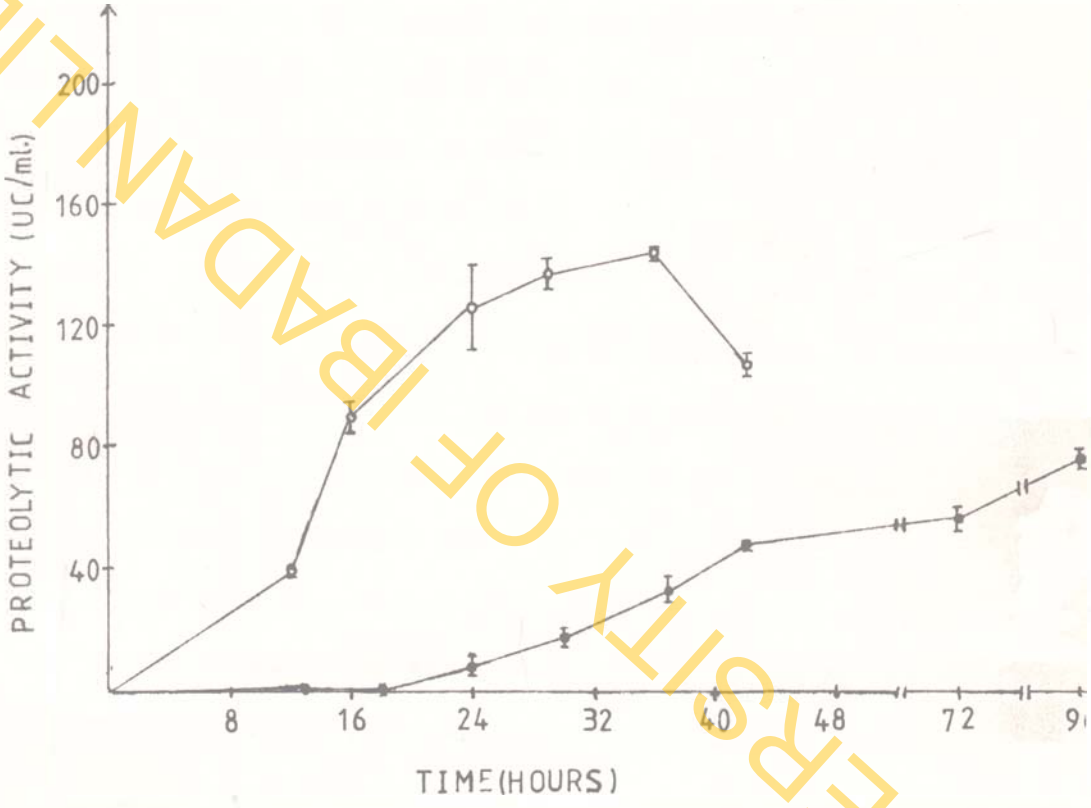
Fig. 8: Gas chromatograms of short-chain fatty acids. (a), peaks from strain BS2; (b), peaks from strain BL2; (c), peaks of the standard short-chain fatty acids isobutyric acid (RT 1.43), 2-methylbutyric acid (RT 3.47) and isovaleric acid (RT 4.05).



	Peaks (RT)	% area
Fig 8a	A (1.42)	11.395
	B (3.43)	5.394
	C (4.01)	7.318
Fig 8b	A (1.45)	13.971
	B (3.47)	6.337
	C (4.06)	19.591
Fig 8c	A (1.43)	20.798
	B (3.47)	19.984
	C (4.05)	19.779

Peaks A,B and C are isobutyric acid, 2-methylbutyric acid and isovaleric acid respectively.

Fig. 9 : Effect of agitation on proteolytic activity of strain BS2. o, agitated medium; ●, non-agitated medium.



in amount of proteinases produced, but this represented only 53% of the amount of enzyme obtained in 36h under agitation.

A similar effect of agitation on proteolytic activity was confirmed with B. licheniformis BL2. Non-agitation of culture medium casued a reduction in the amount of proteinases produced by strain BL2 (Fig. 10). Optimum proteolytic activity level had been attained within 18h of fermentation (290 UC/ml) when flasks were agitated as compared to 24UC/ml when flasks were not agitated. After the optimum level had been attained in agitated flasks the proteinases level decreased, but in non-agitated flasks, proteinase level continued to increase till 96 hours of fermentation.

There were significant effects of the carbon sources on proteinase production (at $\alpha = 0.05$) by the strain BS2 when control was compared with each of the different carbon sources. Starch and raffinose gave the greatest support for enzyme production (Fig. 11). Though sucrose enhanced proteinase production, its effect was not significant. On the contrary, glucose and fructose repressed proteinase production. Among the carbon sources which favoured production of proteinases are, in the order:

raffinose > starch > arabinose > galactose > sucrose.

When the effects by individual carbon-sources were compared, there were no significant differences in most cases.

There were also significant effects of nitrogen-sources on proteinase production (at $\alpha = 0.05$) by strain BS2. Using

Fig. 10: Effect of agitation on proteolytic activity of strain BL2. o, agitated medium; ●, non-agitated medium.

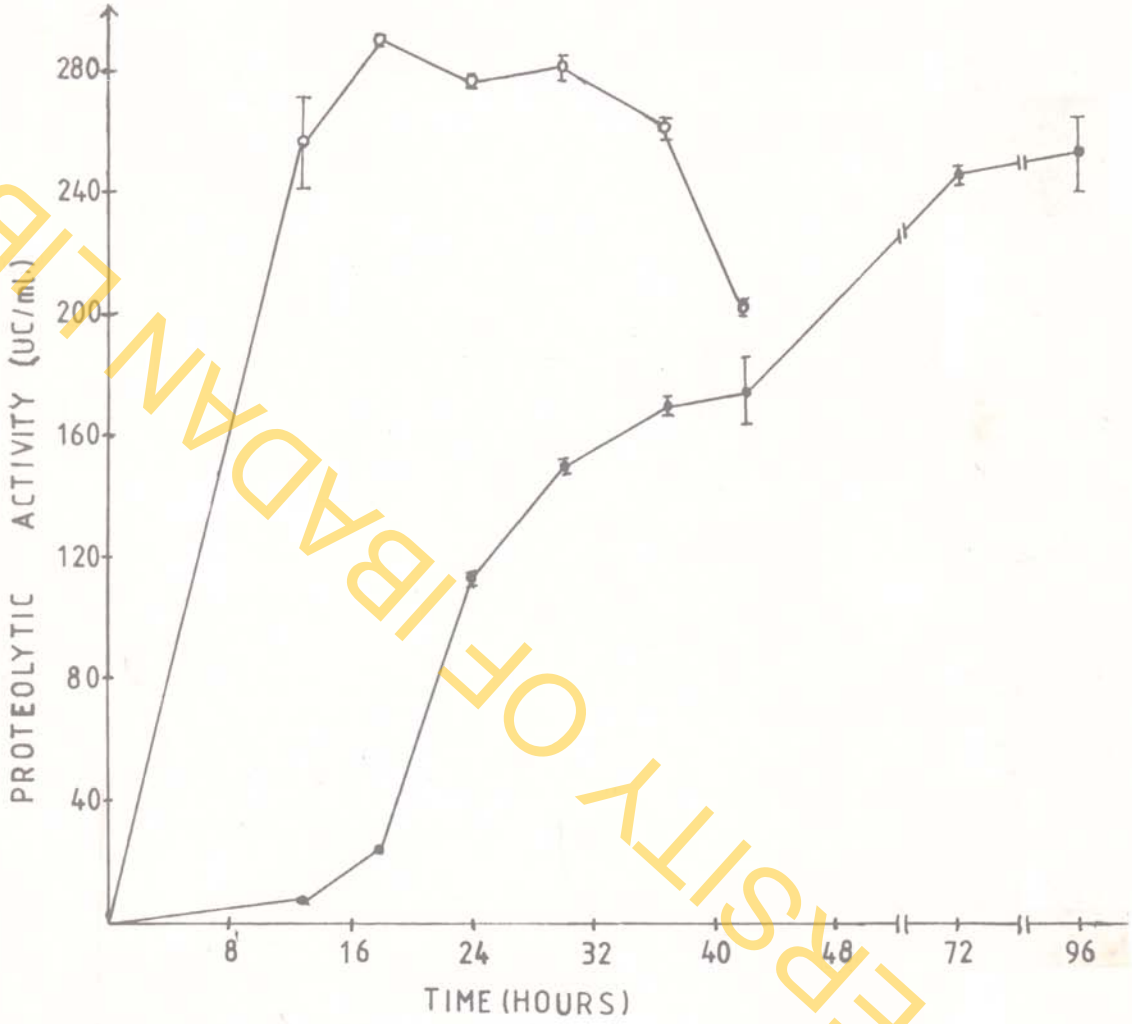
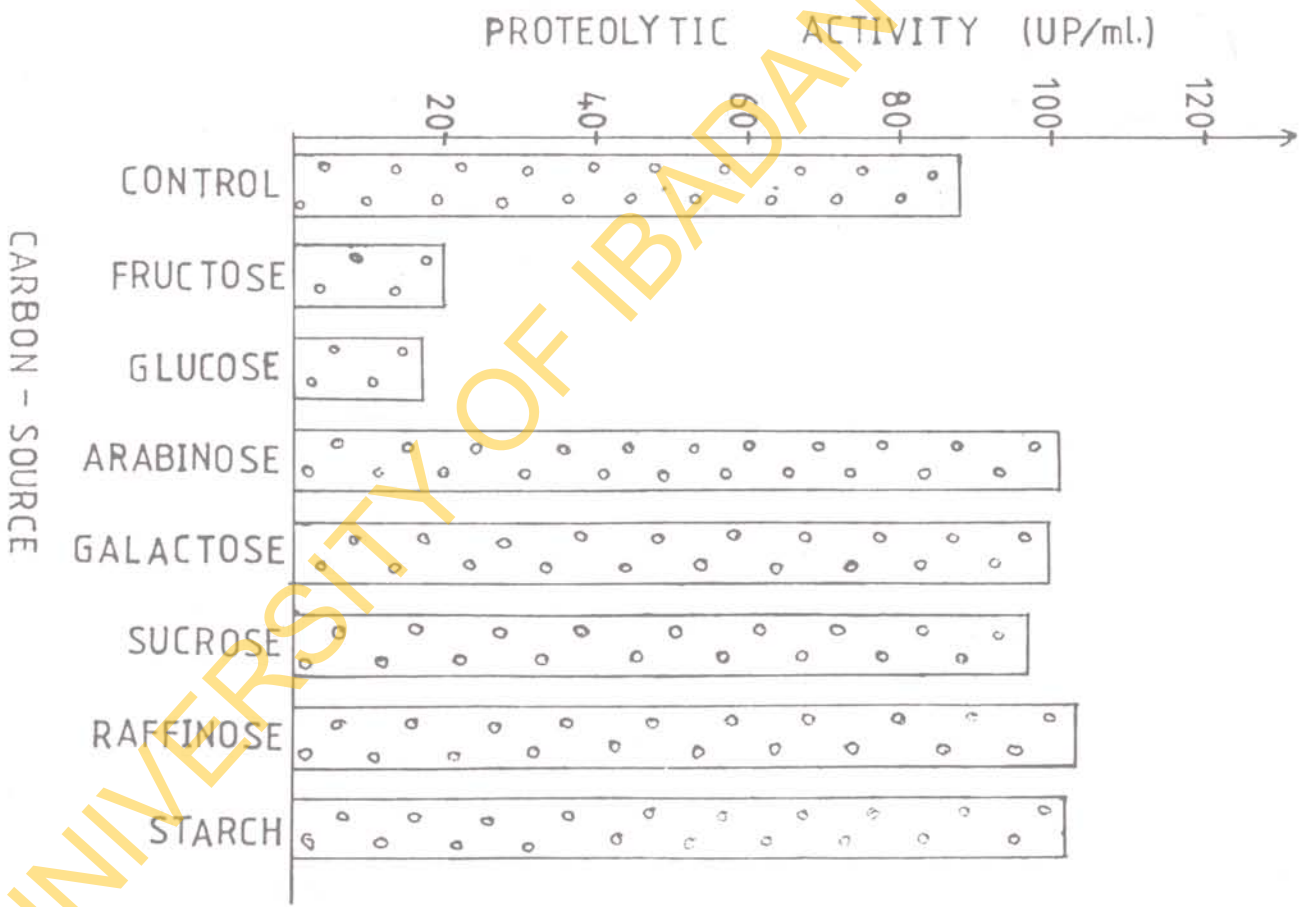


Fig. 11: Effects of carbon sources on proteinase production by strain BS2. Each medium contained 0.5% Tryptone and 0.2% of the respective carbon-source, while control did not have any carbon-source.



tryptone as the reference nitrogen source, casein enhanced proteinase production, but the effect was not significant. Other nitrogen sources repressed proteinase production significantly (Fig. 12). The nitrogen sources repressed proteinase production in the order:

Leucine > Urea > KNO_3 > NH_4NO_3 > Aspartic acid > Glutamic acid > Alanine

The presence of African locust bean in nutrient broth (1.3%) enhanced proteinase production in the three strains; (Fig. 13) but the effect was not significant (at $\alpha = 0.05$).

(h) Characteristics of crude extracellular proteinases

(i) Effects of pH and temperature (strain BS2)

Optimal pH for activity of the crude proteinases of strain BS2 was pH 7.5 (Fig. 14). The enzyme was stable in the pH range 6-10 for several hours. The enzyme was relatively stable at pH 6.0 because 88.5% of total activity was still detectable after 20 minutes when compared to the reference standard (REF.,pH 8.2) (Fig. 15). At pH 5, 81.8% of enzyme activity was lost within 15 minutes while only 7.5% of activity was detectable at pH 4 in 10 minutes. At pH 2 and 3, all activities were lost before 5 minutes.

Optimum temperature for activity was 35°C (Fig. 16). At 50°C, the proteinases were still very active, having 94.6% of the activity at 35°C (enzyme reaction mixture in buffer at pH 7.2). The enzyme was relatively stable at 60°C as it retained

Fig. 12. Effect of nitrogen sources on proteinase production by strain BS2. Each medium contained 0.2% starch and 0.5% of the respective nitrogen-source.

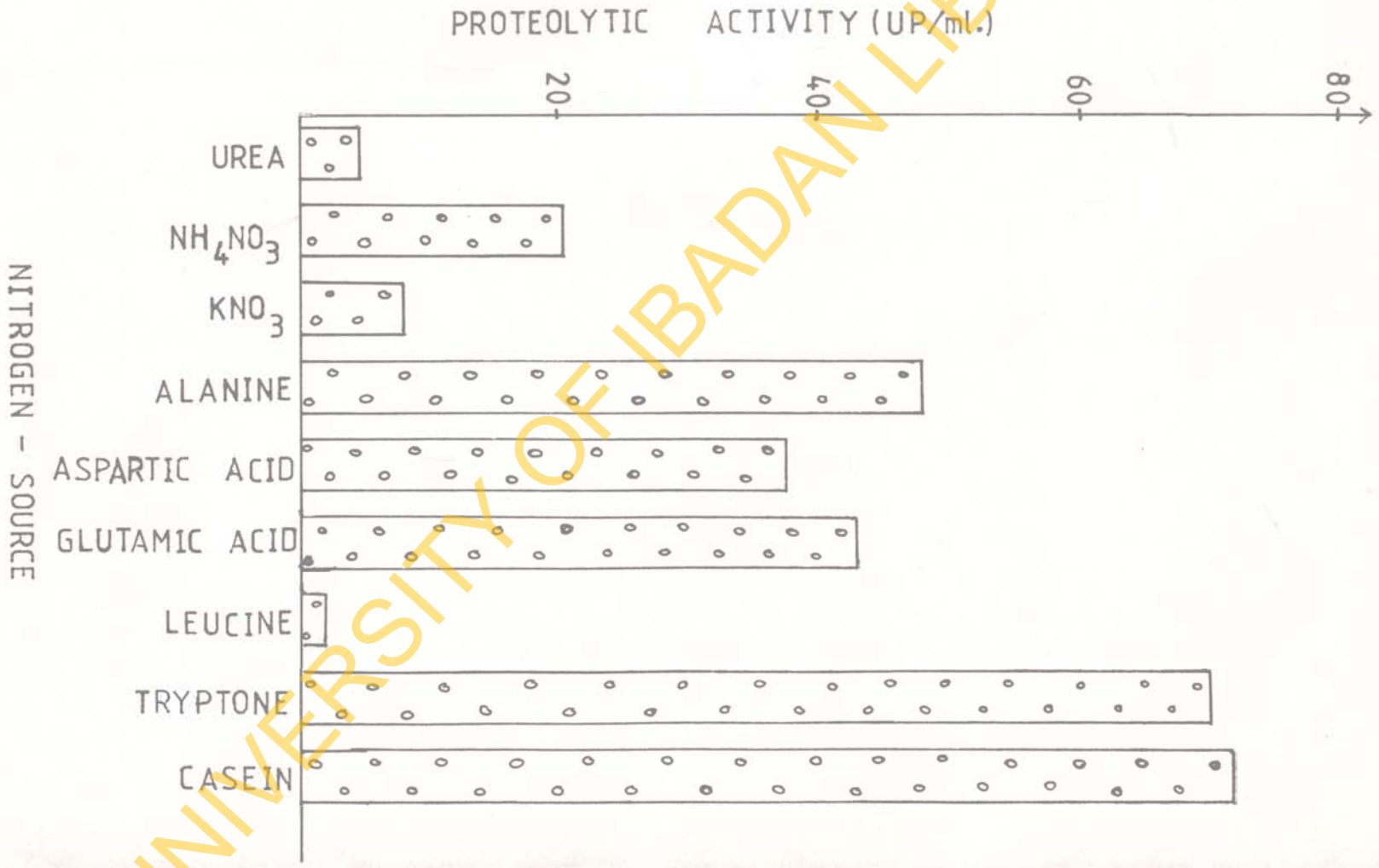


Fig. 13: Effect of presence of African locust bean in culture medium on proteinase production by the three strains BS2, BL2 and BP2.

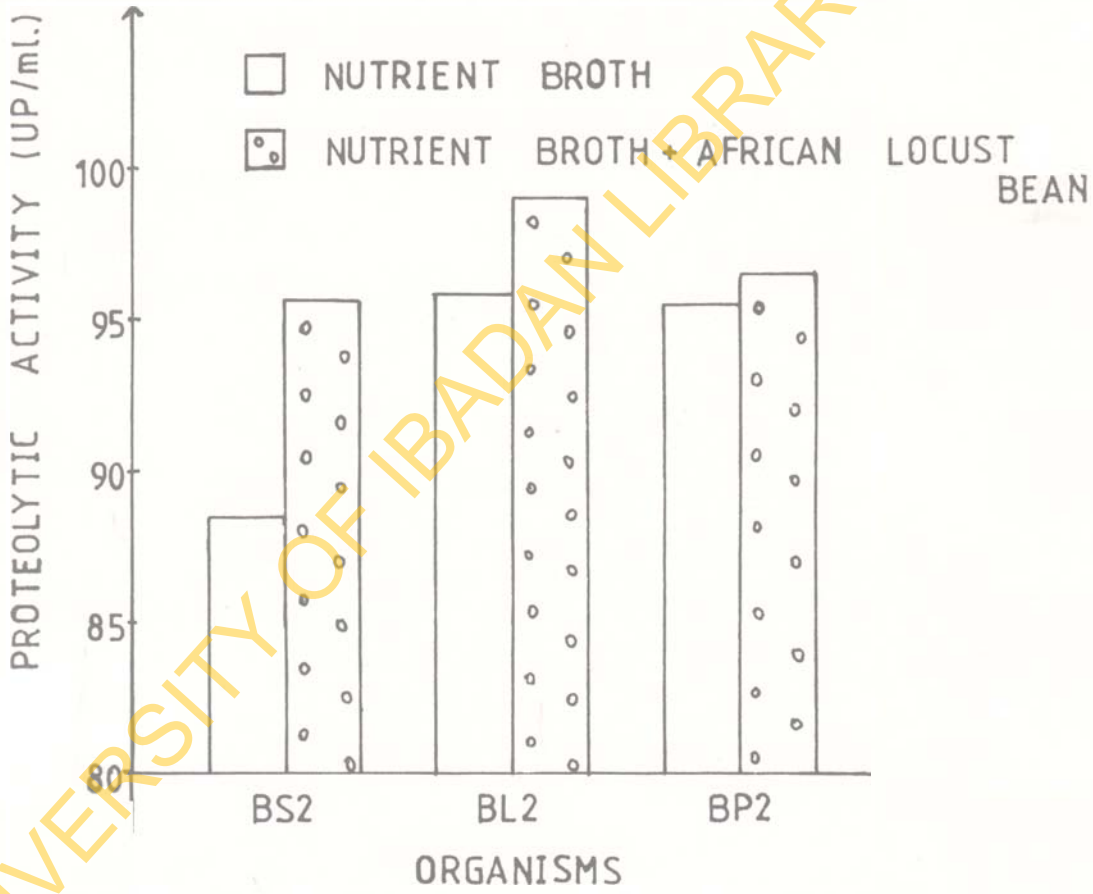


Fig. 14: Effect of pH on activity of crude proteinases of strain BS2.

Fig. 15: Effect of pH on stability of crude proteinases strain BS2.

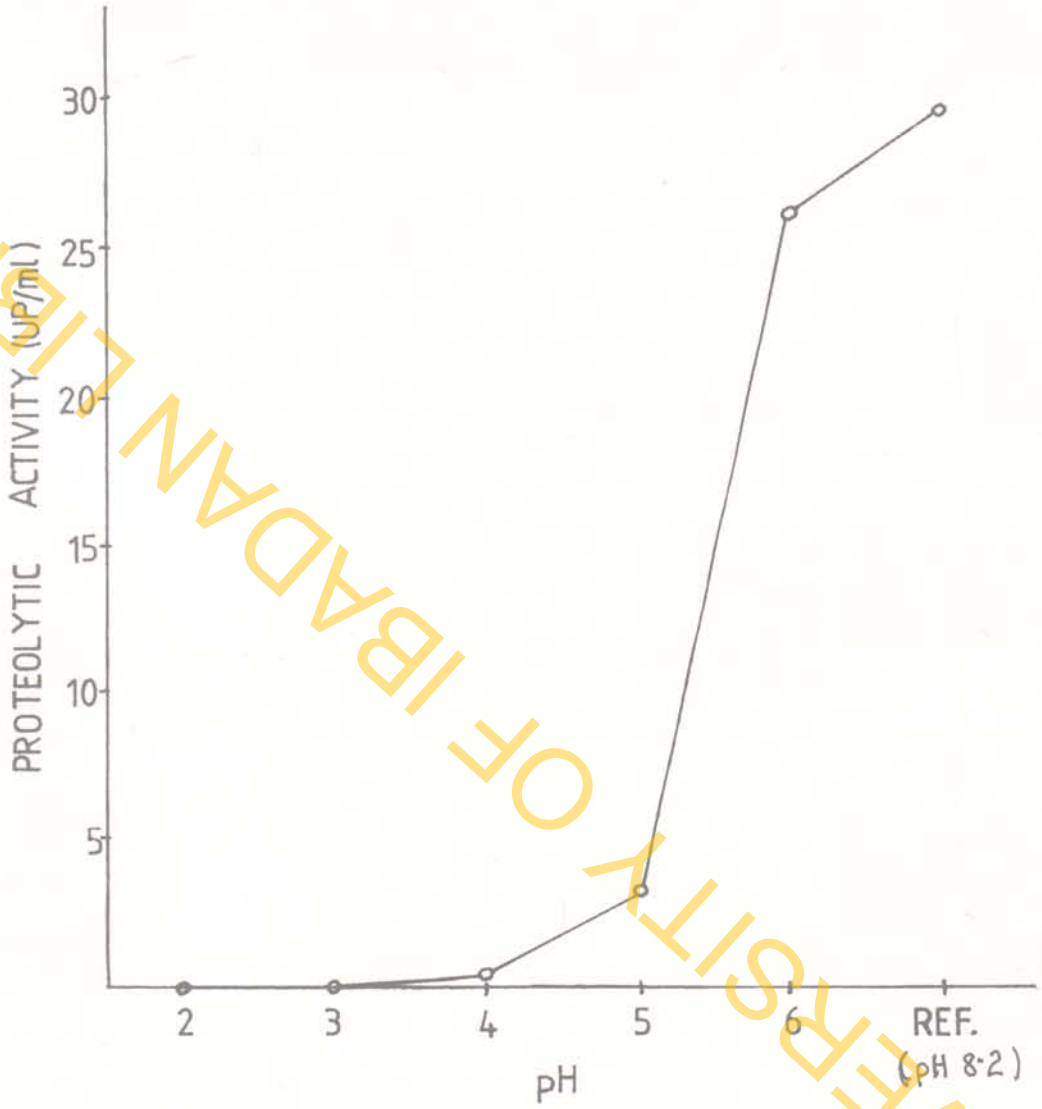
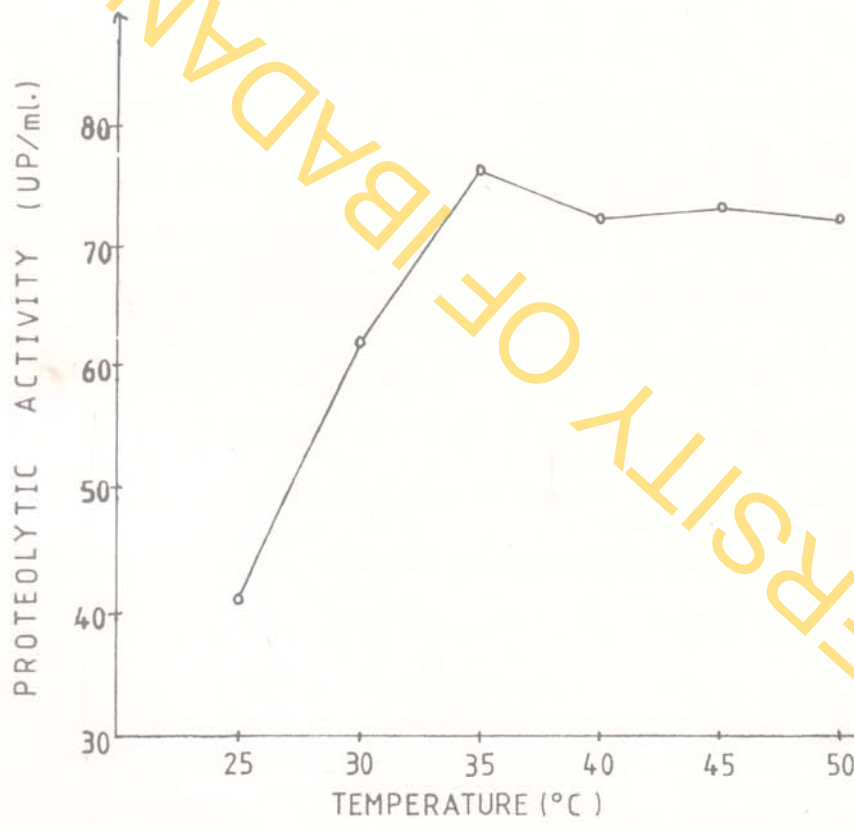


Fig. 16 : Effect of reaction temperature on activity of crude proteinases of strain BS2.



78.1% activity after 30 minutes (Fig. 17). At 70°C and above, the proteinases were quickly denatured, completely losing activity.

(ii) Proteinase kinetics of the strains BS2, BL2 and BP2

Figure 18 shows the effect of time of incubation on proteolytic activity of the three strains. The concentration of the casein substrate used was 1% (w/v). Strains BL2 and BP2 had higher rates of casein hydrolysis than strain BS2. The effect of substrate (casein) concentration on proteolytic activity of the three strains is shown in Figure 19. From the Lineweaver-Burke plot (Fig. 20), the apparent Km values for casein hydrolysis by the strains BS2, BL2 and BP2 were 39.14mg/ml, 33.29mg/ml and 44.1mg/ml respectively.

(iii) PAGE of crude proteinases of the seven strains of Bacillus

Plates 7 - 9 show the proteinase bands obtained after electrophoretic separation of the crude enzymes of strains BS2,BS3 & BP2 respectively on 5-20% polyacrylamide gradient slab gels. The proteinase bands appeared as colourless bands against a blue background containing Coomassie-blue stained casein. Multiple proteinase bands were obtained in all strains, both in the presence and absence of African locust beans in the growth medium. Proteinase bands patterns obtained for strains BS1, BS2 and BL1 were similar (Plate 7) while BS3 had more proteinase bands than the two B. subtilis strains (Plate 8). Strains BL2, BL4 and BP2 had similar proteinase bands patterns (Plate 9).

Fig. 17 : Thermal stability (at 60°C) of crude proteinases of strain BS2.

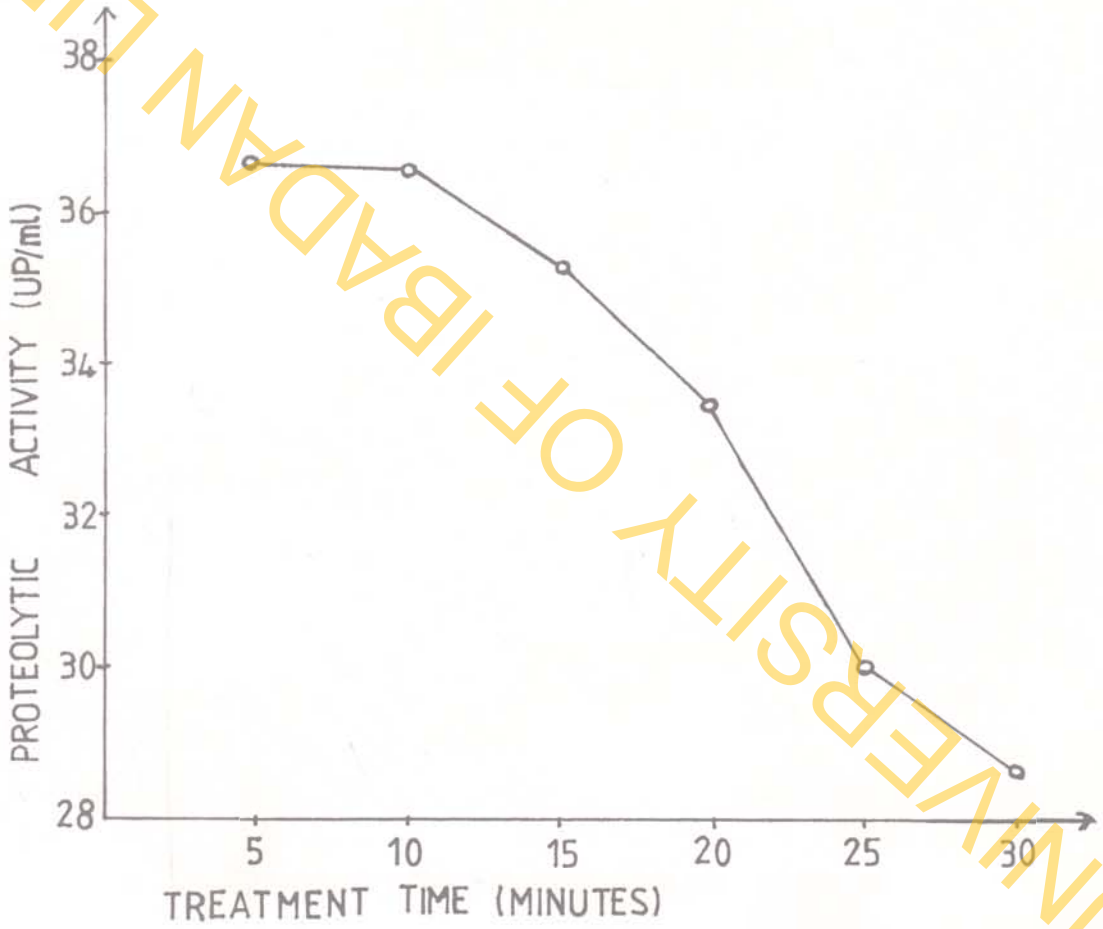


Fig. 18 : Effect of length of incubation on activity of crude proteinases of strains BS2, BL2 and BP2 (reaction temperature = 35°C).

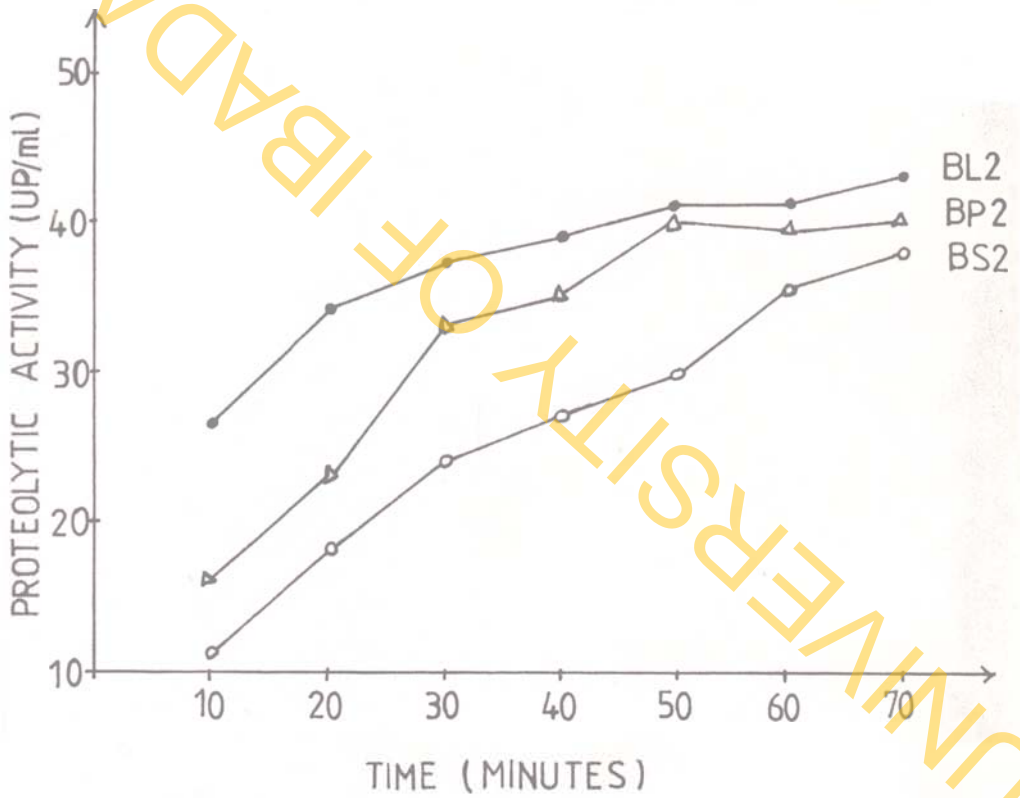


Fig. 19: Effect of substrate concentration on activity of crude proteinases of the strains BS2, BL2 and BP2.

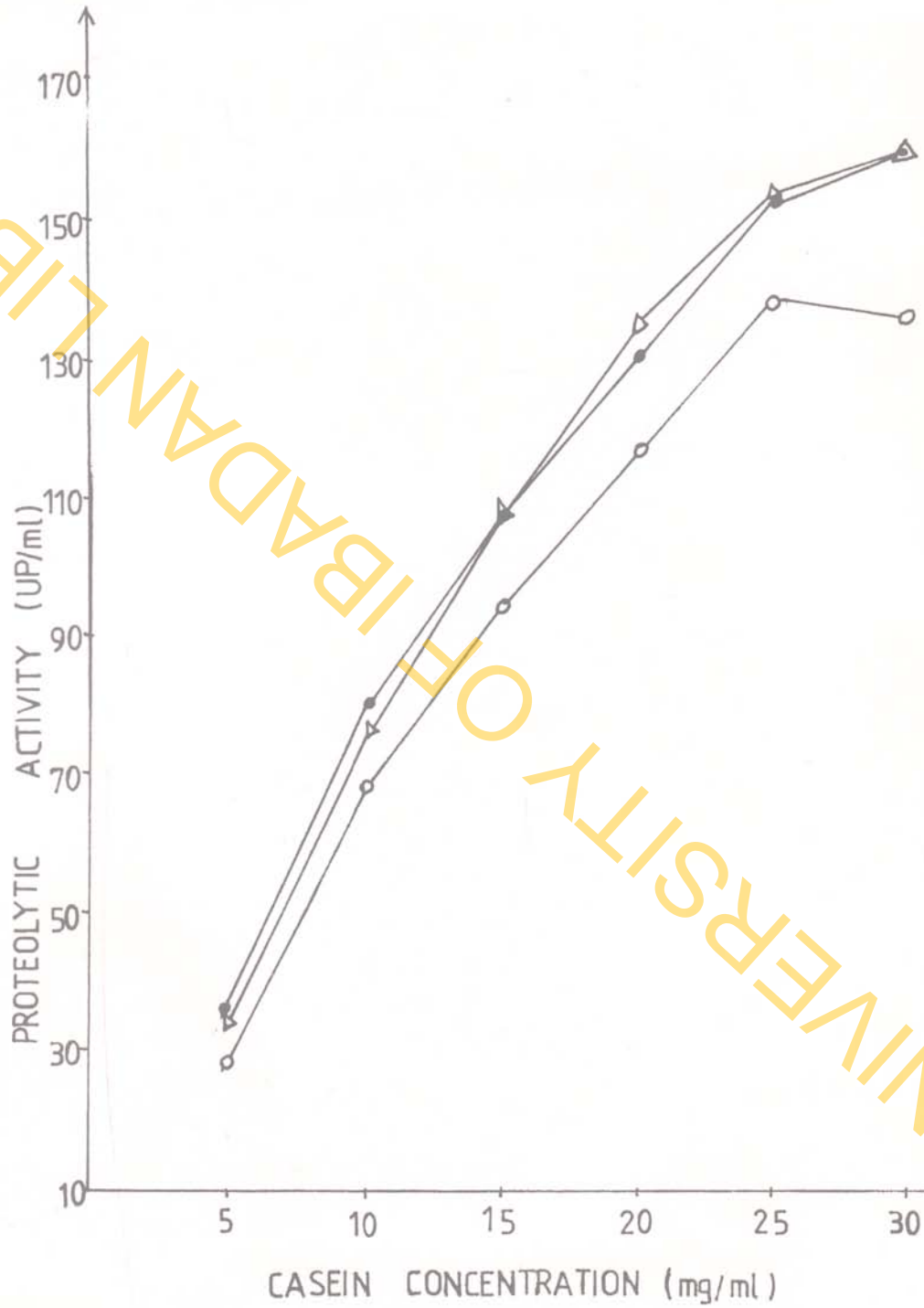


Fig. 20 Lineweaver-Burke plot of activity of crude proteinases of the three strains BS2, BL2 and BP2.

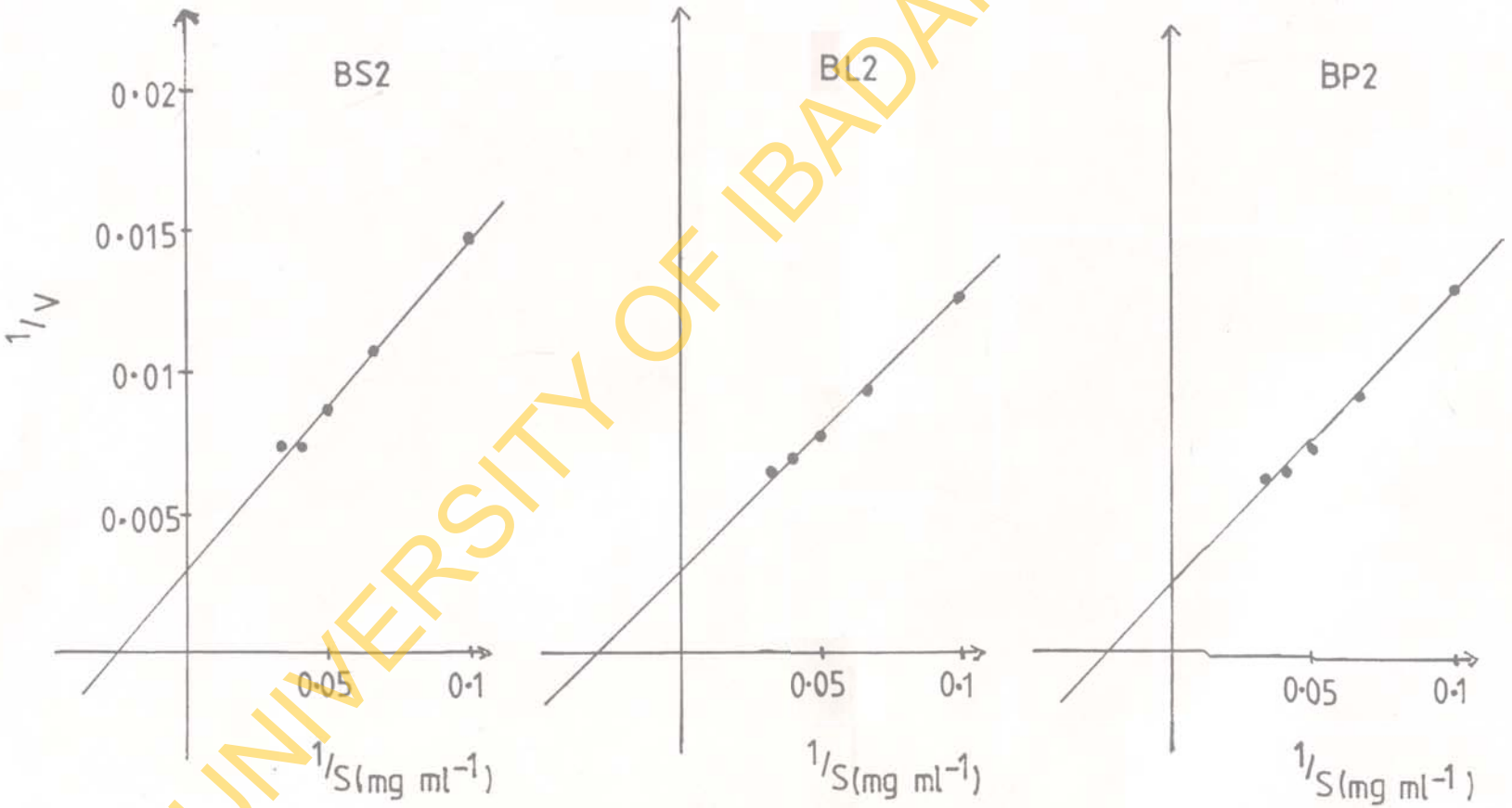


Plate 7: Proteinase bands (P_1 - P_8) of B. subtilis BS2 and marker proteins on polyacrylamide gradient slab gel.

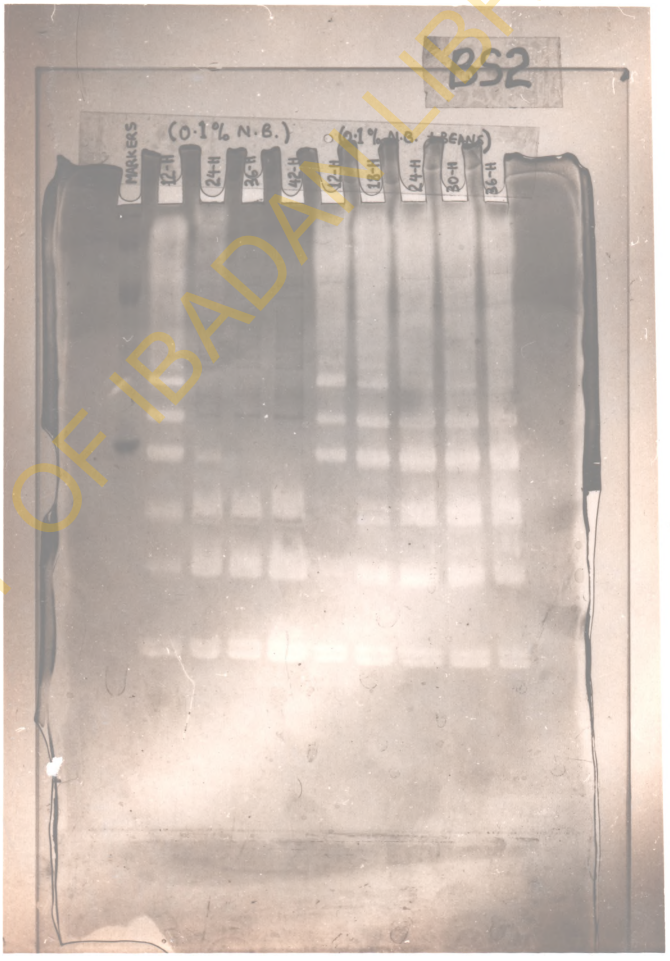


Plate 8 : Proteinase bands of B. subtilis BS3 and marker proteins on polyacrylamide gradient slab gel.

Plate 9: Proteinase bands of B.pumilus PB2 and marker proteins on polyacrylamide gradient slab gel.

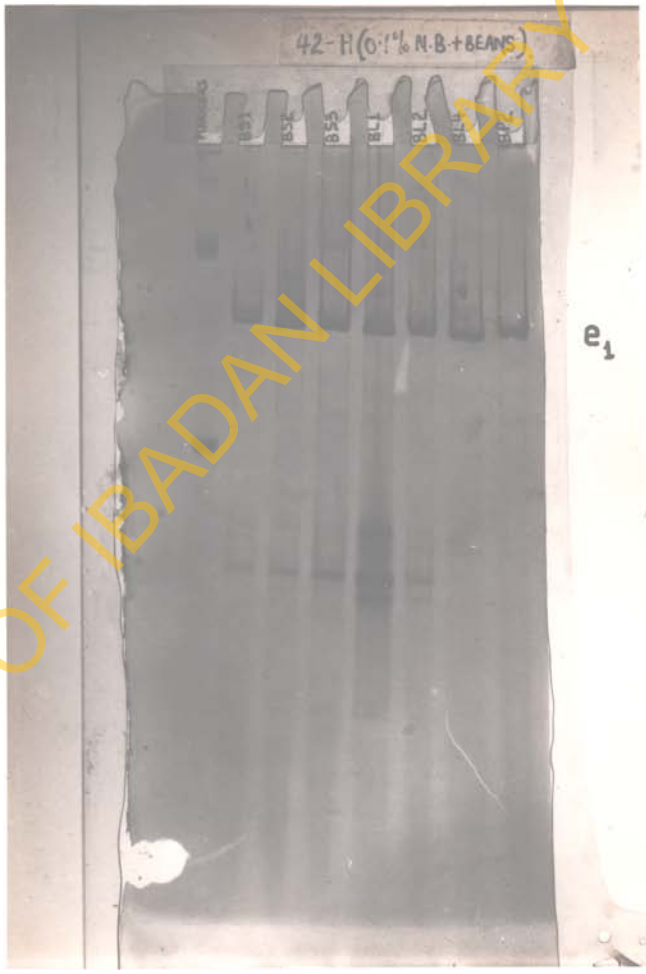
had similar proteinase bands patterns (Plate 9).
A large proteinase band at the upper end of the gel was observed in the samples' runs, especially samples from media containing locust bean. It was also noted that the intensity of proteinase bands P_2, P_3 and P_4 which were observed in the 12h samples (Plate 7) decreased with time of fermentation while the intensities of proteinase bands P_5, P_6, P_7 and P_8 increased.

The esterase bands patterns of the 7 strains of Bacillus species in media with nutrient broth only and nutrient broth with African locust bean are shown (Plates 10 and 11 respectively). A single esterase band e_1, which had same electrophoretic mobility was common to all the isolates, while some strains e.g. BS1, BS2, BS3 and BL4 had a few other esterase bands. In medium containing locust bean, a major esterase band was also common to all strains (Plate 11) except that the intensity of the bands was more than those from medium without locust bean. Most of the strains had other esterase bands.

The molecular weights of the standard proteins used, thyroglobulin, ferritin, catalase and BSA were 655, 455, 240 and 68 KD respectively. The distance of migration, R_f and respective molecular weights of the proteinase bands (read from the standard curves) are shown in Table 5. Each strain had at least seven distinct proteinase bands from 12h samples. These proteinase bands had molecular weights ranging from 7 kilodaltons (KD) to 720KD on the basis of their electrophoretic

Plate 10: Esterase bands of crude enzymes solutions (nutrient broth medium) of the 7 *Bacillus* spp. and marker proteins on polyacrylamide gradient slab gel.

Plate 11: Esterase bands of crude enzymes solutions (nutrient broth with African locust bean medium) of the 7 Bacillus spp. and marker proteins on polyacrylamide gradient slab gel.



e_1

Table 5a: Distance moved, R_f and the molecular weights of proteinase bands of Bacillus species from 'iru'.

Strain	BS1			BS2			BS3			BL1		
TD	16.6			16.4			16.15			15.35		
P	D	R_f	MW	D	R_f	MW	D	R_f	MW	D	R_f	MW
P_1	0.8	0.0482	530	0.5	0.03	530	0.3	0.0186	660	0.4	0.026	720
P_2	5	0.301	114	4.9	0.299	110	4.8	0.297	137.5	4.9	0.319	116
P_3	6	0.361	79	5.8	0.354	84	5.3	0.328	115	5.8	0.3778	82
P_4	7	0.422	54	6.7	0.409	56.5	6.1	0.378	86	6.6	0.43	59
P_5	8.6	0.518	30	8.2	0.500	33	6.7	0.415	69.5	8.0	0.521	33.6
P_6	10.2	0.614	16.5	9.6	0.585	19.6	7.2	0.446	58.5	9.5	0.619	18
P_7	12	0.723	8.5	11.4	0.695	10.2	8.7	0.539	34.4	11.0	0.7166	10
P_8	12.2	0.735	7.9	11.7	0.713	9.1	10.2	0.632	20.4	11.2	0.7296	9.2
P_9							12.1	0.749	10.5			
P_{10}							12.4	0.768	9.4			

TD = Distance of dye migration (cm); D = Distance of proteinase band migration (cm)

P = Proteinase band; R_f = Relative mobility; MW = Molecular weight (kilodaltons)

Table 5b: Distance moved, R_f and the molecular weights of proteinase bands of Bacillus species from 'iru'.

Strain	BL2			BL4			BP2		
TD	17.0			16.7			15.3		
	D	R_f	MW	D	R_f	MW	D	R_f	MW
P_1	0.7	0.0412	530	0.3	0.018	708	0.3	0.0196	425
P_2	5.8	0.341	80	1.2	0.072	505	3.5	0.229	125
P_3	6.8	0.4	55.25	6.1	0.365	77.5	5.5	0.359	59
P_4	9.3	0.547	22	8.6	0.515	29.6	7.8	0.51	24
P_5	10.2	0.6	15.7	10.1	0.598	17.2	9	0.588	15
P_6	11.1	0.653	11.3	10.4	0.623	14.7	9.7	0.634	11.5
P_7	11.9	0.7	8.3	11.9	0.713	8.4	10.7	0.699	7.8
P_8	12,2	0.712	7.7	12.2	0.731	7.4	10.9	0.712	7.4

TD = Distance of dye migration (cm); D = Distance of proteinase band migration (cm)

P = Proteinase band; R_f = Relative mobility; MW = Molecular weight (kilodaltons)

mobilities. For example, the molecular weights of the proteinase bands from samples of strain BS2 were 530, 110, 84, 56.6, 33, 19.6, 10.2 and 9.1KD respectively in order of their R_f in the gel.

Table 6 shows the distance moved, R_f and the corresponding molecular weights of the esterase bands from the 7 strains, both in the presence and absence of locust bean. The molecular weights of the main esterase bands common to all the strains in medium containing African locust bean and medium without locust bean (e_1) are 164 KD and 157 KD respectively. It was noted that these main esterase bands in the two media do not have corresponding proteinase bands on the basis of R_f and molecular weights. Some of the other esterase bands are proteinase(s) with esterolytic activity.

Plates 12 and 13 show the proteinase bands of strains BS2 and BP2 respectively when crude enzyme samples were further incubated at 37°C. Similar proteinase bands pattern were obtained for strains BL2 and BP2. There were no changes in the proteinase bands pattern for samples incubated sterile from BL2 and BP2 culture medium flasks (Plate 13). Samples taken after further incubations of the 12h sample had similar proteinase bands patterns. The same was true of the 18h and 24h samples. This showed that there were no autolysis of 'higher molecular weight' proteinases to 'lower molecular weight' proteinases in the absence of the organisms. In case of BS2 (Plate 12) a

Table 6: Distance moved, R_f and the molecular weights of esterase bands of *Bacillus* species from 'iru'.

		Nutrient broth (NB) medium (TD = 17.9)			NB + locust bean medium (TD = 19)		
Strain	e	D	R_f	M.W	D	R_f	M.W
BS1	e_1	4.1	0.229	157	4.3	0.226	163
	e_2	9.2	0.514	23.9	10.3	0.542	25.3
BS2	e_1	4.1	0.229	157	4.3	0.226	164
	e_2	2.1	0.117	360	10.3	0.542	25.3
	e_3	3	0.167	236	-	-	-
BS3	e_1	4.1	0.229	157	4.3	0.226	164
	e_2	2.1	0.117	360	2.4	0.126	298
	e_3	8.9	0.497	26.7	10.3	0.542	25.3
BL1	e_1	4.1	0.229		4.3	0.226	164
		-	-		9.3	0.489	34.2
		-	-		10.3	0.542	25.3
		-	-		10.9	0.574	20.0
BL2	e_1	4.1	0.229	157	4.3	0.226	164
					10.3	0.542	25.3
BL4	e_1	4.1	0.229	157	4.3	0.226	164
	e_2	9.3	0.519	23	-	-	-
BP2	e_1	4.1	0.229	157	4.3	0.226	164

TD = Distance of dye migration (cm), e = esterase band, D = Distance of esterase band migration, R_f = relative mobility, M.W = Molecular weight (kilodaltons).

Plate 12: Proteinase bands of B. subtilis BS2 at different times of sampling in polyacrylamide gel.

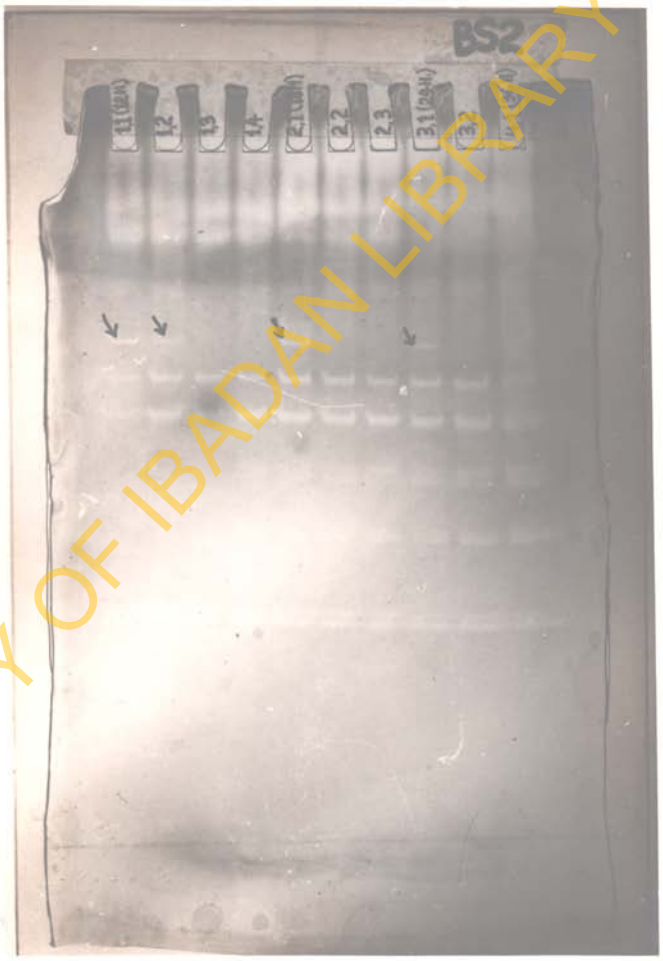


Plate 13: Proteinase bands of B. pumilus BP2 at different times of sampling in polyacrylamide gel.

proteinase band (arrowed) was observed to be present in samples freshly taken, but not in samples which were further incubated sterile. Other proteinase bands were consistent throughout the time of incubation.

The effect of buffers on proteinase bands pattern and proteinase activity was tested with strain BS2. Similar proteinase bands were obtained in media A, B and C containing different buffers (Plate 14). The pH and proteolytic activity detected in each medium are stated in Table 7. Highest proteolytic activity (476.1UC/ml) was observed in flask C which contained higher nutrient broth concentration, and the least activity (109.7UC/ml) in flask B which contained 20mM of the alkaline salt Tris. High pH values affected the proteolytic activity of this strain. A single esterase band was observed for each sample, except for medium B which had very low-intensity esterase band. Thus buffers did not affect proteinase bands pattern, but medium with high pH led to reduction in proteolytic activity of strain BS2.

(i) Purification and characterization of proteinases

(i) Ammonium sulphate ($(NH_4)_2SO_4$) precipitation

Figure 21 shows the proteinase activities of proteins from crude enzyme solution precipitation with ammonium sulphate at different saturation levels. Most of the proteinases were precipitated out between 55 - 70% $(NH_4)_2SO_4$ - saturation. There was significant loss in activity during the salting out process.

Plate 14 : Effect of buffers on proteinase bands pattern of strain BS2.

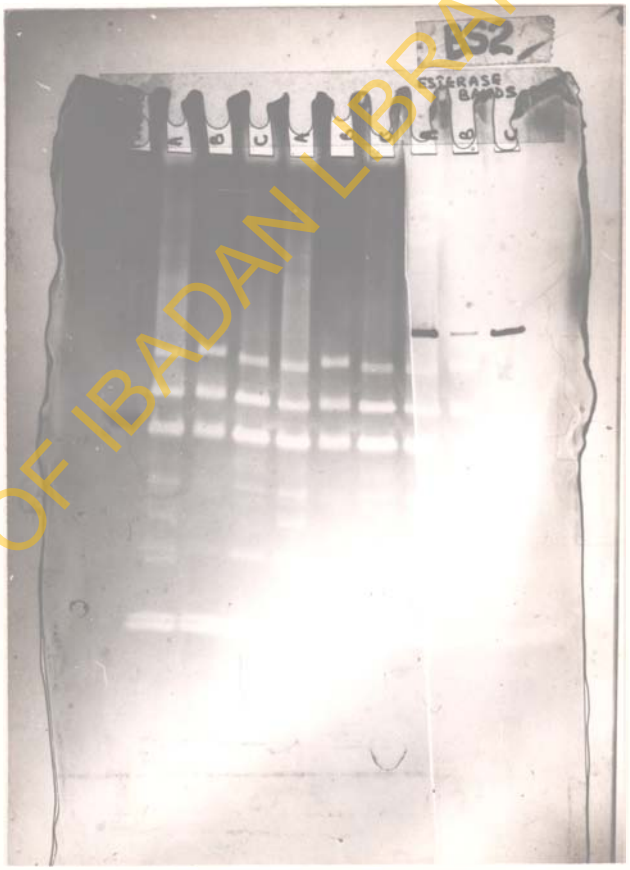


Table 7: Effect of buffers on pH and proteolytic activity in media.

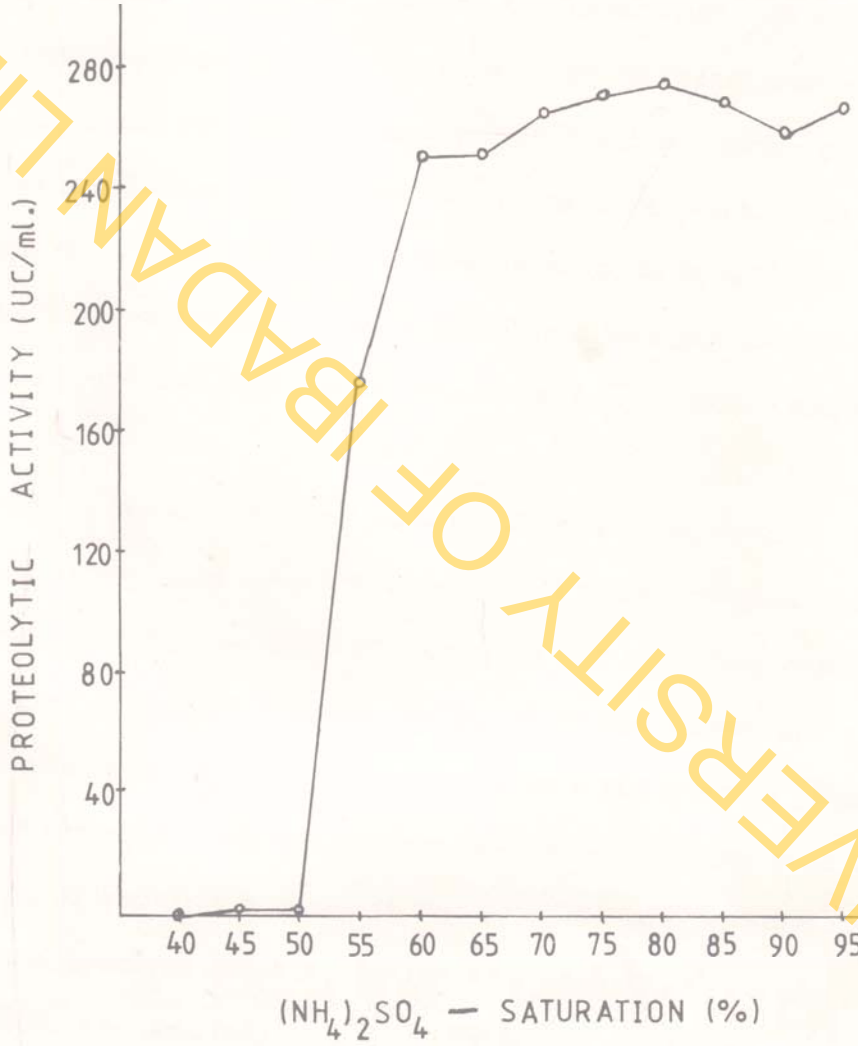
Buffer	A	B	C
pH	8.21	8.9	8.49
Proteolytic activity (UC/ml)	238.6	109.7	476.1

A: Medium contains 0.2% NB + 20mM phosphate buffer pH 7.0

B: Medium contains 0.2% NH + 20mM Tris

C: Medium contains 0.8% nutrient broth only

Fig. 21 : Ammonium sulphate precipitation of proteinases at different levels of saturation.



The proteolytic activity in various fractions of $(NH_4)_2SO_4$ - precipitation is shown in Table 8.

Only 58.5% of the total activity in the crude enzyme could be detected in precipitates and supernatants after $(NH_4)_2SO_4$ precipitation. Though some of the proteinases were precipitated between 50-70% saturation, a substantial percentage (41.5%) of total activity was lost.

(ii) Ion-Exchange Chromatography (IEC)

Figure 22 shows the chromatogram of the proteinases of strain BS2 after IEC on DEAE Sephadex A-50 (1.5 x 20cm). Three proteinase activity peaks: peaks I, II and III were obtained. The first and major peak (peak I) was eluted before application of the salt gradient. The other two peaks (peak II & peak III) were eluted at about 0.2M NaCl and 0.4M - 0.5M NaCl respectively. Peak I was eluted in fractions 17-29, Peak II in fractions 49-57 and Peak III in fractions 75-91. These peaks were pooled separately.

Three peaks were also obtained when a column with different dimensions 1 x 47cm (Fig. 23) was used. Peak I constituted the major peak again, and was eluted before the NaCl elution gradient was applied. The three peaks I, II and III were eluted in fraction 13-36, 48-80 and 86-100 and pooled respectively. The third proteolytic activity peak had a brown pigment. The proteolytic activity, protein content and specific activity of the peaks in Figures 22 and 23 are shown in Table 9. In both IEC runs; Peak I had the highest specific activity followed by peak II, while peak III had the least specific activity.

Table 8: Proteolytic activity in various fractions of $(NH_4)_2SO_4$ - precipitated enzymes of strain BS2.

Fraction	Volume (ml)	Proteolytic activity (UC/ml)	Total proteolytic activity UC
Crude enzyme filtrate	70	238.6	16702
Precipitate from saturation	7	81.9	573.3
Dialysed 70% - saturated supernatant	85	54.6	4641
Precipitate from 70% saturation	15	304	4560

Fig. 22: Chromatography of $(NH_4)_2SO_4$ - precipitated enzyme (5ml) of strain BS2 on DEAE Sephadex A-50 (1.5 x 20cm column). Flow rate: 15ml/h, fractions of 1.5ml. ●, protein absorbance at 280nm; x, proteolytic activity.

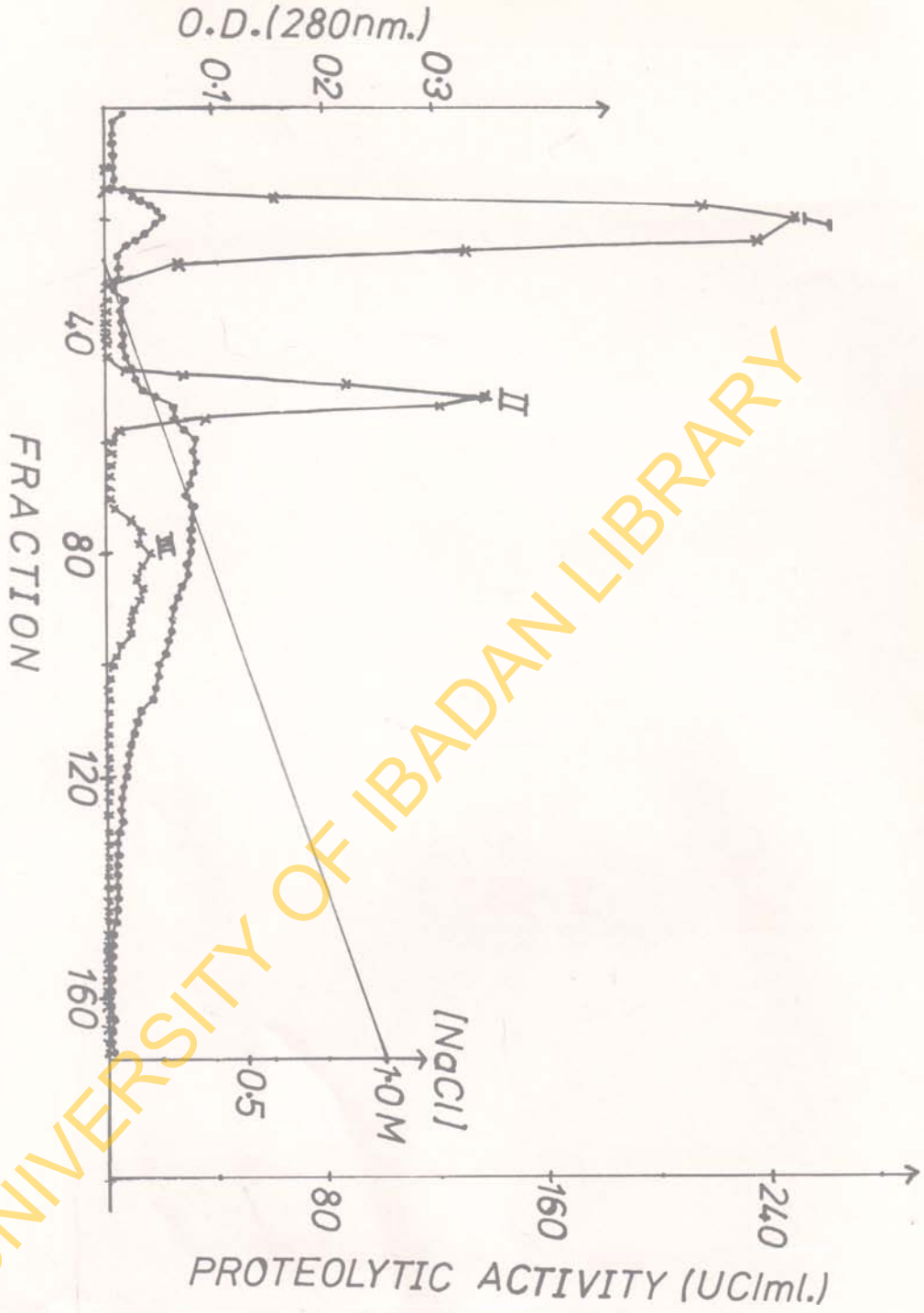


Fig.23 : Chromatography of $(NH_4)_2SO_4$ - precipitated enzyme (20ml) of strain BS2 on DEAE Sephadex A-50 (1 x 47cm column). Flow rate: 10ml/h, fractions of 3.5ml. ●, protein absorbance at 280nm; x, proteolytic activity; o, esterolytic activity.

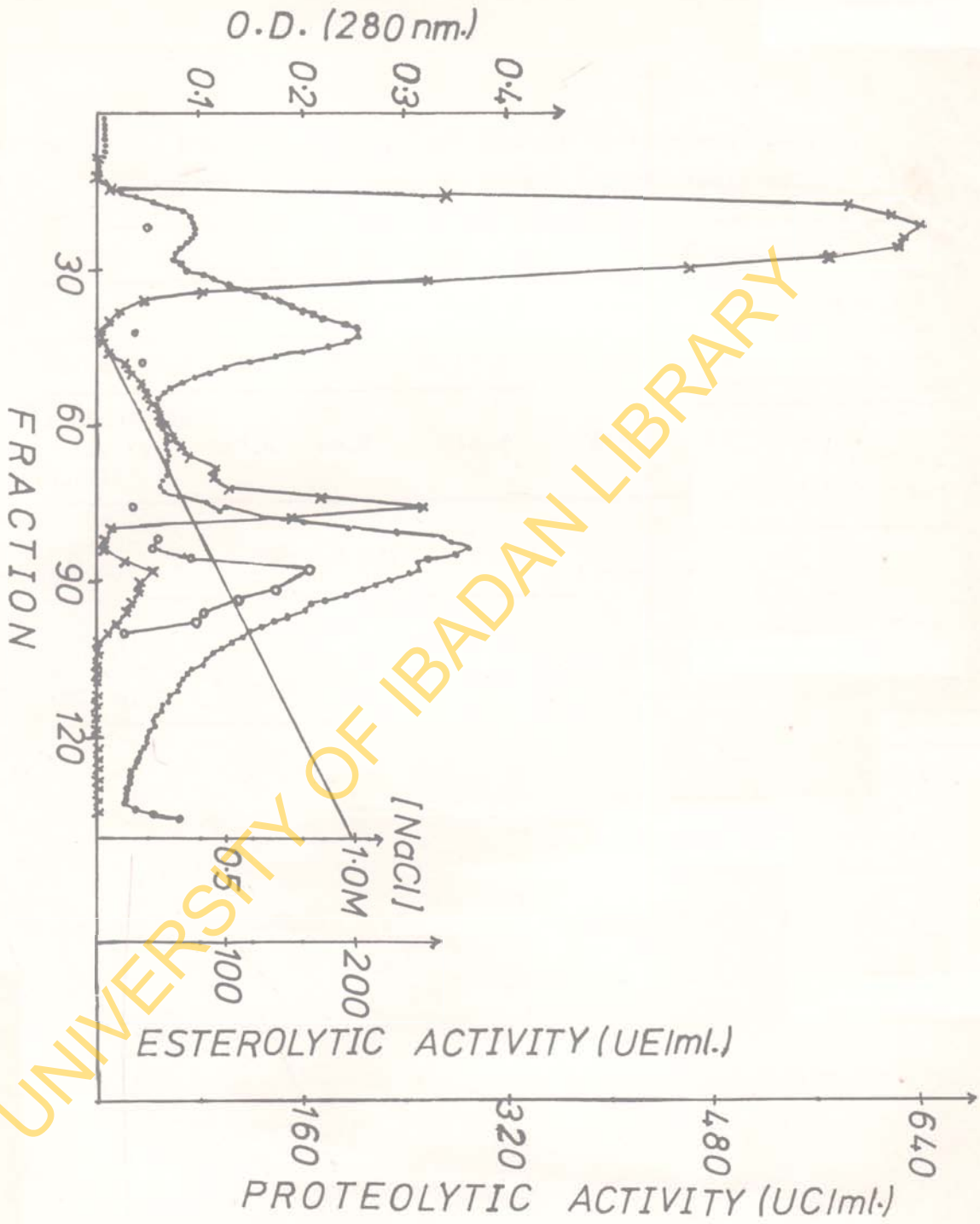


Table 9: Proteolytic activity, protein content and specific activity of peaks I, II and III of strain BS2

	Figure 24			Figure 25		
	Peak I	Peak II	Peak III	Peak I	Peak II	Peak III
Volume (ml)	11	9	17.5	30	54	45
Proteolytic activity (UC/ml)	217.6	90.4	14.4	608	93.2	24.2
Protein (mg/ml)	0.038	0.031	0.012	0.068	0.029	0.059
Specific activity (UC/mg)	5726.3	2916	1200	8941.2	3213.8	410.2

The chromatogram of IEC of strain BL2 proteinases on DEAE Sephadex A-50 (1.5 x 20cm) is shown in Figure 24. Three peaks were eluted in fractions 14 - 32, 38 - 62 and 102 - 112 respectively. Proteinase activity, protein content and specific activity of the pooled fractions of enzyme activity peaks are shown in Table 10. Chromatography of the precipitated enzymes of BL2 on anion exchanger column dimensions 1 x 47cm also yielded three proteolytic activity peaks.

The $(NH_4)_2SO_4$ -precipitated enzymes of BP2 was applied on DEAE Sephadex A-25 column and DEAE Sephadex A-50. The first and major peak was eluted before application of the NaCl salt gradient on DEAE Sephadex A-25 (Figure 25). The second enzyme activity peak was eluted at about 0.3 molar NaCl. The elution profile was different on DEAE Sephadex A-50 (Figure 26). The first proteolytic activity peak was minor while the main proteolytic activity peak was eluted later in fractions 36 - 46. Thus apparently, only two proteolytic activity peaks were detectable in IEC runs of BP2 enzymes. Proteolytic activity, protein content and specific activity of the major peak in each run is as shown in Table 11.

When the binding capacity of the cation exchanger CM 52 cellulose was tested with $(NH_4)_2SO_4$ - precipitated enzyme of strain BP2, most of the enzyme did not bind to the cation exchanger. The proteolytic activity before and after adding enzyme to the

Fig. 24 : Chromatography of $(NH_4)_2SO_4$ - precipitated enzyme (10ml) of strain BL2 on DEAE Sephadex A-50 (1.5 x 20cm column). Flow rate: 15ml/h, fractions of 2ml. ●, protein absorbance at 280nm; x, proteolytic activity.

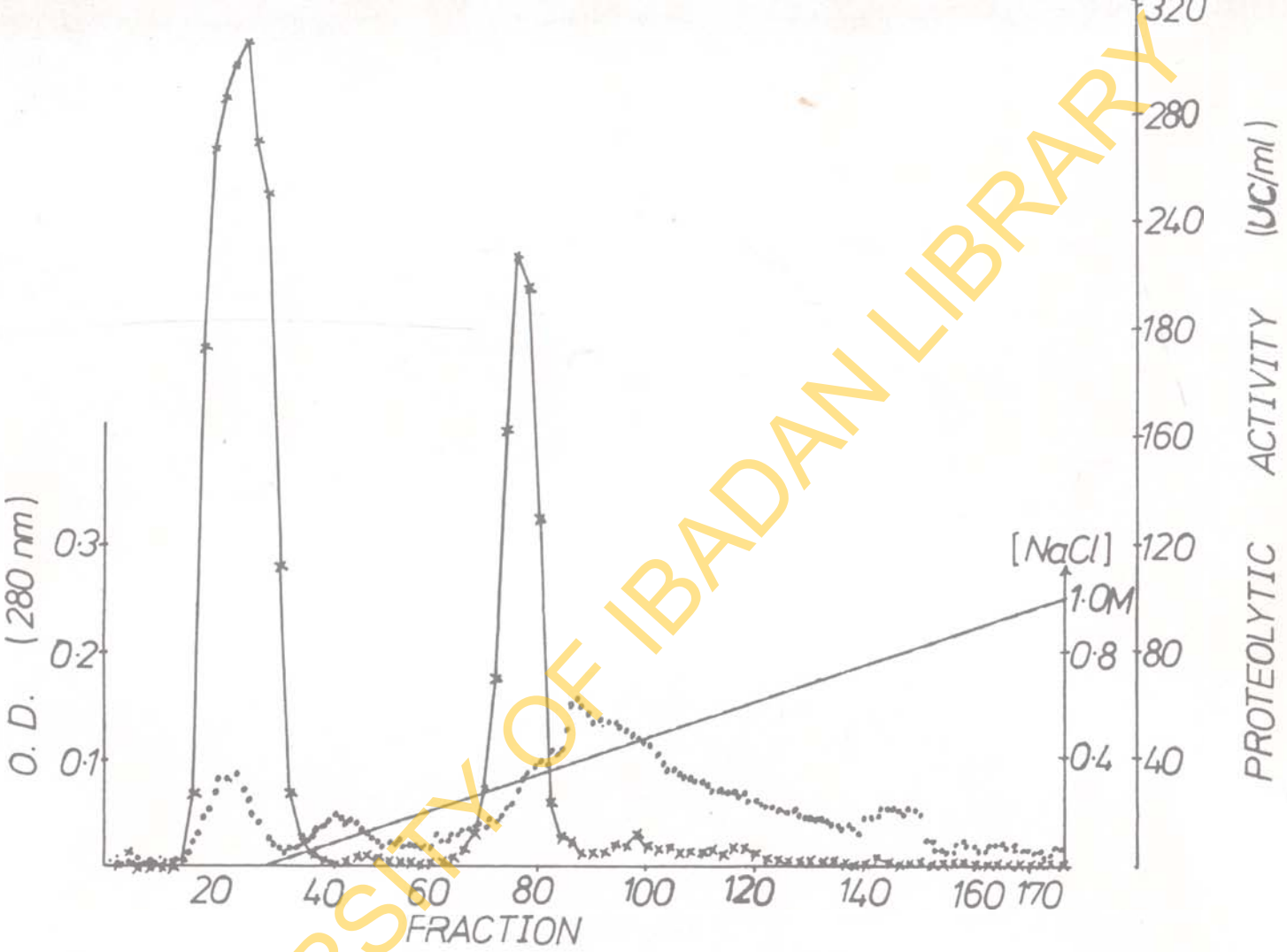


Table 10: Proteolytic activity, protein content and specific activity of peaks I, II and III of strain BL2.

	Peak I	Peak II	Peak III
Volume (ml)	20	16	31
Proteolytic activity (uC/ml)	252.8	176.2	7
Protein (mg/ml)	0.0587	0.0428	0.035
Specific activity (UC/mg)	4306.6	4116.8	198.3

Fig. 25 : Chromatography of $(NH_4)_2SO_4$ - precipitated enzyme (5ml) of strain BP2 on DEAE Sephadex A-25 (1.5 x 10cm column). Flow rate: 15ml/h, fractions of 2ml. ●, protein absorbance at 280nm; x, proteolytic activity.

Fig. 26 : Chromatography of $(NH_4)_2SO_4$ - precipitated enzyme (4ml) of strain BP2 on DEAE Sephadex A-50 (1.5 x 20cm column). Flow rate: 10ml/h fractions of 2ml. ●, protein absorbance at 280nm; x, proteolytic activity.

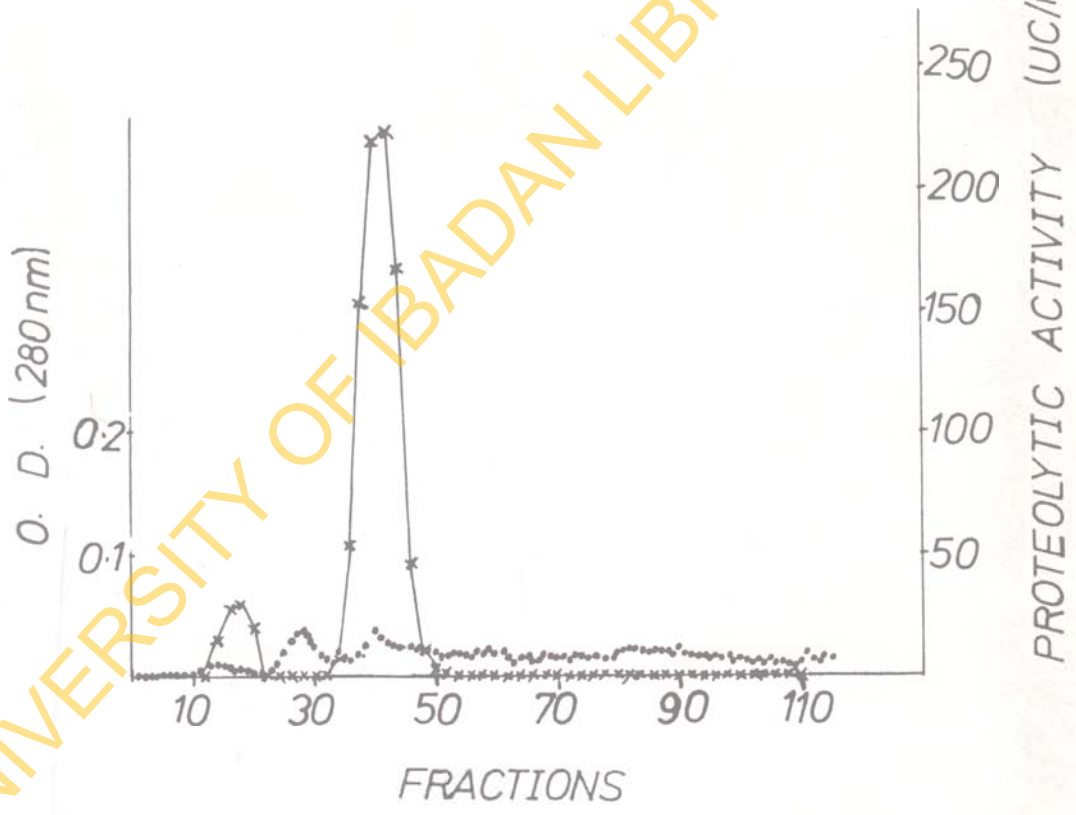


Table 11: Proteolytic activity, protein content and specific activity of main enzyme peaks of strain BP2.

	Figure 27	Figure 28
	Peak I	Peak II
Volume (ml)	9	19
Proteolytic activity (UC/ml)	287.6	227.6
Protein (mg/ml)	0.0495	0.01
Specific activity (UC/mg)	5810.1	22,760

cation exchanger were 444.5UC/ml add 399UC/ml respectively. Less than 10% of total enzyme was bound to the column.

(iii) Gel filtration and Inhibition studies.

Chromatography of strain BS2 peak I gave a symmetrical protein peak and a corresponding proteinase activity peak (Figure 22) on Sephadex G-75. Inhibition tests of single fractions confirmed homogeneity of the peak. Fractions 25 - 33 were pooled. The effects of inhibitors on proteolytic activity of the pooled peak I are shown in Table 12. There was complete inhibition of enzyme activity in the presence of 5mM PMSF which strongly indicated that peak I was a serine proteinase.

Much extraneous proteins were eliminated during chromatography of peak II on Sephadex G-75 (Figure 28). A single peak of proteinase activity was obtained which was confirmed to be homogenous by inhibition tests of single fractions. The effect of inhibitors on proteolytic activity of pooled peak II is also shown in Table 12. Peak II was strongly inhibited by EDTA but partially by PMSF, indicating that it was a neutral proteinase. Figure 31 shows the chromatogram of gel filtration of peak III on Sephadex G-75. Some extraneous proteins were eluted along with the enzyme as there was an overlap between the protein peak and the proteolytic activity peak. Peak III had a corresponding esterolytic activity peak. This peak was identified as an esterase with low proteolytic activity. Fractions 21 - 32 were pooled which had proteolytic and esterolytic activities of 52.5UC/ml and 130UE/ml respectively.

Fig. 27: Gel filtration chromatography of strain BS2 proteolytic activity peak I on Sephadex G-75 (1 x 97cm). Flow rate: 10ml/h fractions, 1.6ml. o, protein absorbance at 280nm; x, proteolytic activity.

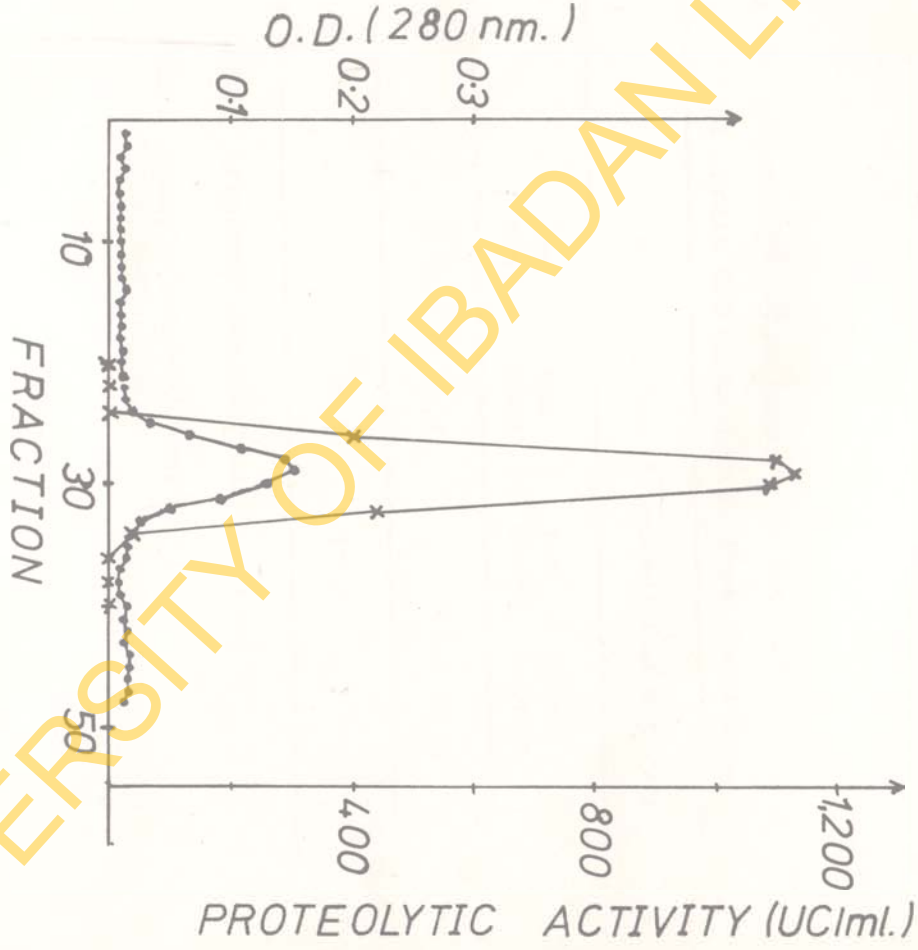


Table 12: Effects of inhibitors on proteolytic activity of strain BS2 proteinases, Peak I and Peak II

	% inhibition of proteolytic activity			
	2mM EDTA	5mM EDTA	1mM PMSF	5mM PMSF
Peak I	0.1	19.3	-	99.9
Peak II	-	99.1	18.3	-

Key: EDTA: Ethylenediaminetetraacetic acid

PMSF: Phenylmethanesulpnonyl fluoride

Fig. 28: Gel filtration chromatography of strain BS2 proteolytic activity peak II on Sephadex G-75 (1 x 97cm). Flow rate: 12.8ml/h, fractions, 1.6ml. ●, protein absorbance at 280nm; o, proteolytic activity.

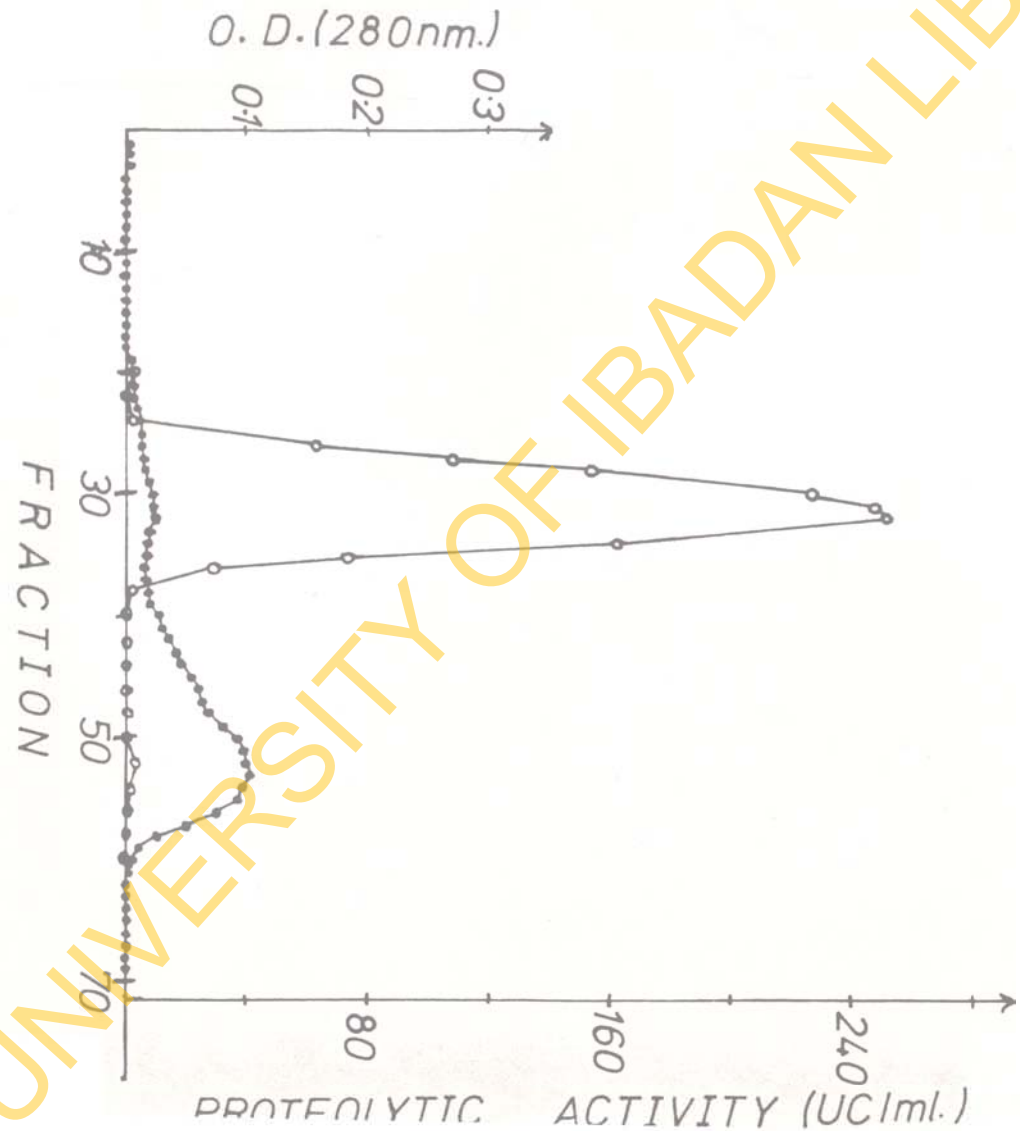
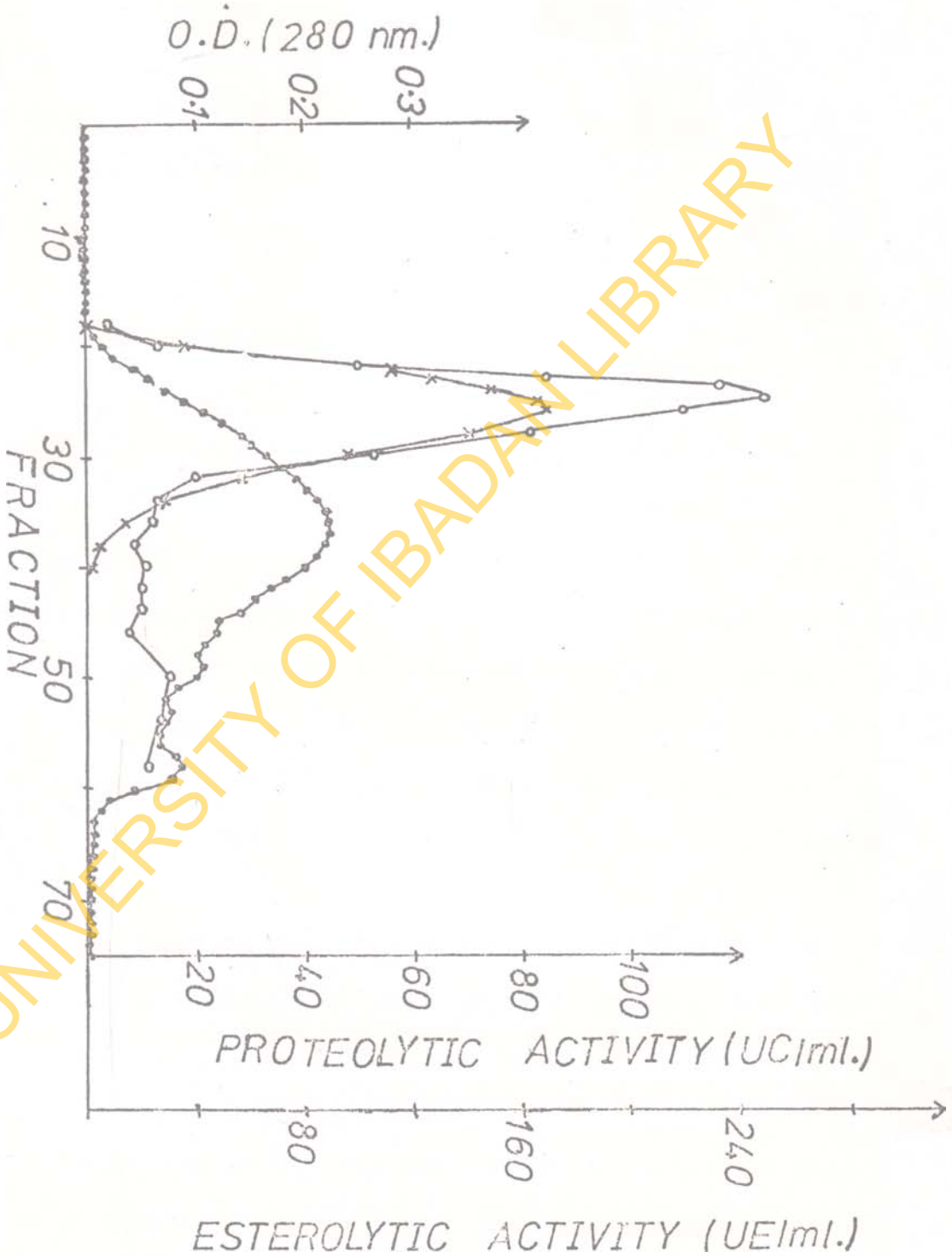


Fig. 29: Gel filtration chromatography of strain BS2 proteolytic activity peak III on Sephadex G-75 (1 x 97cm). Flow rate: 12ml/h, fractions, 1.6ml. ●, protein absorbance at 280nm; x, proteolytic activity; o, esterolytic activity.



When peaks II and III were rechromatographed on Sephadex G-50, the chromatograms in Figures 30 and 31 were obtained respectively. A single symmetrical peak of proteolytic activity was obtained in peak II. Homogeneity was confirmed by the sensitivity of individual fractions to PMSF and EDTA. Fractions 32 -35 were pooled, and inhibition tests confirmed peak II was a neutral/ metalloproteinase. A single peak of proteolytic/esterolytic activity was obtained in peak III. Fractions 27 - 32 were pooled. Its proteolytic and esterolytic activities were found to be 57.5UC/ml and 214UE/ml respectively. Thus BS2 peak III was confirmed as an esterase with low proteolytic activity.

Figure 32 shows the chromatogram of BL2 peak I (from DEAE Sephadex A-50) on Sephadex G-75. Two protein peaks were obtained. The first protein peak had a corresponding proteolytic activity peak. Fractions 29 - 38 were pooled. When the pooled peak was rechromatographed on Sephadex G-50, the chromatogram in Figure 33 was obtained. A single protein peak and corresponding proteolytic activity peak was obtained. Fractions 34 - 40 were pooled. Effects of inhibitors on the proteolytic activity of the peak are shown in Table 13. The enzyme was strongly inhibited by PMSF while EDTA had only little effect on its activity. This indicated that BL2 peak I was a serine enzyme.

The gel filtration chromatogram of BL2 peak II on Sephadex G-50 is shown in Figure 34. Inhibition tests on single fractions

Fig. 30 : Gel filtration chromatography of strain BS2 proteolytic activity peak II on Sephadex G-50 (1 x 93cm). Flow rate: 6.6ml/h, fractions, 1ml. ●, protein absorbance at 280nm; o, proteolytic activity.

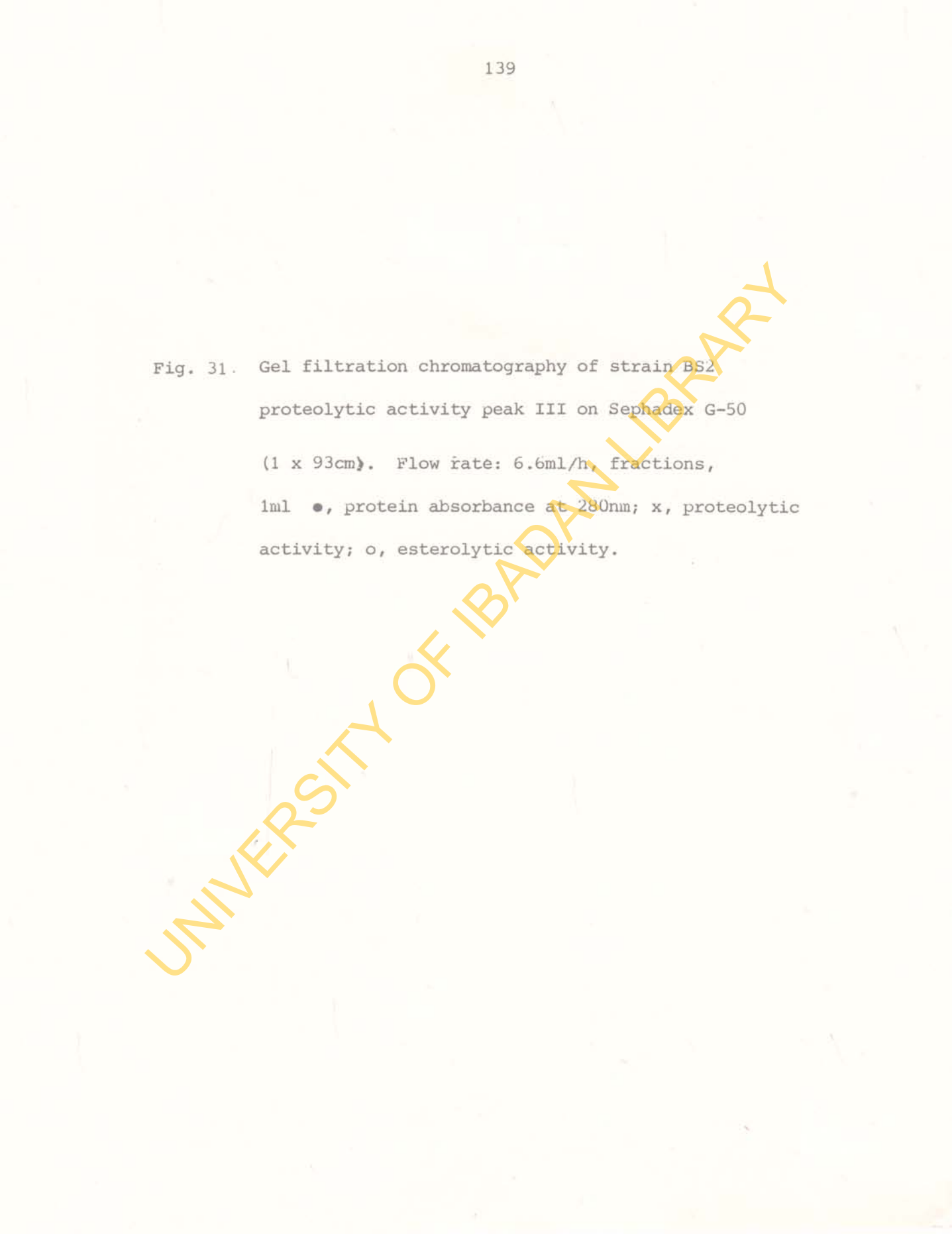


Fig. 31. Gel filtration chromatography of strain BS2 proteolytic activity peak III on Sephadex G-50 (1 x 93cm). Flow rate: 6.6ml/h, fractions, 1ml ●, protein absorbance at 280nm; x, proteolytic activity; o, esterolytic activity.

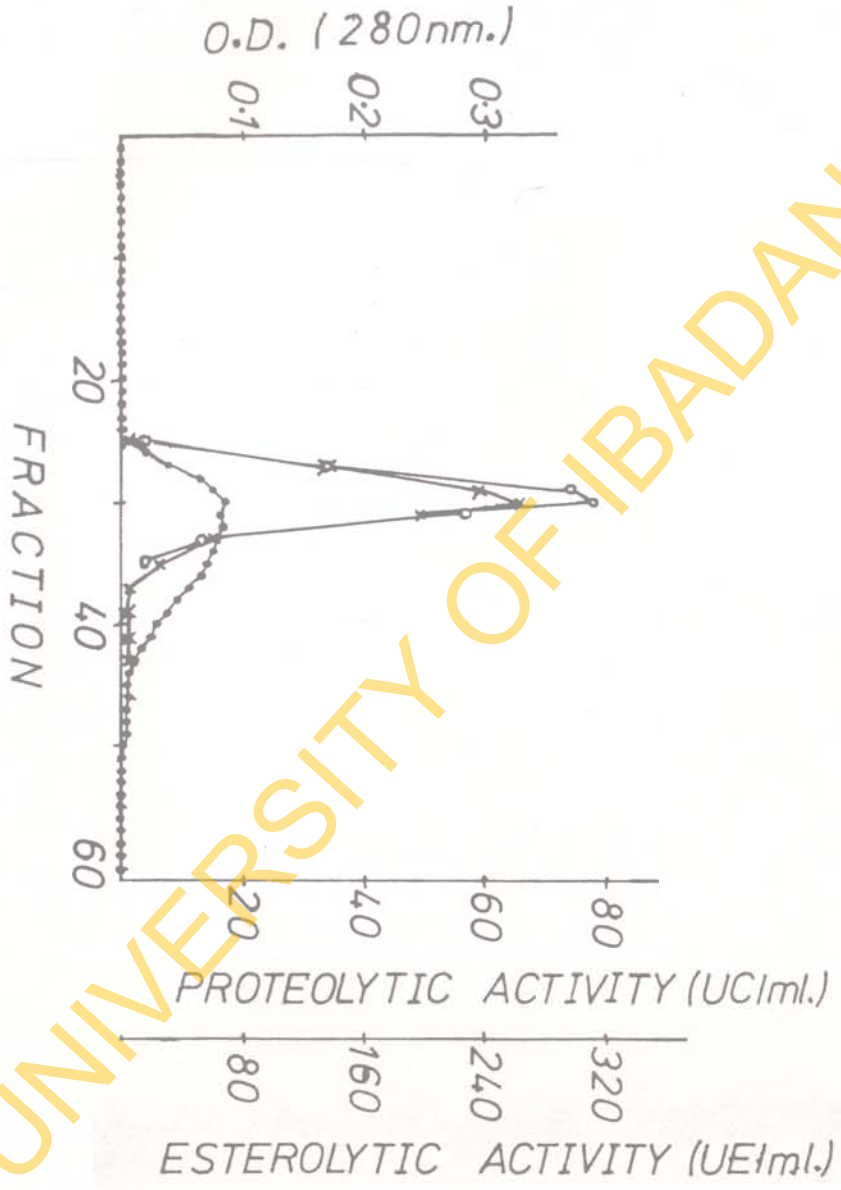


Fig. 32. Gel filtration chromatography of strain BL2 proteolytic activity peak I on Sephadex G-75 (1 x 97cm). Flow rate: 12ml/h, fractions, 1.6ml ●, protein absorbance at 280nm; o, proteolytic activity.

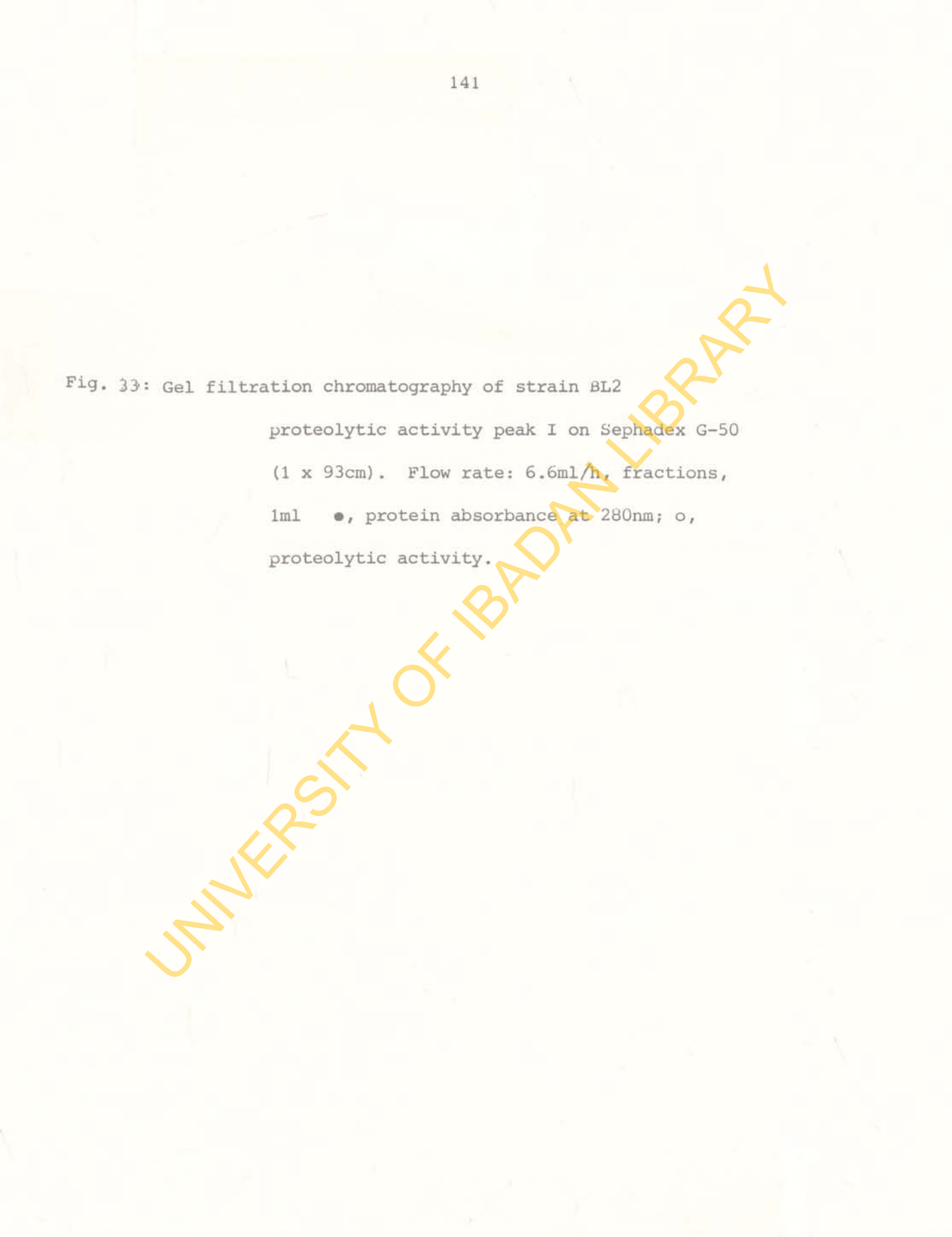


Fig. 33: Gel filtration chromatography of strain BL2 proteolytic activity peak I on Sephadex G-50 (1 x 93cm). Flow rate: 6.6ml/h, fractions, 1ml ●, protein absorbance at 280nm; o, proteolytic activity.

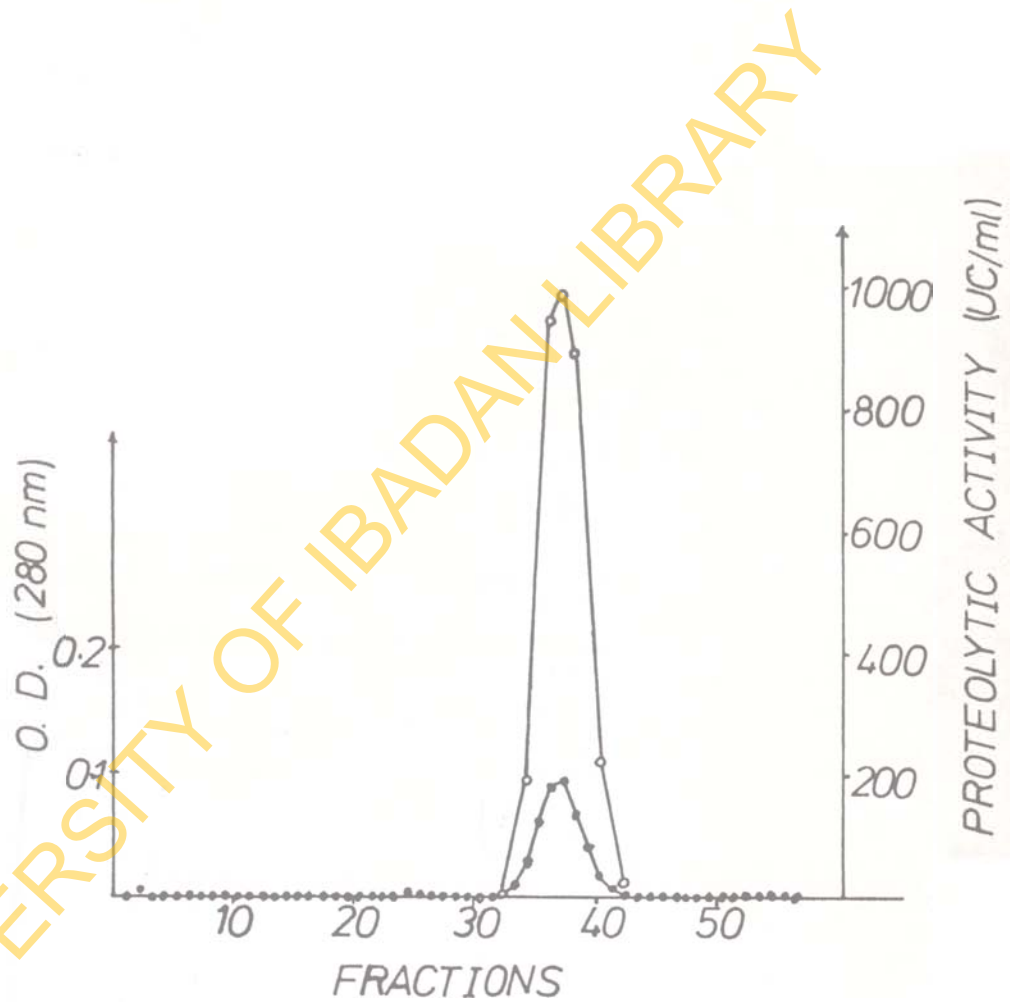


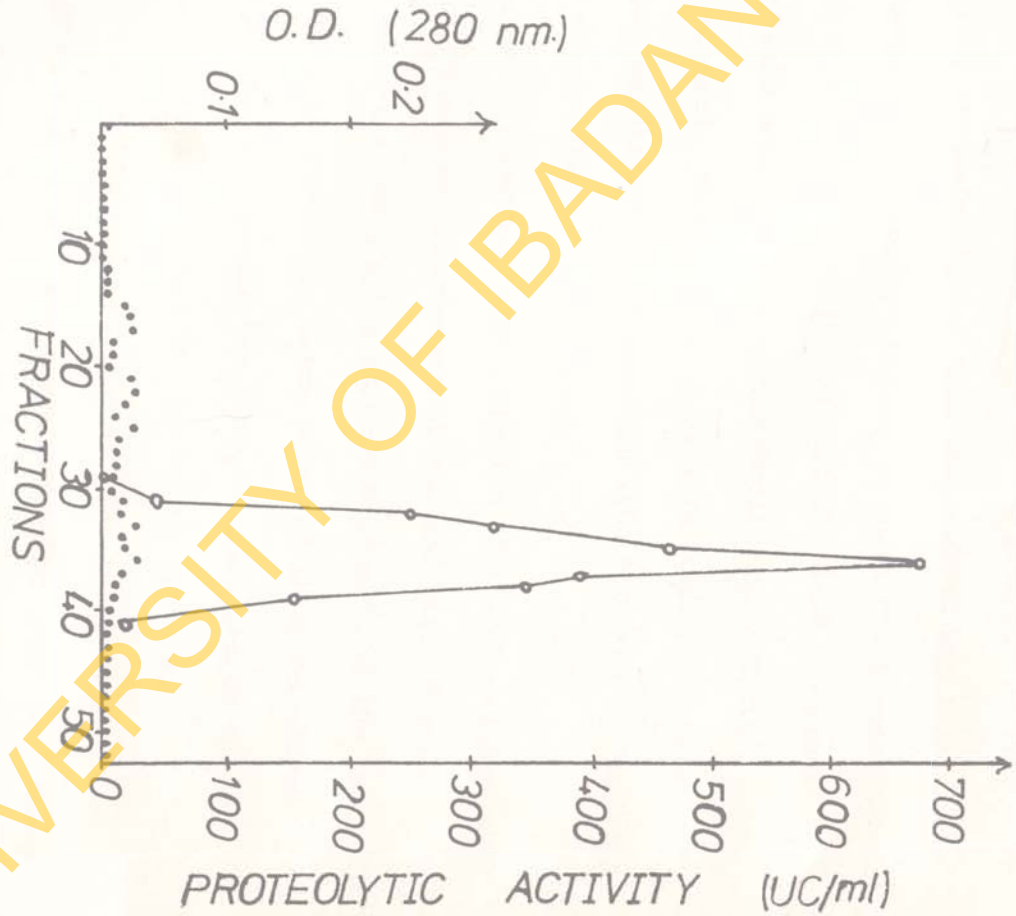
Table 13: Effects of inhibitors on proteolytic activity of strain BL2 proteinases, Peak I and Peak II.

	% inhibition of proteolytic activity			
	2mM EDTA	5mM EDTA	1mM PMSF	5mM PMSF
Peak I	13.6	-	98.5	97.1
Peak II	-	100	3.7	-

Key:- EDTA: Ethylenediaminetetraacetic acid

PMSF: Phenylmethanesulphonyl fluoride

Fig. 34 : Gel filtration chromatography of strain BL2 proteolytic activity peak II on Sephadex G-50 (1 x 93cm). Flow rate: 6.6ml/h, fractions, 1ml. ●, protein absorbance at 280nm; o, proteolytic activity.



confirmed homogeneity of the peak. Fractions 32 - 39 were pooled. The effects of the inhibitors EDTA and PMSF on proteolytic activity of the enzyme are as shown in Table 13. Enzyme activity was completely inhibited by 5mM EDTA while in the presence of PMSF, 96.3% of activity was detectable. Thus BL2 peak II was identified as a neutral/metallo proteinase. Figure 35 shows the chromatogram of BL2 peak III after gel filtration on Sephadex G-50. A single peak of proteolytic activity with corresponding esterolytic activity was obtained. Fractions 27 - 32 were pooled. Strain BL2 peak III was identified as an esterase with proteolytic activity.

(iv) PAGE of enzymes during purification

Figure 36 is a schematic representation of the proteinase and esterase bands patterns of proteinases of BS2 and BL2. Similar proteinase and esterase bands patterns were obtained for the two strains. The multiple proteinase bands observed in crude enzyme samples were also observed in the $(NH_4)_2SO_4$ - precipitated enzymes, both having a large proteinase band at the top of the gel. Separation of the proteinases by IEC showed that the serine proteinase (peak I) was largely responsible for the large proteinase band at the top of the gel (Plate 15). The direction of migration of neutral proteinase (peak II) could not be ascertained, while peak III had multiple proteinase bands (Plate 15), which also showed corresponding esterase bands. In addition there was a major esterase band e_1 which was also found in crude enzyme runs,

Fig. 35: Gel filtration chromatography of strain BL2 proteolytic activity peak III on Sephadex G-50 (1 x 93cm). Flow rate: 6.6ml/h, fractions, 1ml. ●, protein absorbance at 280nm; x, proteolytic activity; o, esterolytic activity.

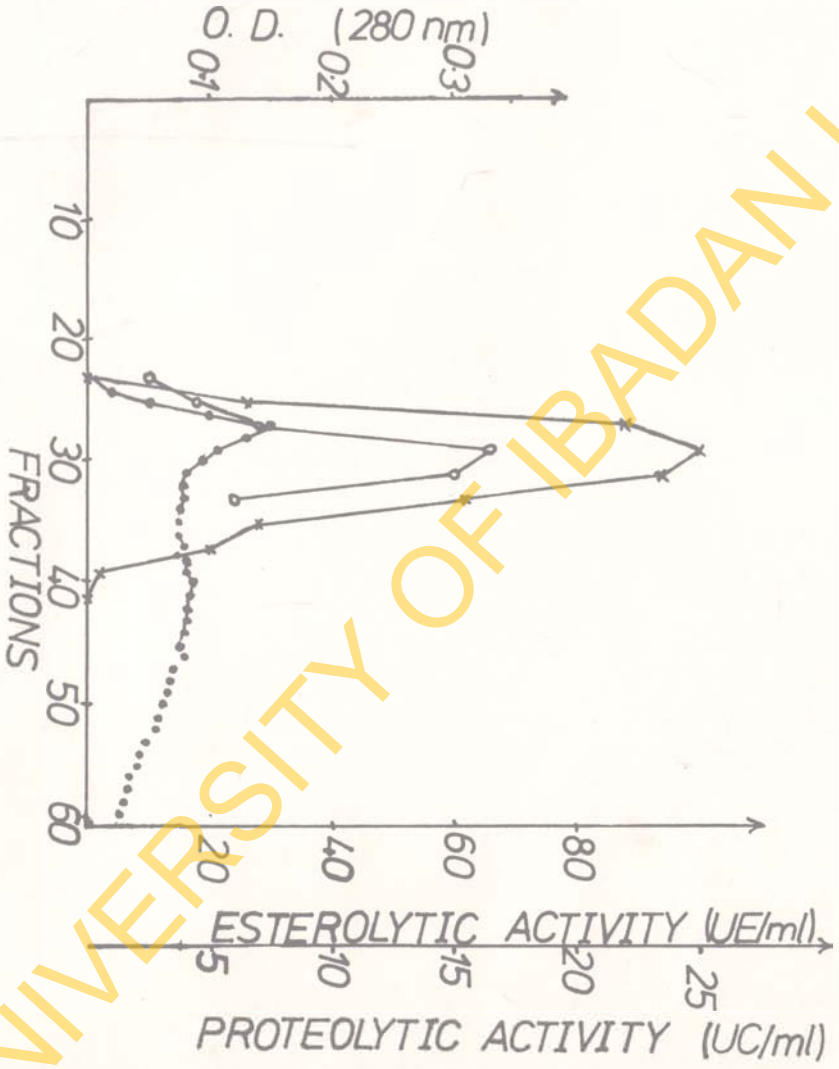


Fig. 36: Schemmatic representation of proteinase and esterase bands on polyacrylamide gradient slab gels 1: proteinase bands of crude enzyme,

2: proteinase bands after $(NH_4)_2SO_4$ precipitation,

3: proteinase band of peak I after gel filtration,

4: proteinase band of peak II after gel filtration,

5: proteinase band of peak III after gel filtration,

6: esterase bands of peak III,

p: proteinase band, e: esterase band. NB: No esterase bands were observed in peaks I and II.

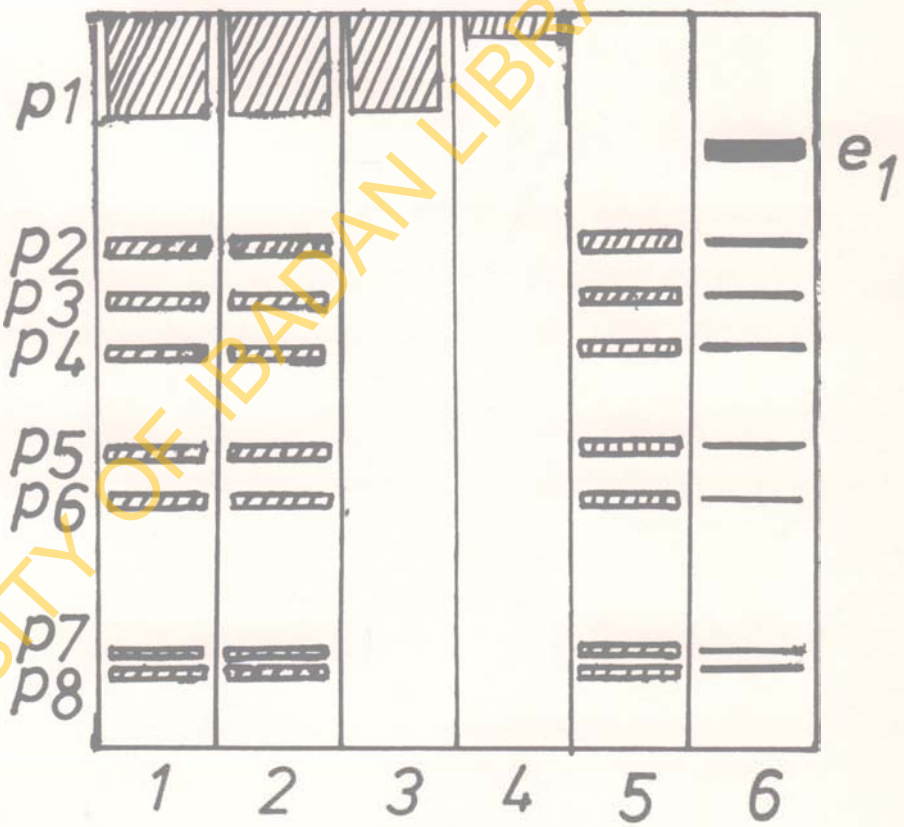
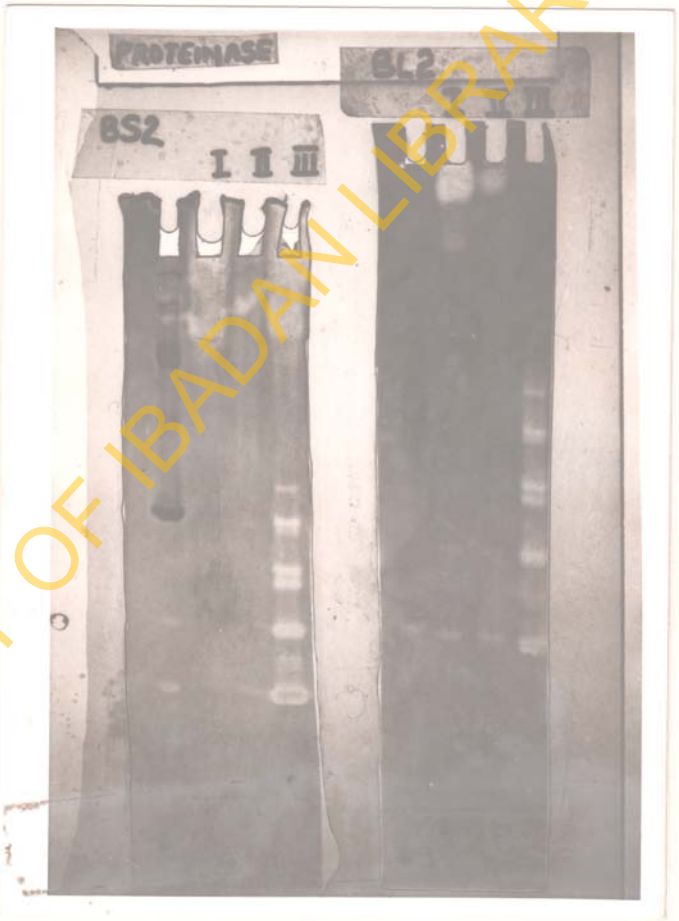


Plate 15: Proteinase bands of proteolytic activity peaks I, II, and III of the strains BS2 and BL2.



that had no corresponding proteinase band. This showed that e_1 is an esterase eluted along with peak III (Plate 16) which had esterolytic activity on α-naphthyl acetate but had no proteolytic activity on casein.

(v) Hydrophobic Interaction Chromatography (HIC)

After peak I was bound to the HIC column (Phenyl Sepharose CL-4B), all attempts to elute the enzyme failed. Elution with buffer containing negative salt gradient of 2 - 0M NaCl coupled with positive gradient solution of 0 to 0.1% Triton X-100 failed. Also attempt to have the enzyme eluted with 10% methanol, then 20% methanol failed. Enzyme activity was detected after the column was unpacked and washed with 0.2% Triton X-100, confirming that the enzyme was actively bound to the column.

(vi) Molecular weights of proteinases (by gel filtration)

Figure 37 shows the chromatogram of gel filtration of the standard proteins BSA, CTA and Cyt, C on Sephadex G-75. The BSA peak was eluted in the void volume (along with dextran blue 2000). The elution volume (V_e) and other parameters of the standard proteins CTA and Cyt, C were calculated thus:

V_o = 30.4ml

V_t = 76.1ml

V_x = 45.7ml

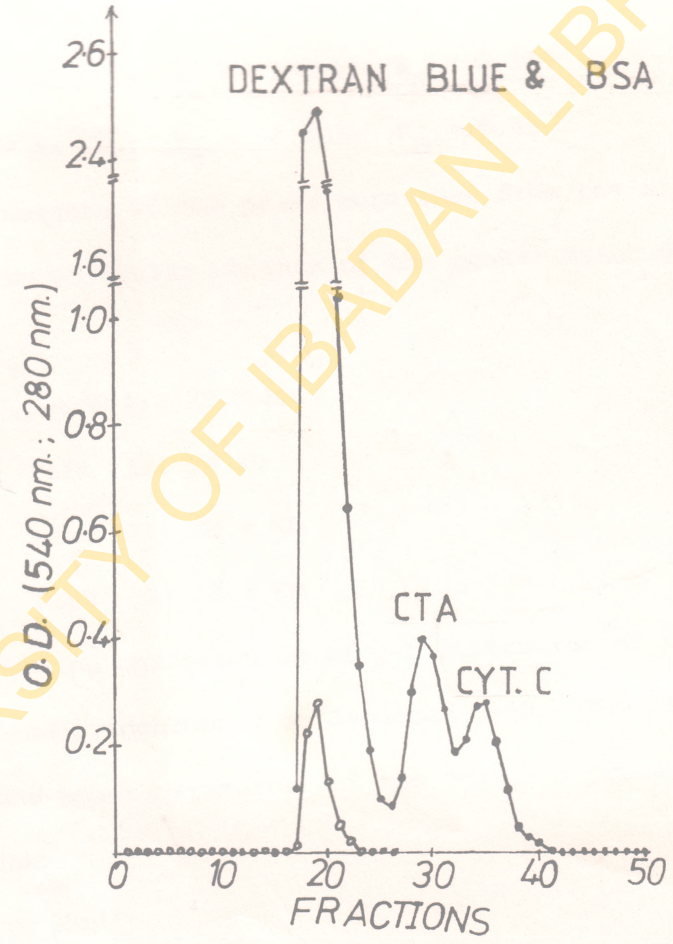
V_e (CTA) = 48ml

V_e (Cyt.C) = 56ml

$$K_d \text{ (CTA)} = \frac{V_e - V_o}{V_x} = \frac{48 - 30.4}{45.7} = 0.385$$

Plate 16 : Esterase bands of proteolytic activity peaks I, II and III of the strains BS2 and BL2.

Fig. 37: Gel filtration chromatography of standard proteins on Sephadex G-75 o, absorbance at 540nm; ●, protein absorbance at 280nm.



$$K_d \text{ (Cyt.C)} = \frac{V_e - V_o}{V_x} = \frac{56 - 30.4}{45.7} = 0.56$$

The standard curve of K_d versus molecular weight of the standard proteins was plotted. The V_e and K_d values for the different proteinases chromatographed on Sephadex G-75 are as follows:

BS2 Peak I: V_e = 46.4ml; K_d = 0.35

Peak II: V_e = 49.6ml; K_d = 0.42

Peak III: V_e = 41.6ml; K_d = 0.245

BL2 Peak I : V_e = 52.8ml; K_d = 0.49

The molecular weights of the peaks were read from the standard curve. Thus the molecular weights of the peaks (using Sephadex G-75) were:

BS2 Peak I: 29.8 KD

Peak II: 24 KD

Peak III: 38.4 KD

BL2 Peak I: 18.2 KD

Figure 38 shows the chromatogram of gel filtration of the standard proteins CTA and cytochrome C on Sephadex G-50. The elution volume (V_e) and other parameters of the standard proteins were calculated thus:

V_o = 25ml

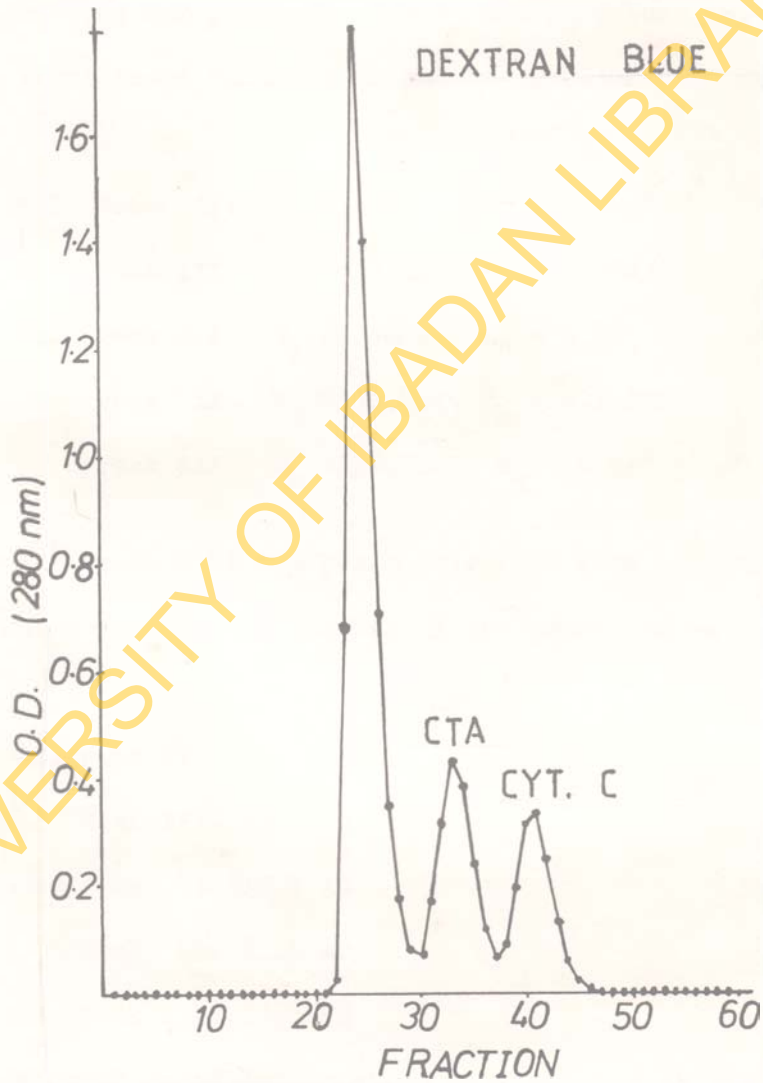
V_t = 75ml

V_x = 48ml

V_e (CTA) = 33.25ml

V_e (Cyt.C) = 40.75ml

Fig. 38: Gel filtration chromatography of standard proteins on Sephadex G-50.



$$K_d \text{ (CTA)} = \frac{V_e - V_o}{V_x} = \frac{33.25 - 25}{48} = 0.171875$$

$$K_d \text{ (Cyt. C)} = \frac{V_e - V_o}{V_x} = \frac{40.75 - 25}{48} = 0.328$$

The standard curve of K_d versus molecular weight of the standard proteins was plotted. The V_e and K_d values for the different proteinases chromatographed on Sephadex G-50 are as follows:

BS2 Peak II: V_e = 33ml; K_d = 0.1667

Peak III: V_e = 29.5ml; K_d = 0.094

BL2 Peak I: V_e = 37ml; K_d = 0.25

Peak II: V_e = 35.5ml; K_d = 0.21875

Peak III: V_e = 29.75ml; K_d = 0.99

The molecular weights of the peaks were read from the standard curve. Thus the molecular weights of the peaks (using Sephadex G-50) were:

BS2 Peak II: 27.4 KD

Peak III: 33.9 KD

BL2 Peak I: 19.7 KD

Peak II: 22.6 KD

Peak III: 33.5 KD

(vii) Summary of purification steps

Table 14 gives a summary of the purification steps of strain BS2 proteinases. The salting out process with $(NH_4)_2SO_4$ resulted

Table 14: Summary of purification steps of strain BS2 proteinases

Purification Step		Volume (ml)	Total proteolytic Activity (UC)	Total protein (mg)	Specific activity (UC/mg)	% Yield	Purification fold
Crude enzymes		250	66025	67.5	978.15	100	1
$(NH_4)_2SO_4$ - precipitation		28	15694	25.004	627.66	23.77	0.642
IEC	i	11	2393.6	0.418	5726.32	3.62	5.854
Peak I	ii	30	18240	2.04	8941.17	27.62	9.141
Peak II	i	9	813.6	0.279	2916.13	1.23	2.981
	ii	54	5032.8	1.566	3213.79	7.62	3.285
Peak III	i	17.5	252	0.21	1200.00	0.38	1.227
	ii	45	1089	2.655	410.17*	1.65	0.419
Gel Filtration (Sephadex)							
Peak I		13	10959	1.1537	9499.00	16.60	9.711
G-75 Peak II		7	1701	0.1435	11853.66	2.58	12.118
Peak III		12	630	0.498	1265.06	0.95	1.293
G-50 Peak II		2.5	265	0.02125	12470.59	0.40	12.749
Peak III		4.5	259.65	0.16425	1580.82	0.39	1.616

in a slight decrease in specific activity. Subsequent purification steps gave higher specific activity except for peak III during the second run of IEC (Figure 23). At the end of purification step, the neutral proteinase had higher specific activity than the serine proteinase while the esterase/proteinase enzyme had the least.

Table 15 is a summary of the purification steps of strain BL2 proteinases.

There was also reduction in the specific activity during $(NH_4)_2SO_4$ - precipitation of BL2 proteinases. Proteinase peak III was characterized by low specific activity, but other peaks had higher specific activities with each step of purification.

Table 15: Summary of the purification steps of strain BL2 proteinases

Purification Step		Volume (ml)	Total proteolytic Activity (UC)	Total protein (mg)	Specific activity (UC/mg)	% Yield	Purification fold
Crude enzymes		250	76750	61.875	1240.40	100	1
$(NH_4)_2SO_4$-precipitation		26	14996.8	23.53	637.35	19.54	0.514
IEC	Peak I	20	5056	1.174	4306.64	6.59	3.472
	Peak II	16	2819.2	0.6848	4116.82	3.67	3.319
	Peak III	31	217	1.0943	198.30	0.283	0.16
Gel Filtration (Sephadex) G-75	Peak I	10.5	6027	0.79275	7602.65	7.85	6.129
G-50	Peak I	6.5	4511	0.455	9914.28	5.88	7.99
	Ppeak II	4.5	2097	0.092	22793.48	2.73	18.37
	Peak III	6	207	0.4623	446.89	0.27	0.36

CHAPTER 4

DISCUSSION

The results of this study confirmed the variability among the strains of Bacilus subtilis group. Though strains belonging to the B. subtilis group have some common features (Gibson & Gordon, 1974), they differ considerably in their biochemical activities (Ottow, 1972; Fogarty et al., 1974a; Priest, 1977). There were significant differences in growth and the amounts of extracellular enzymes produced by the strains used. This variation among the strains elucidates the need to use attested culture(s) as starter cultures for fermentation of African locust bean.

The strains of B. subtilis (BS1, BS2 & BS3) differed in growth and extracellular proteinase production. A similar trend was observed with the strains of B. licheniformis (BL1, BL2 & BL4). Within each organism, growth with time was accompanied by increase in proteolytic acticity, with peak at about 36h of fermentation. Korculanin et al. (1972) also found that enzyme synthesis was associated with growth in the B. subtilis var. amyloliquefaciens.

Strains BS1 and BS3 which had the highest growth rates between 36-42h) had the least proteolytic activities. Strain BP2, which had the least growth, had the second highest proteinase production. Markkanen and Bailey (1974) reported a strain of B. subtilis K_{160} among other strains which had the highest production of extracellular enzymes, but produced very few heat stable pores in the culturing conditions. The metabolic turnovers of the seven strains in this study varied. Some of the strains could have metablized substrates primarily for cellular growth, while some others produced higher yields of extracellular enzymes. Thus 'iru' samples having increased digestibility could be obtained depending on the strains involved in the fermentation of the beans.

The strains BS2 and BP2 had optimum growth between pH 7.0 - 7.5 while strain BL2 and BP2 had optimum pH between pH 7.4 - 9. Strain BS2 was more tolerant of alkaline pH than strain BP2 while the latter tolerated acidic pH. This variability in sensitivity to pH of medium by the strains is important with reference to the fermentation of the beans. The pH of fermenting beans increased from about 6.04 to 8.26 when fermented for 36h (Odunfa & Adewuyi, 1985a). Thus strain BP2 will be useful in initiating the fermentation. As the pH increases with time, strains BS2 and BL2 will be useful at the later stages of fermentation process.

Comparison of the best three strains (on the basis of proteinase production) showed further variation in the production of other extracellular enzymes. The amounts of amylase and polygalacturonase (PG) produced by strain BS2 were significantly higher than those of strains BL2 and BP2. The three strains produced amylase constitutively, as it has been reported for some other strains of B. subtilis (Coleman, 1967). Odunfa and Oyewole (1986) identified B. pumilus strains on the basis of inability to hydrolyse starch. In this study strain BP2 produced low amounts of amylase when compared to the other two strains BS2 and BL2.

The strain BS2 attained highest exoamylase production level early (by 36h of incubation) when compared to 60h as observed for a B. subtilis strain by Eka and Fogarty (1972). The difference in the time of highest amylase production may be due to the differences in history of the culture organisms and the culture media used by the latter authors and those used in this study.

Exoamylase and exoproteinase production is known to be associated with the sporulation phase (Coleman, 1967; Schaeffer, 1969). The BS2 strain started sporulating at about 18h of growth in the nutrient broth medium thus its attaining highest exoamylase production in 36h confirms the association between the two physiological processes in the organism. Dod et al. (1978) reported normal production of extracellular α-amylase

by a spore control mutant of B. subtilis in which spore formation was delayed for hours.

The presence of African locust bean in the culture medium had significant effects on amylase production by the three strains BS2, BL2 and BP2. African locust bean enhanced amylase production in strains BS2 and BL2, while production was repressed in strain BP2. African locust bean does not contain starch, but contains some oligosaccharides and monosaccharides (Odunfa, 1983). The initial delay in amylase production in medium containing locust beans by the strain BS2 may be due to repression by glucose which is present in the locust beans (Odunfa, 1983). The same repressive effects by glucose might have been responsible for the reduction in activity observed in strain BP2. Glucose and some low molecular weight metabolizable sugars have been reported to repress amylase production in B. subtilis and B. licheniformis (Glenn, 1976; Meers, 1972).

None of the strains BS2, and BP2 produced pectin - methylesterase (PME); while they all produced varied amounts of PG constitutively. The amounts of PG produced by strain BS2 were significantly higher than those of strains BL2 and BP2 whether in the presence or absence of locust bean. The presence of locust bean in the culture medium caused a significant increase (at $\alpha = 0.05$) in the amount of extracellular PG excreted by the three strains. Most pectinolytic bacteria produce mainly PG and/or

polygalacturonate lyase (PGL) but not PME (Fogarty & Kelly, 1979; Priest, 1984). Strains of B. subtilis and B. pumilus which produce PGL constitutively have been isolated (Dave & Reese, 1971; Ward & Fogarty, 1974; Dave et al., 1976; Kurowski & Dunleavy, 1976). The most commonly implicated pectinolytic bacteria associated with vegetable rots Erwinia carotovora, produce only PG, PGL and oligogalacturonate lyase (Fogarty & Kelly, 1979).

Some few bacterial species which produce pectin esterase have been identified. They include Pseudomosas marginalis, Clostridium multifermentans and Cl. aurantbutyricum (Fogarty & Kelly, 1979). Chesson and Codner (1978) isolated a strain of B. subtilis B3 (from carrot) which produced extracellular pectin esterase and pectate lyase in culture medium. Thus pectinolytic bacteria such as the strains BS2, BL2 and BP2 that could not produce PME will require a PME - producer before they can successful attack a plant material containing the intact pectin molecule (Kurowski & Dunleavy, 1976). It could also be inferred that the pectic substances present in African locust bean are likely to be de-esterified chains of polygalacturonic acid, since the addition of the beans into the culture medium enhanced production of PG in the three strains.

The three strains showed lipolytic activity on glycerol tributyrate agar. Traces of lipolytic activity were detected in the crude enzymes solutions of the three strains when the culture

medium contained African locust bean. The bean contains 26.6% fats (Oyenuga, 1969). This indicated that the lipase in B. subtilis is an inducible enzyme secreted extracellularly by the strains BS2, BL2 and BP2. Jonsson and Snygg (1974) reported that emulsified olive oil in nutrient broth medium repressed production of lipase in a strain of B. licheniformis. Though lipolytic strains of B. subtilis group have been isolated (Singh et al., 1976; Garutskas et al., 1977; Kennedy & Lennarz, 1979), not all strains are capable of producing the enzyme (Obi, 1980). In most cases, phospholipase D (also known as lecithinase) is produced (Berkel & Hadlok, 1976).

The fatty acids produced during fermentation may have contributed to the odour given off by the fermenting beans. In this study, the short-chain fatty acids detected in the culture broth were isobutyric acid, 2-methylbutyric acid and isovaleric acid. Odunfa and Adesomoju (1985; 1986) also detected some fatty acids in fermenting locust beans. The short-chain fatty acids identified in this study may be due to proteolysis of nitrogenous materials in the medium or may be part of the hydrolysis products of lipase activity.

Raffinose is an oligosaccharide present in leguminous seeds such as soybean (Tanaka et al., 1975; Rackis et al., 1967), cowpea (Onigbinde & Akinyele, 1983) and African locust bean (Odunfa, 1983). Its presence in the beans cause flatulence when ingested (Steggerda, 1967; Rackis et al., 1970). Thus its hydrolysis during fermentation

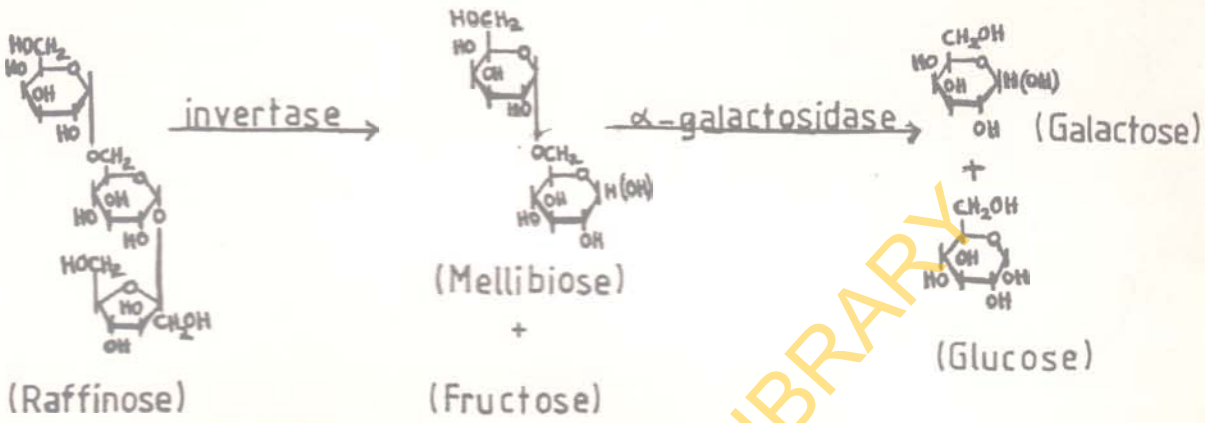
of African locust bean (Odunfa, 1983) is nutritionally beneficial. Mital and Steinkraus (1975), Hesseltine et al., (1977) also reported the utilization of the oligosaccharide in soybean by lactic acid bacteria.

Though the three strains differed in their ability to hydrolyse raffinose, the enzymatic hydrolysis products in each strain were mellibiose and fructose indicating that the point of cleavage was at the sucrose moiety. Levansucrase (EC 2.4.1.10) is an extracellular enzyme that acts on sucrose and low molecular weight levan to form high molecular weight levans and glucose (Pascal & Dedonder, 1972; Berthou et al., 1974). It has been reported to be secreted by some strains of B. subtilis (Gonzy-Treboul et al., 1975; Tanaka et al., 1978; 1979; Petit-Glatron et al., 1980). Loitianskaya and Uspenskaya (1976) reported the metabolism of raffinose in Gluconobacter oxydans to be catalyzed by levansucrase. Levan was synthesized and mellibiose and small quantities of fructose were liberated.

Raffinose $\xrightarrow{\text{levansucrase}}$ levan + mellibiose + fructose

On the other hand, the other enzymes that can act on raffinose are α-galactosidase and invertase. Silman et al. (1980) found that crude enzymes extract of Aspergillus awamori NRRL 4869 contained α-galactosidase and invertase which hydrolysed raffinose to mellibiose, sucrose, galactose, glucose and fructose. Sugimoto & VanBuren (1970) also noted that the two enzymes required to hydrolyse raffinose are invertase (which hydrolyse the sucrose moiety) and

α -galactosidase (which hydrolyse the remainder).



The detection of mellibiose and fructose as the hydrolytic products suggested invertase/sucrase activity, but lack of α -galactosidase activity in the extracellular crude enzyme solutions of the strains BS2, BL2 and BP2. Silman et al. (1980) found that the invertase produced by Aspergillus awamori NRRL 4869 could attack raffinose with the liberation of mellibiose and fructose. Alpha-galactosidase activity has been reported in microorganisms and are in some cases intracellular (Dey & Pridham, 1972; Mital et al., 1973; Delente et al., 1974; Thananunkul et al., 1976). The isozymes of α -galactosidase isolated and purified from Bacillus stearothermophilus was also found to be intracellular (Pederson & Goodman, 1980). A mold which produced α-galactosidase that hydrolysed raffinose had most of the enzyme in the mycelium when compared to that detected in culture filtrate (Suzuki et al. 1969).

Akiba and Horikoshi (1976a) isolated a strain of B. subtilis

No. 7 - 5 from soil which produced extracellular α-galactosidase constitutively. Recently Ikura and Horikoshi (1987) isolated some strains of bacteria from soil which belong to the genera Flavobacterium and Corynebacterium and produced α-galactosidase extracellularly. The extracellular enzymes solutions of the three strains (BS2, BL2 & BP2) lacked α-galactosidase activity. The enzyme is inducible in some organisms (Akiba & Horikoshi, 1976a) and exhibit some degree of substrate specificity (Akiba & Horikoshi, 1976b). Since mellibiose failed to induce production of α-galactosidase, it is also possible that the organisms could not utilize mellibiose as source of carbon (Loitianskaya & Uspenskaya, 1976).

This study has confirmed the presence of arabinogalactan in African locust bean (Parkia biglobosa). Some uncommon sugars are known to be present in certain seeds. Arabinogalactan has been reported to be found in soybean (Morita, 1965; Kawamura, 1967; Clarke et al., 1979) and wheat endosperm (Fincher & Stone, 1974), while galactomannan is known to be present in locust bean seeds (Ceratonia siliqua). Information is available on the proximate analysis of African locust bean (Oyenuga, 1968), which did not specify the absence nor presence of any of the two disaccharides above.

The arabinogalactan extracted from African locust bean was hydrolysed by crude enzyme solutions of the three strains BS2,

BL2 and BP2, indicating galactanase activity. Strains of Bacillus subtilis group that produce galactanase have been reported (Emi & Yamamoto, 1972; Emi et al., 1971). There was variation among the three strains in galactanase production. The strain BP2 had the highest galactanase activity, while strain BS2 had the least.

The differences in ability to produce extracellular enzymes by the strains will affect the nutritional quality of the fermented African locust bean product. Strains that produce large amounts of the extracellular enzymes will be desirable to effect maximum biochemical changes in the beans. Such strains as BS2, BL2 and BP2 are therefore recommendable for development as starter cultures for industrial scale production of 'iru'. The attested starter cultures can be improved further by genetic engineering. Increased yields of ∝-amylase and proteinases have been obtained by genetic manipulations of strains of B. subtilis (Higerd et al., 1972; Uehara et al., 1974; Yamaguchi et al., 1974; Dod et al., 1978).

Studies on the culture conditions required for optimal production of extracellular proteinases have shown that agitation of culture medium is essential for strain BS2. This may be due to the aerobic nature of the strains of B. subtilis (Gibson & Gordon, 1974). Thus to maximize proteolytic fermentation of the beans, the oxygen level in the fermentation vessel is an important

factor to be monitored.

Significant repressions of proteinase production in the strain BS2 were observed in media containing glucose, fructose and amino acids as carbon and nitrogen-sources. Similarly, catabolite repression of exoenzyme production by glucose has been reported in strains of Bacillus spp (Coleman, 1967; Doi, 1972; Meers, 1972; Sekiguchi & Okada, 1972; Priest, 1975). Repression of enzyme production in strains of Bacillus spp. by amino acids had also been documented (Chaloupka & Kreckova, 1966; Levisohn & Aronson, 1967; Laishley & Bernlohr, 1968; Schaeffer, 1969). The repressive effect of glucose is not a general phenomenon in exoenzyme production by strains of Bacillus spp. (Priest, 1977). High levels of pectinase was produced by a strain of B. subtilis when glucose was supplied as carbon source (Kurowski & Dunleavy, 1976). Kole et al. (1988) found that proteinase production by B. subtilis CNIB 8054 was improved when oxygen - controlled, glucose fed system was used in batch fermentation.

The presence of African locust bean in medium increased the amount of proteinases produced by the three strains, but the effect was not significant (at $\alpha = 0.05$). Casein a milk protein equally enhanced proteinase production but its effect was not significant too. Thus it can be deduced that the proteins (which are nitrogen-sources) present in the beans are balanced to give optimum fermentation of the beans. The only source of repression of the proteolytic fermentation of beans will be the effect of

fructose and glucose which were found to be present in the fermenting beans (Odunfa, 1983). Since starch had a significant positive effect on proteinase production by the strain BS2, the addition of starch to the beans (ratio 1:200-500) before fermentation will likely enhance the fermentation.

The inactivation of the crude proteinases of strains BS2 at temperatures $\geq$ 70°C and pH values $\leq$ 5 indicates that the serine proteinases constituted a large proportion. Subtilisins are quickly inactivated at temperatures $\geq$ 70°C and pH values $\leq$ 5 (Priest, 1984). Mantsala and Zalkin (1980) noted that 99% of the extracellular proteinase activity is accounted for by the serine and neutral proteinases. Dod et al. (1978) also reported that the serine and neutral proteinases contributed about 70% and 30% of total activity respectively in crude enzyme solutions of B. subtilis spore control mutants. The relative stability of the proteinases at 60°C makes the strain BS2 a potential source of heat - stable proteinases in food fermentations (Gusek & Kinsella, 1988).

The optimum pH and temperature for activity of the crude proteinases of strain BS2 were 7.5 and 35°C respectively. The optimum temperature for fermentation of African locust beans were also found to be 35°C (Odunfa & Adewuyi, 1985a). The result confirms earlier finding that proteolysis is one of the major biochemical processes taking place during fermentation (Odunfa, 1985b). Thus for industrial fermentation of the beans, optimum proteolytic activity may be achieved by incubating at 35°C.

The pH of the fermenting beans was found to increase from about 6.04 to 8.26 when fermented for 36h (Odunfa & Adewuyi, 1985a). Stability of the proteinases in the pH range of the fermenting beans is desirable. This is necessary in order to ensure maximal breakdown of the proteins to readily digestible forms. The stability of the crude proteinases of strain BS2 in the pH range 5.91-10.0 indicates that throughout fermentation, the proteinases are likely to be active. These properties of strain BS2 proteinases suggest that the strain BS2 is recommendable as one of the strains to use as starter cultures for large scale production of 'iru'.

Gel electrophoreses of the crude proteinases of all the seven strains revealed multiple proteinase bands. Since similar proteinase bands were obtained in nutrient broth media with and without locust beans (Plate 7) the presence of locust beans did not induce the production of novel proteinases. Rather, the presence of locust beans in the medium stimulated higher yield of the proteinases. This was reflected in the higher intensity of proteinase band of crude enzymes supernatants (Plate 7).

There was significant loss in activity during the $(NH_4)_2SO_4$ - precipitation step. Similar observation of substantial loss in activity by precipitation with dry $(NH_4)_2SO_4$ was also reported by Boyer and Carlton (1968). The results obtained in this study confirmed that DEAE Sephadex A-50 is a better choice for IEC in separating the neutral and serine proteinases than DEAE Sephadex

A-25. Millet (1970) used DEAE Sephadex A-25 for IEC during which the two enzymes (neutral and serine proteinases) were eluted in the void volume. This is due to the fact that the DEAE Sephadex A-25 was based on gel material with lower exclusion limit. Uehara et al. (1974) succeeded in separating the three kinds of proteinaes produced by B. subtilis 6160 using DEAE Sephadex A-50.

Factors that affected the elution profile and the separation of the proteinases include: choice of ion-exchanger, dimensions of the packing column, amount of enzyme solution loaded, and the capacity of the ion exchanger. The enzyme activity peak I that did not bind to the anion exchanger (Figures 22, 23, 24) was either neutral of cationic in net charges. The 1.5 x 20cm column gave better resolution of peaks than the 1 x 47cm (Figures 22 & 23). The binding capacity of the anion exchanger is limited since larger volume of enzyme solution loaded affected the elution profile. Failure of the $(NH_4)_2SO_4$ - precipitated enzymes to bind on the cation exchanger CM-52 cellulose, showed that the latter would not be effective in separating the proteinases.

Most of the extracellular proteolytic activity was accounted for by the serine proteinase (Fig. 22). The serine proteinase (with optimum pH at pH 7 - 9 (Priest, 1977)) is likely to be largely responsible for the fermentation of the beans as the pH of the latter changes from neutrality to alkalinity. The serine proteinase of strain BS2 had a molecular weight of 29,800 daltons (by filtration on Sephadex G-75) which falls in the range 25,000-30,000

recorded for serine proteinases (Palunbiskas et al., 1976; Priest, 1977). Different values have been reported as molecular weight for the subtilisins. Rappaport et al. (1965) recorded a minimum molecular weight of 28,812 calculated from amino acid analysis. These figures have been reconciled by Perlmann and Lorand (1970) giving the molecular weights of 27,287 (subtilisin Carlsberg) and 27,532 (subtilisin BPN'/Novo). According to Fogarty et al. (1974b) the subtilisins have an average molecular weight of 27,500. The serine proteinase purified by Mantsala and Zalkin (1980) had a molecular weight of 28,500. The serine proteinase of strain BL2 had unusually low molecular weights of 18.2 KD and 19.7 KD (by gel filtration chromatography on Sephadex G-75 and Sephadex G-50 respectively).

Most serine proteinases reported in literature have esterolytic activity especially on p-tosyl-L-arginine methyl ester (TAME), BTEE and some other esters (Farmer & Hageman, 1975; Millet, 1970; Perlmann & Lorand, 1970). The serine enzymes isolated from the strains BS2 and BL2 did not show any esterolytic activity on BTEE nor on α-naphthylacetate (in gel electrophoresis). Such serine proteinase devoid of esterolytic activity has been reported by Boyer and Carlton (1968). The hydrophobic nature of the serine proteinases of strains BS2 and BL2 is a novel dimension in their characterization. Also the possibility of their having a different mechanism for secretion cannot be ruled out. Hydrophobic extracellular penicillinase has been reported

in B. licheniformis 749/C (Lampen, 1976) by conversion of the hydrophilic membrane-associated enzyme to the hydrophobic extracellular penicillinase (Wouters & Buysman, 1977). A similar mechanism for secretion of the serine proteinases is postulated in this study.

The neutral proteinase of strain BS2 had a molecular weight of 24,000 (by Sephadex G-75) and 27,400 (by Sephadex G-50) while that of strain BL2 was 22,600 (by Sephadex G-50). These values are lower than the 36,000 - 40,000 range recorded for B. subtilis neutral proteinase by Vaganova et al. (1976) and Priest (1977). The metalloenzyme aminopeptidase isolated from a strain of B. licheniformis by Rodriquez-Absi and Prescott (1978) had a molecular weight of 37,500 and 36,000 (by two polyacrylamide electrophorectic procedures). Yasunobu and McConn (1970) recorded a value of 44,700 ± 800 for B. subtilis neutral proteinase. It is noteworthy that Sohoni and Joshi)1982) isolated a metalloenzyme proteinase which had a molecular weight of 25,120. In both strains BS2 and BL2, the specific activities of the neutral proteinases were higher than those of the serine proteinases as it has been previously reported of other strains of the B. subtilis group (Priest, 1984).

The third enzyme activity peaks of strains BS2 and BL2 (Figures 22 & 24) contained a mixture of at least two enzymes the esterase with low proteolytic acticity but high esterolytic activity, and an esterase (which had activity on α-naphthylacetate)

with no proteolytic activity on casein. The proteinase/esterase enzyme of strain BS2 had molecular weights 38,400 (by Sephadex G-75) and 33,900 (by Sephadex G-50) while that of strain BL2 was 33,500 (by Sephadex G-50). Their molecular weights are close to the value of 35,000 recorded for the esterase/proteinase isolated by Mantsala and Zalkin (1980). The esterases in this study also displayed multiple electrophoretic mobilities on polyacrylamide gel thus confirming their similarities to the esterase isolated by Mantsala and Zalkin (1980).

The esterase (band e_1, plates 10 & 11) without proteolytic activity which was eluted along with the third enzyme activity peak could infer that the enzyme had similar physico-chemical properties to the esterase/proteinase enzyme. The ability of this enzyme to cleave α-naphthylacetate an ester, and not casein in gels shows that further investigation is needed to verify the exact role of this enzyme.

The strains BS2 and BL2 are closely related to other known strains of B. subtilis and B. licheniformis respectively in terms of the types of extracellular proteinases excreted. The few differences in the physico-chemical properties of their proteinases and esterases could be contributive factors in the success of the organisms to ferment African locust bean cotyledons. The beans are fermented in moist solid state with the fermenting organisms growing and forming a bacterial film on the surface (Odunfa, 1981). This close interaction between the bacteria and

bean cotyledons could be contributive to the hydrophobic nature of their serine proteinases.

Selection of proper strain of B. subtilis capable of producing high quantities of protein could be the choice of organism for producing a variety of new and improved products of fermentation (Ingle & Boyer, 1976). Development of mutant strains from BP2, BL2 and BS2 which can produce higher yields of proteinases and pectinases will provide starter cultures for larger scale production of improved product, 'iru'. Combination of the attested strains BS2, BL2 and BP2 as starter cultures to ferment the beans may also bring about 'iru' of higher quality. These three strains have physiological properties that complement each other, such that the fermentation process might be optimized by their combined biochemical activities.

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A P P E N D I X 1

Composition of trace elements (SL 10) solution:

HCl (25%)	10ml
$FeCl_2.4H_2O$	1.5g
$ZnCl_2$	0.07g
$MnCl_2.4H_2O$	0.10g
H_3BO_3	0.006g
$CoCl_2.6H_2O$	0.19g
$CuCl_2.6H_2O$	0.002g
$NiCl_2.2H_2O$	0.024g
$Na_2MoO_4.H_2O$	0.036g

Distilled water (add to make up volume to 1000ml)

Reference: WIDDEL, F., KOHRING, G.W. & MAYER, F. (1983) Studies on dissimilatory sulphate - reducing bacteria that decompose fatty acids. III. Characterization of the gliding Desulfonema limicola gen. nov. sp. nov. and Desulfonema wagnum sp. nov. Arch. Microbiol. 134, 286-294.

APPENDIX 2

Composition of vitamins solution:

D+ Biotin	10mg/1
4-Aminobenzoic acid	50mg/1
Ca-Pantothenate	50mg/1
Pyridoxamine	250mg/1
Nicotinic acid	100mg/1
Thiamine. 2HCl	100mg/1
B_{12} vitamine	50mg/1

Reference: WIDDEL, F. & PFENNIG, N. (1981) Studies on dissimilatory sulphate - reducing bacteria that decompose fatty-acids. I. Isolation of new sulphate -reducing bacteria enriched with acetate from saline environments. Description of Desulfobacter postgatei gen. nov. sp. nov. Arch. Microbiol. 129, 395 - 400