Selective comparability and physiological studies of lactic acid bacteria protease and *Calotropis procera* (linn) extracts

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The West African soft cheese (*wara*) is traditionally produced using latex extracts of the leaves of *Calotropis procera*. Even though no death has been apparently traced to it, the extract is said to be poisonous causing death by paralysis of the heart. This project, therefore, aims at obtaining an alternative (in this case lactic acid bacteria protease) to the latex extract of *C. procera* in order to rule out possible poisoning effect. The physiological characteristics of such alternative protease was studied and compared to that of the extracts from *C. procera*. Seven lactic acid bacteria species were isolated from samples of the West African soft cheese (*wara*) obtained from local retailers. They were identified as *Streptococcus lactis* , *Streptococcus pyogenes*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Streptococcus faecalis.* The isolates were subjected to growth and protease production studies. Optimization of protease production was carried out on three selected isolates - *Streptococcus lactis*, *Lactobacillus acidophilus* and *Lactococcus lactis.* Enzymes obtained were further characterized and separated using molecular exclusion procedures. Results obtained were compared to those of different extracts of *C. procera.* It was observed that *Streptococcus lactis* had the highest (4.25 units/ml) protease production at 28°C after 72 hours with peptone and casein as nitrogen sources in the presence of glucose as carbon source. The ethanolic extract of *Calotropis procera* had the highest protease activity (12.60 units/ml) at 5% substrate concentration. *Lactococcus lactis* and *C. procera* extracts were inhibited (0.00 units/ml) at 0.2M and 0.3M concentrations EDTA respectively thus suggesting the presence of a metallic protease. Na^{2+} ion was seen to enhance protease activity in all the extracts. Comparative results show that *Streptococcus lactis* protease had a K_m of 0.42 mg/ml for casein at a V_{max} of 2.0; *Lactobacillus acidophilus* protease had a K_m of 0.77 mg/ml for casein at a V_{max} of 2.50; *Lactococcus lactis* protease had a K_m of 3.3 mg/ml for casein at a V_{max} of 0.87; The crude extract of *Calotropis procera* had a K_m of 5.0 mg/ml for casein at a V_{max} of 1.54; the ethanolic extract of *C. procera* had a K_m of 2.44 mg/ml for casein at a V_{max} of 8.77 while the methanolic extract of *C. procera* had a K_m of 5.0 mg/ml for casein at a V_{max} of 4.17. In conclusion, the lactic acid bacteria protease from *Streptococcus lactis* was found to fare better physiologically, while the protease from the ethanolic extract of *C. procera* faired better amongst the plant extracts.

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INTRODUCTION

Lactic Acid Bacteria (LAB): Classification, Characteristics and Properties

Lactic Acid Bacteria (LAB) are gram-positive usually non-motile, non-spore forming rods and cocci that belong to the family *Lactobacteriaceae*, they are obligate fermenters that do not contain haemins (cytochromes, catalase) (Hans, 1993). In spite of this, they are able to grow in the presence of oxygen (especially streptococcal species), thus their distribution in nature is related to their high nutritive demand and energy generation purely by fermentation. They are hardly ever found in soil or water; their natural habitats include milk and milk-related places, intact and rotting plants as well as intestinal tracts and mucous membranes of animals and humans (Hans, 1993).

They are characterized by a fermentative sugar metabolism in which lactic acid is a major end-product. According to Hammes and Vogel (1995), Lactic acid bacteria are classified by the glucose fermentation pathway and by their cell morphology. Those belonging to the genera *Lactococcus, Leuconostoc, Pediococcus, Streptococcus* and *Lactobacillus* are commonly found in fermented foods and feeds that are regularly consumed (Campbell – Platt, 1987). Among the lactic acid bacteria groups are:

- *Lactococcus* includes strains that are gram-positive, spherical cells occurring in pairs and chains. They have a strictly homofermentative metabolism and are found in dairy and plant products. *Lactococcus lactis* is an example of a strain used as starter culture in the production of cheese (Sneath, 1986).
- *Leuconostoc,* are avoid cocci appearing often in chains. All members of this genus have an heterofermentative mode of metabolism when grown in media containing sucrose. Copious amounts of a slimy polysaccharide called dextran are produced. Dextran has found use in medicine as a plasma extender in biotechnology.

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Leuconostoc species are useful in the fermentation of cabbage to sauerkraut (Collins *et al.*, 1993).

- *Pediococcus* are cocci often found in pairs and tetrads, they are strictly homofermentative. Their habitat is restricted mainly to plants. *Pediococcus cerevisiae* has been used as starter culture for the fermentation of sausages. They are also found in beer (in which the produce sarcine sickness), meat-curing brines and fermenting vegetable juices. They are distinguished from *Leuconostoc* species by their inability to produce slimy colonies on carbohydrate containing media and carbon dioxide from glucose (Gibson and Abd-el-malek, 1945).
- *Lactobacillus,* vary in morphology from long, slender rods to short coccobacilli which frequently form chains. Some species are aerotolerant and may utilize oxygen through the enzyme flavoprotein oxidase, while others are strictly anaerobic. Growth is optimum at pH 5.5 – 5.8 and the organisms have complex nutritional requirements for amino acids, peptides. They are either homo or heterofermentative. They are widespread and can be isolated from plant and animal sources. They are more tolerant to acid than the other genera of lactic acid bacteria and this properly makes them important in the final phases of many food fermentations when other organisms are inhibited by low pH. (Salaminiene *et al.*, 1998). *L. bulgaricus* is commonly used in yogurt production.
- *Streptococcus,* are cocci in chains that are distinguished from *Leuconostoc* by their strictly homofermentative metabolism. These organisms can be isolated from oral cavities of animals, the intestinal tract, skin and any foods that come in contact with these environments. While the other genera rarely cause diseases, *Streptococcus pyogenes* is a common troublesome pathogen causing sore throat and rheumatic fever.

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Streptococcus thermophilus is used in the production of yogurt and as starter culture in cheese production. One of the most important identification characteristic is the ability to lyse red blood cells when grown on a medium containing sheep or ox blood (Sneath, 1986).

Lactic acid bacteria have limited biosynthetic ability, requiring preformed amino acids, B-vitamins, purines, pyrimidines and typically a sugar as carbon and energy source. A rich medium is usually employed when cultivating them. These multiple requirements restrict their habitats to areas where the required compounds are abundant like plants, animals and other multicellular organisms. Lactic acid bacteria grow at temperatures from 5^{o}C to 45^{o}C and not surprisingly are tolerant to acidic conditions with most strains able to grow at pH 4.4.

They can be divided into two groups based upon the products produced from glucose fermentation:

Homofermentative organisms ferment glucose to 2 moles of lactic acid, generating a net of 2ATP (Adenosine Triphosphate) per mole of glucose metabolized, lactic acid is the major product of this fermentation (Axellsson, 1998).

Heterofermentative organisms ferment 1 mole of glucose to 1 mole of lactic acid, 1 mole of ethanol and 1 mole of CO_2. A mole of ATP is generated per mole of glucose, resulting in less growth per mole of glucose metabolized. Due to the low energy yields, lactic acid bacteria often grow more slowly than microbes capable of respiration and thus produce smaller colonies of between 2 – 3mm (Axellsson, 1998).

Since they do not use oxygen in their energy production, lactic acid bacteria readily grow under anaerobic conditions, but they can also grow in the presence of oxygen, and are thus microaerophiles. They are protected from oxygen by products like hydrogen peroxide

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because they have peroxidases, they are also differentiated from other organisms by their ability to ferment hexoses to lactic acid, hence their name (Eugene *et al.*, 1998).

Cellular Metabolism and Fermentation

Lactic acid bacteria at any point in time break down various substances to produce new ones through different processes, one of such is Glycolysis. According to Lin *et al.*, (1995), eight reactions each catalyzed by a specific enzyme make up the glycolytic process. At steps 1 and 2, ATP is converted into ADP; inputting energy into the reaction as well as attaching a phosphate to the glucose. At steps 5 and 8, ADP is converted into the higher energy ATP while NAD^+ is converted into NADH + H^+.

The reaction takes place to yield 2 pyruvic acid molecules having 3-carbon sugar each. Under anaerobic conditions, pyruvic acid in routed into one of three pathways; lactic acid fermentation, alcohol fermentation or cellular (anaerobic) respiration.

Tricarboxylic Acid Cycle is another set of reactions coupling one reaction to the other. In this transition reaction, pyruvic acid is first altered by the removal of a carbon and two oxygen molecules that forms carbondioxide. When the carbondioxides is removed, energy is given off and NAD^+ is converted into the higher energy form NADH. Coenzyme A attaches to the remaining 2-carbon acetyl unit forming acetyl co-A, which is then attached to a 4-carbon chemical, called oxaloacetic acid. The co-A is released and returns to await another pyruvic acid. The 2-carbon and 4-carbon molecule forms another chemical known as citric acid, a 6-carbon compound. The process after citric acid is essentially removing CO_2 and releasing energy in the form of ATP, GTP, NADH and $FADH_2$, and lastly regenerating the cycle.

According to Eugene *et al.*, 1998), fermentation is defined as a gradual change in raw material by the enzymes of some bacteria into products with acceptable rheological and organoleptic properties and also an increase in consumption period. A large number of acids,

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including acetic acid, lactic acid and butyric acids, as well as a large number of alcohols are identified as products of fermentation. Fermentating organisms have been used in food processing and preservation (Hans, 1993). Representative examples include silage, cheese, yogurt, sauerkraut, in the brewing industries for the manufacture of wine, beer, alcohol and lactic acid. Many fermentative organisms are obligate and facultative anaerobes. Food fermentation has great economic value and has been accepted that its products contribute to improvement of human health (Ray and Daeschel, 1992).

Lactic acid bacteria are of great significance in fermentation due to their widespread benefits in preparation of foods. Such benefit includes their ability to produce desired flavour, discourage spoilage and contamination by other organisms through the production of inhibitors such as bacteriocins. Bacteriocins are polypeptide antimicrobials that inhibit the growth of pathogens. Bacteriocins produced by gram-positive bacteria are small peptides of 3 – 6Kda in size. (Nes *et al.*, 1996). They fall into two broad classes namely the lantibiotics (Jack *et al.*, 1995) and the non-lantibiotics (Nes *et al.*, 1996). Many bacteriocins of lactic acid bacteria are safe because these group of organisms are generally regarded as safe (GRAS) organisms (Soomra *et al.*, 2002).

According to Vandenberg (1993), lactic acid bacteria cultures perform the following activities:

- Lipolysis which involves the breakdown of complex fat into digestible components: Triglycerides (fat) $\xrightarrow{\text{lipases}}$ Fatty acids + glycerol.
- Lactose metabolism where the enzymes β-galactosidase glycolases and lactic dehydrogenase (LDH) produces lactic acid from lactose.

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Lactic acid is produced from pyruvate by the enzyme lactic acid dehydrogenase. Lactic acid bacteria then take up lactose (lactose permease) and hydrolysis it to glucose and galactose by the enzyme galactosidase through lactose operon.

Lactic acid has some physiological benefits such as:

- Enhancing the digestibility of milk proteins by precipitating them in fine curd particles.
- Improving the utilization of calcium, phosphorus and iron.
- Stimulating the secretion of gastric juices.
- Accelerating the onward movement of stomach contents.
- Serving as a source of energy in the process of respiration.

Proteases in LAB Metabolism

Types of Proteases, Occurrence and Roles

Proteases are defined as enzymes that break peptide bonds between amino acids of proteins in a process called proteolytic cleavage (Hooper, 2002; Barrett *et al.,* 2003). The process involves the use of a molecule of water, thus classifying proteases as hydrolases. Proteases are grouped into six classes namely; serine proteases (e.g. trypsin, chymotrypsin, elastase, enterokinase B); Threonine protease; cysteine proteases (e.g. cathepsins, calpains, caspases, some lysosomal proteases); methalloproteases (e.g. collagenase, gelatinases, stromelysins); Aspartic acid proteases (e.g. Pepsin, Plasmepsin) and Glutamic acid proteases.

They occur naturally in all organisms and constitute one to five percent of the gene content. These enzymes are involved in a multitude of physiological reactions and can break either specific peptide bonds (limited proteolysis), depending on the amino acid sequence of a protein. They could also breakdown a complete peptide to amino acids (unlimited, proteolysis). Proteases are inhibited by the class of serpins (serine proteases or peptidase

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inhibitors) such as alpha-1-antitrypsin, neuroserpin, complement 1-inhibitor, antithrombin, alpha 1-antichymotrypsin and plasminogen activator inihibitor 1.

Proteolytic enzymes are the most industrial enzymes, representing a worldwide sale of about sixty-percent of total enzyme market (Woods *et al.*, 2001). They find commercial applications in a number of industries like the leather industry where serine proteases are used to dehair hides and softer leather due to their collagenolytic activity (George *et al.*, 1995), pharmaceutical industry in combination with broad spectrum antibiotics for treating festering and wet wounds by offering a gentle and selective debridement while supporting natural healing process, and also as an antiplaque and antitartar components of toothpastes (Hernandez and Maria 1996), cosmetics (Ohta *et al.*, 1996), also for the recovery of silver from used X-ray films (Ishikawa *et al.*, 1993).

Due to their ability to remain stable in the presence of surfactants, they aid the removal of proteinaceous dirt from laundries (Godfredson, 1990). Metalloproteases have found immense use in the brewery industries as well as in alcohol production while the acid proteases find uses in the manufacture of cheese and the baking industries (Bjorkling *et al.*, 1991). Solid substrate fermentation has the potential for higher protease yield because economically this type of fermentation processes many advantages including superior volumetric productivity, use of simpler machinery, use of an inexpensive substrate, simpler down-stream processing, low energy requirements and low waste water output (Malathi and Chakraborty, 1991).

Protease Purification

Several research works have been carried out on lactic acid bacteria proteases, likewise several types have been purified and characterized. It has been reported that the protease activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* reaches its maximum during the log

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phase (Tamine and Robinson, 1983; Hartley and Denariaz, 1993; Oberg and Broadbent, 1993). Several studied using *Streptococci* and *Lactococci* extracellular or cell wall-associated proteases have also been conducted (Thomas and Mills, 1981; Kunji *et al.*, 1996). Julliard *et al.*, (1995) found that cell wall associated extracellular protease produced by *Lactococcus lactis* subsp. *cremoris* is a P-1 type that hydrolyses casein. A 96kDa aminopeptidase was purified and characterized from *Streptococcus salivarius* subsp. *thermophilus* NCDO 573 with ion-exchange chromatography using sephacryl 5300 and arginine sepharose 4B (Pharmacia) equilibrated with 20mmol^{-1} phosphate buffer at pH 7.0. The procedure purified a yield of 6 – 7% 300 – 500 fold of the aminopeptidase (Robyn and Pritchard, 1994).

In another instance, an extracellular cysteine proteinase produced by *Micrococcus* sp. INIA 528 was also purified by chromatography using sephadex G-100 and G-50 pre-equilibrated with 50mmol^{-} sodium phosphate buffer to achieve a 29-fold increase and 28% recovery of the proteinase activity (Fernandez *et al.*, 1996). An endopeptidase and three exopeptidases were purified by gel chromatography using Bio-Gel P-100, P-200 or sepharose 6B equilibrated with 0.01m potassium phosphate buffer and affinity chromatography using agarose – Gly – Tyr (Obz) – Arg equilibrated with 0.0066m potassium phosphate buffer respectively (Morsi *et al.*, 1978). A surface bound aminopeptidase of *Lactobacillus lactis* was also purified and characterized by streptomycin sulfate precipitation ammonium sulfate fractionation (using 0.05m tris hydroxymethyl amino methane buffer), chromatography on sephadex G-100 and diethyl amino ethyl-sephadex A-50 equilibrated with 0.05M Tris buffer at pH 7.0 to obtain a single polypeptide chain with molecular weight between 78,000 to 81,000 (Bernhard and Marc, 1980).

Microbial proteases play an important role in biotechnological processes accounting for approximately 59 percent of total enzymes used (Maugh, 1984). The core of biotechnology

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is the use of enzymes in several industries and also for diagnostic, scientific and analytical purposes. They are greatly used due to their catalytic activity and high degree of specificity. The use of microorganisms to produce enzymes has enormous economic and technical advantages, thus methods of production, extraction and purification are of much concern to scientists (Alvarez-sanchez *et al.*, 2000; Gianna *et al.*, 2001; Thangam and Suseela, 2002). At present industrial enzymes are extracted from bacteria, fungi and actinomycetes species (Kumar, 1998).

Enzyme Immobilization and Proteases

Modification of biotechnology processes using immobilized biocatalysts has gained attention of biotechnologists. Application of immobilized enzymes is advantageous because such biocatalysts display better operational stability (Fortin, 1990) and higher efficiency of catalysis (Ramakrishna *et al.*, 1992). Immobilization is considered to be the conversion of enzymes from water-soluble mobile state to a water insoluble immobile state. Immobilization makes it possible for the activity of the enzyme to be retained even after removal and subsequent re-use, and also to prevent diffusion of the enzyme in the reaction mixtures. It also facilitates their recovery from the product stream by simple solid/liquid separation techniques. Immobilization according to Smith (1986) can be achieved by the following procedures:

(1) Covalent attachment to the surface of a water insoluble solid material like porous glass and ceramics, alumina, synthetic polymers, nylon and cellulose. Formation of covalent bonds has the advantage of forming an attachment that is not reversed by pH, ionic strength or substrate.

(2) Entrapment in a matrix or gel that is permeable to the enzyme substrate and products. This method is very mild and gel formation occurs either by changing the temperatures

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or by adding a gel-including chemical. Entrapment materials include silica gel, silicone rubber, starch and polyacrylamides.

(3) Encapsulation with semi-permeable membranes that ate impermeable to enzymes and other macromolecules but permeable to low molecular weight substrates and products. Materials used include cellulose derivates polystrene and nylon. These materials can be used to form thin, spherical semi-permeable membrane, which form microcapsules with the enzyme inclusion.

(4) Adsorption of enzymes on solid supports or surface. The most commonly used adsorbants include many organic and inorganic materials such as alumina, cellulose, clays, glass, hydroxylapatite, carbon and various silicaceous materials. Ion exchange readily absorbs proteins and thus have been widely employed.

(5) Cross linking with suitable agents to give an insoluble particle. Enzymes are usually crosslinked after adsorption onto a suitable carrier. Among the commonly used crosslinkers are aliphatic diamines, dimethyladipimate, dimethyl suberimidate and glutaraldehyde. Crosslinking can be intermolecular (creating water-insoluble aggregates) and intramolecular.

Transforming Milk to Cheese

Cheese is made from the milk of goats, sheep, buffalo, reindeer, camel, ilama and yak but is usually made from cow's milk, which is about 88% water and the remainder is fat, protein, sugar, minerals and vitamins. Milk is a white fluid secreted by female mammals for the purpose of rearing their offspring (Kosikowski and Mistry, 1997). Its role is to nourish and provide immunological protection for the mammalian young because its nutritional value is high (O'Connor and Tripathi, 1991).

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The origin of cheese making is lost in unrecorded history. There is evidence of suggest that cheese was made as far back as 7,000BC. There are numerous references to cheese making in the Bible while the writings of Homer and Aristotle indicate that cheese was made from the milk of cows, sheep, mares and asses. Around 300AD trade in cheese between countries especially on sea routes became so great that the Roman emperor Diocletion had to fix maximum prices for the cheese. The earliest tribes of ancient Egypt and South-West Asia discovered sometime around 5,000BC that cow milk was a nourishing human food. While the ancient Egyptians recognized that cow milk was a wholesome and sustaining food. The earliest evidence of knowledge of milk composition is dated at about 350BC when Aristotle wrote "casein, fat and water are all the known substances of milk." (FAO, 1990).

In the process of cheese making, most of the protein, fat and some minerals and vitamins are concentrated and separated as a solid. The remaining liquid called "whey" contains most of the sugar, water, some protein, minerals and vitamins. Whey is utilized in foods and feeds or disposed off as waste. According to Law (1999) and Walstra *et al.*, (1999) there are two principal agents, which bring about the concentration and separation of protein and fat to make cheese:

- **Bacterial Culture:** Bacteria are often responsible for food spoilage but there are also many useful types. During the manufacture of cheese and other cultured dairy products, lactic acid bacteria change the milk sugar to lactic acid. The acid acts as a preservative by inhibiting undesirable types of bacteria, helps remove water from the curd and is important to the development of cheese texture. The lactic acid bacteria and other microorganisms, which happen to be present in the cheese, contribute enzymes, which breakdown fats, proteins and sugar during aging to produce flavors characteristic of particular cheese varieties.

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- **Coagulating Enzyme:** Proteins can be thought of as long microscopic chains. Various food products such as jello, jams and cheese depend on the ability to protein chains to intertwine and form a mesh-like network. The formation of this network is called "coagulation". When proteins coagulate in water, they trap water in the network and change the liquid to a semi-solid gel. In cheese-making gelatin is caused by proteolytic enzymes, which when added to warm milk, transforms it into a soft gel. When firm enough, it is cut into small pieces, 0.5 – 1.0 cm square ($^1/_4$ – $^3/_4$ inch) called "curds". According to Scott *et al.*, (1998), there are three types of curd formation in cheese making:
- **Lactic Coagulation:** due to the fermentation of lactose to lactic acid. The low pH causes the casein micelle to coagulate. The acidity causes a certain demineralization of the micelles as the pH lowers. It is important that the temperature should be around 20^{o}C to destabilize the micelles, the acid should be formed slowly to give a uniform curd and carbondioxide-forming bacteria avoided so that gas released does not interfere with curd formation.
- **Rennet Coagulation:** dependent upon the action of proteolytic enzymes. E.g. Chymosin breaks down bonds in casein but does not proteolyse the other caseins present in rennet. There in formation of floccular-fibrous network that retain moisture and fat globules. The firmness of the gel depends on calcium phosphate. The cheese milk is inoculated with lactic acid bacteria, which must produce some acid for rennet to work. Rennin, a substitute for rennet, is composed of fungal protease from *Endothia parasitica, Mucor mietiei* and *Mucor pusillus*.
- **Mixed Coagulation:** carried out by the combined action of lactic acid forming bacteria in coagulating amounts along with the addition of proteolytic enzymes, this is achieved

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at a temperature of 32°C. Many varieties of soft and semi-hard cheeses are made this way, they can be classified into two groups: (i) those with a traditional form and rind (Gouda, Wara and others) and (2) those that are ripened with molds (Brie, Camembert and others). For the first group, a mixed culture of *Lactococcus* (*Lc. Cremoris* and/or *L. lactis*) plus either *Leuconostoc* or *Lactococcus diacetylactis*.

Certain types of cheeses such as some types of Queso Blanco (Latin American countries) and Paneer (India) are made without bacterial cultures and without rennet. In these types, curd is formed by adding acid juices or vinegar to hot milk. It has the advantage that all the milk proteins including those normally lost in the whey are included in the cheese (Hill, 1999).

Plants and the Protease Supply Pool

Bacteria are not the only source of industrial enzymes like proteases. The use of proteases has been in existence for quite a while as this was the common source in ancient times. The primary necessities of man, which are food, clothing and shelter, are met by plants. Green plants are thus essential for the existence of all kinds of life (Dutta, 2000). In a research carried out by Llorente *et al.*, (1992), the presence of proteinases in the flowers of cardoon (*Cynara cardunculus* L.) – which are traditionally used in the Mediterranean region for cheese making – was investigated by determining the proteolytic and milk clothing activities of its crude extracts from different parts of the inflorescence in various stages of development, as well as of leaves and roots. Although all the preparations showed a certain extent of proteolytic activity, only those of adult leaves, pappus, and immature and mature flowers were able to clot milk.

The extracts of the violet parts of mature flowers exhibited optimum activity at acid pH values with 90 percent of maximum activity at pH 3.5 – 5.0, which was strongly inhibited by

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pepstatine A, suggesting the presence of aspartic proteinases. The extract had low thermal stability at temperatures above 45^{o}C. Different species are described for the genus *Cynara*, but only *C. cardunculus* is referred to be used in cheese making.

Other certain plants have been reported to yield promising proteinase activity, such as *Withania coagulans* (Yeshoda, 1941; Dastur *et al.*, 1948), *Eicus carica* – the Fig tree (Whitakar, 1959), Pumpkin (Rebecca and Leibowtiz, 1963), seeds of *Moringa oleifera* (Dahot, 1985), seeds of *Ricinis communis*, dried Papaya latex, Pineapple, Cucumber, *Benincasa cerifere, Galium verum, Pinquicule vulgaris, Prosera rotindifolia* and *Ranunculus ligua* (Scott, 1973).

Ten plants traditionally known to exhibit milk clotting and protease activities were investigated by Umar *et al.*, (1990). Out of the ten plants namely *Opuntia phylloclades, Cereus triangularis, Aloe* L. sp., *Euphorbia caducifolia, Calotropis procera, Papaya, Ficus bengalensis, Ficus elastica* and *Euphorbia hista*,, It seems that *Papaya* leaves and *Euphorbia caducifolia* stem samples showed a higher ratio of milk clotting to protease activity in comparison to other plants.

Calotropis procera: Classification, Sources and Morphology

Calotropis procera or *Calatrope* belongs to the family Asclepiadaceae known as the milkweed family. This large family of erect and twinning shrubs and perennial herbs consists of 130 genera and some 2,000 species. The plant is widely distributed in the tropics and subtropics (Webb, 1948). The giant milkweed is also known as akund, Sodom apple, French cotton, king's crown, madar, mudar, Roostertree, Swallow wort, small crown flower, Rubber-bush, Sodom's milkweed (English names); Arbrea soie, cotton-france, Bois canon (French names); Algodon de seda, bomba and Calotropis (Spanish names) amongst others (Haward, 1989; Liogier, 1995). The genus name, *Calotropis* means "beautiful keel" referring to the

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specialized structure called the corona in the center of the flower, while the species name, *procera* means "tall", as most milk weeds are much shorter than this plant. The plant is a soft-wooded, evergreen perennial shrub or small tree that grows to a height of 5.4 metres. It has one or a few stems, few branches and relatively few leaves mostly concentrated near the growing tip. The leaves were used during Vedic times in sun worship. The plant was considered sacred and maruts worshipped on Saturdays with garlands of its flowers.

Ancient Arab tribes also had a deep superstitious belief about calotopries in relations to sun worship. The bark is corky furrowed and light-grey, a copious white sap flows whenever the stem or leaves are cut. Its seeds are spread by wind and water over large distances (Smith, 2002). The species favours open habitat with little competition, and as such are commonly found at beach fronts, dunes, roadsides, overgrazed pastures and dry habitats with 150 to 1000 millimetres precipitation. It is also sometimes found in excessively drained soils in areas with as much as 2,000 millimetres of annual precipitation (Parrota, 2001). Flowering and fruiting takes place throughout the year (Little *et al.*, 1974) with its deep, stout taproots branching and reaching depths of 1.7 to 3.0 metres, with few or no near-the-surface lateral roots thus rarely growing in shallow soils (Sharma, 1968). The flowers are shallowly companulate with five sepals, 4 to 5 millimetres long, fleshy and variable in colour from white to pink, often spotted or tingled with purple. The fruits are inflated, obliquely avoid, follicles that split and invert when mature to release flat, brown seeds with a tuft of white hair at one end (Howard, 1989; Liogier, 1995). It is native to West Africa as far as Angola, North and East Africa, Madagascar, the Arabian Pennisula, Southern Asia and Indochina to Malaysia, the species is now naturalized in Australia, many Pacific Islands, Mexico, Central and South America and the Caribbean Islands (Rahman and Wilcock, 1991).

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Economic Importance of *C. procera*

Global shortage of animal-based enzyme and various kinds of aversion of some people to such sources have led to the use of proteases isolated from plants and microorganism, especially in the dairy industries (Scott, 1986). Juice extracts from fruits and plants have long been used as milk coagulants. These include papain extracts from papaya, bromelin from pineapple ricin from castor oil seeds and the latex of the fig tree. Juice from *C. procera* provides a cheap and readily available alternative to rennet.

Rennet is extracted from the stomach of mammals or more easily from the fourth stomach of an unweaned calf. Rennet (chymosin) is a proteolytic enzyme and its role in cheese making is to destabilize casein micelles and make them coagulate (O'Mahony, 1988). Compared with the quantities produced in Europe and North America, the amount of cheese produced in Africa is quite small. Most of the cheese produced in Africa is made on a small scale and generally at farm level.

The plant extracts are suitable for softer curd cheese, which is consumed within a few days. They are not suitable for hard cheese with long maturing periods on account of their excessive proteolytic activity, which leads to bitter flavours in the ripened cheese (FAO, 1990). The West African soft cheese, Warankasi, which is the typical type of cheese found in Nigeria has a shelf-life of 2 to 3 days, wives of Fulani herdsmen often convert the surplus milk into wagashi soft cheese using the vegetable rennet extract of *C. procera* (Belewu *et al.*, 2005).

Fruits of *Calotropis* are also used in the production of Tannin, which is produced from the fruit, bark, leaves and roots of many arid-zone shrubs and trees. It is readily soluble in water or alcohol giving strongly astringent solutions also useful in medicines. Tanning permits the processing and protection of the locally made raw materials adding utility and commercial

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value to a major by product of meat production. *Calotropis* yields a durable fibre commonly known as Bowstring of India, useful for ropes, carpets, fishing nets and sewing threads. Floss obtained from the seeds is used for stuffing purposes in pillow making. Fermented mixture of *Calotropis* and salt is used to remove the hair from goat skins for production of "nari leather" and of sheep skins to make leather, which is much used for inexpensive book binding (FAO, 1989).

Latex Composition and Health Implications of *C. procera*

It has been discovered that shade-dried giant milk weed leaves contain 94.6 percent dry matter, 20.9 percent ash, 19.6 percent crude protein, 2.2 percent fat, 43.6 percent acid detergent fiber and 19.5 percent neutral detergent fibre (Abbas *et al.*, 1992). Its leaves contain several active chemicals with mudarine as the principal active constituent, besides a yellow bitter acid resin, including three toxic glycosides namely calotropin, uscharin and calotoxin. Procerain, a stable cysteine protease with amidolytic activity was purified to homogeneity by Ammonium sulphate precipitation and cation exchange chromatography. It was found to have a molecular mass of 28.8KDa and an isoelectric point of 9.32.

Proteolytic and amidolytic activities of the enzyme have been reportedly activated by thiol protease activators and inhibited by thiol protease inhibitors, the enzyme cleaves N-succinyl-Ala-Ala-Ala-p-nitroanilide but not L-Ala-Ala-p-nitroanilide, L-Ala-p-nitroanilide and N-d-Benzoyl-DL-Arg-p-nitroanilide. The enzyme appears to be peptide length-dependent and unlike most of the plant cysteine proteases, has blocked N-terminal residue. It contains 8 tryptophan, 20 tyrosine and 7 cysteine residues forming three disulfide bridges with the remaining one being free. Procerain retains full activity over a broad range of pH 3.0 – 12.0 and temperatures up to 70^{o}C, besides being stable at very high concentrations of chemical denaturants and organic solvents (Vikash and Jagannadham, 2003).

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The latex is similar to that of *Calotropis gigantea* in its content of proteinase and also calotropin and other cardiac glycosides (Watt and Breyer-Brandwijk, 1962). Atal and Sethi (1962) isolated calotropain from the latex and showed it to be a mixture of at least five proteinase with proteolytic activity greater than that of papain, ficin or bromelain. Hussein *et al.*, (1994) reported the presence of alkaloids, flavonoids, cardiac glycosides as well as sterols and uscharin in the entire plant of calotropis. Ansara and Ali (2001) isolated a norditerpenyl ester named calotropterpenyl ester and two unknown pentacyclic triterpenoids, namely calotropursenyl acetate and cahetropfriedelenyl acetate from the root bark of the plant on the basis of spectral data analyses and chemical reactions.

Abraham and Joshi (1979a, 1979b) described two carbohydrate containing proteinases, cahotropain F1 and F11, the former being very similar in properties to chymopapain whilst the latter more closely resembles papain. Pal and Sinha (1980) also described the isolation of two other papain-like proteinases, calotropains D1 and D11 that do not contain carbohydrate. Arul *et al.*, (2004) evaluated the activity of the ethanolic extract of *Calotropis* on Dalton's ascitic lymphoma (DAL) in Swiss albinomice and found a significant enhancement of mean survival time of the tumor bearing mice and peritoneal cell count in normal mice. When the ethanolic extract of *Calotropis* (ECP) treated animals underwent inoculation with DAL cells, tumor cell growth was found to be inhibited. ECP is able to reverse the changes in the hematological parameters, protein and packed cellular volume consequent to tumor inoculation. Likewise Arya and Kumar (2003) evaluated the anti-inflammatory potential of the methanolic extract and dry latex extract and found both to inhibit oedema induced by histamine, carrageenin and bradykinin at oral doses ranging from 50 – 100mg/kg. The inhibitory effects were comparable with antihistaminic drug chlorphenidramine.

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Health Implications of *C. procera* extract

The plant is poisonous, but has been useful in small amount as folk remedies for various ailments with fatal effects. It also serves as an anti-coagulant and anti-cancer remedy with the bark being used as a local drug for malaria, snake bite, menorrhagia, elephantiasis, leprosy and chronic eczema, the juice has also been used as a blistering agent (Nadkarni, 1976). Jain *et al.*, (1996) described its use in traditional medicine as a purgative, appetizer, expectorant, antihemintic and as an abortifacient. It is also used to treat asthma, cholera, leucoderma, ulcers, piles and diseases of the spleen. In an experiment carried out by Akinloye *et al.*, (2002) on Wister rats using extracts of *Calotropis*, it was observed that the extract was potentially injurious to the body especially after prolonged or chronic.

Histopathological observations showed varying degrees of testicular lesions, which were more severe in Wister rat groups that received the extract for longer periods. Histological changes observed in the testis were due to the presence of cardiac glycosides found in the extract. The extract affects sperm volume negatively thus confirming its use as an anti-fertility agent. The extract also has destructive effect on actively dividing germ cells also explaining its anti-tumor effects (Jain *et al.*, 1996). In higher close it causes nausea, vomiting and diarrhea, increased heartbeat and respiration, which can lead to death.

Advantages of LAB Protease over *C. procera* extract

In contrast, lactic acid bacteria are part of the normal human microflora and they exert a positive effect on human health. Lactic acid bacteria are widely used in the food industry due to its probiotic effects and activities. Probiotics are defined as living organisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition (Schaafsma, 1996). The possible takeover of the role of *Calotropis* protease by lactic acid bacteria protease has gone a long way in imparting enormous benefits with no side effects when consumed.

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Furthermore, since the traditional use of probiotic strains in the food industry confirms their lack of pathogenicity, the use of these bacteria as live vehicles for the production and delivery of heterologous proteins of vaccinal, medical and technological interest has been extensively investigated (Seegers, 2002). Hence, the justification for this project.

Aims and Objectives of the Current Work

Bearing in mind all the aforementioned points, the current project aims to:

(i) Extract, purify and characterize the proteases of selected Lactic Acid Bacteria.

(ii) Extract, purify and characterize the proteases in the different extracts of the plant *Calotropis procera.*

(iii) Subject the two extracts to physiological and other characterization studies.

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MATERIALS AND METHODS

Sample Collection

West African soft cheese samples "Wara" were purchased from Bodija Market in Ibadan metropolis and Owode in Oyo town both in Southwest Nigeria. They were steeped in the whey and kept in a sterile sample bottle for a week to allow bacterial colonization to occur.

Isolation and Culture Methods

Medium for Isolation

De Man – Rogosa – Sharpe (MRS) medium was used (De Mann *et al.*, 1960). It was prepared according to the manufacturer's direction and sterilized by autoclaving at 121^{o}C for 15 minutes. The pH of the medium was adjusted to 5.5 for the purpose of this study (Sneath, 1986), an optimum pH for the growth at lactic acid bacteria.

Isolation of Lactic Acid Bacteria

The serial dilution method of Harrigan and McCance (1966) was used. Here, 6 sterile test tubes were prepared, each containing 9ml of sterile distilled water. 10g Wara sample was mashed in 100ml whey until a suspension was formed. 1.0ml aliquot of the mixture was taken with a sterile pipette and homogenized with 9ml of sterile distilled water in the first test tube repeated for others, until a dilution of 10^{-6} was achieved. 1ml of the dilutions 10^{-4}, 10^{-5} and 10^{-6} was pipetted into sterile petri plates and simultaneously 10ml of already autoclaved molten MRS agar was aseptically poured into the inoculated plates and swirled to ensure mixture. The plates were allowed to set before being incubated at 37^{o}C for 24 hours under anaerobic conditions.

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Maintenance of Pure Culture

At the end of incubation period, isolated colonies were sub-cultured and repeatedly streaked to obtain pure cultures, which were stored on MRS slants at 4^{o}C.

Biochemical Characterization of Bacterial Isolates

In each case, a 24-hour old culture was used for characterization of pure isolates obtained. These include:

Gram Staining

The method of Pelczar and Chan (1977) was used. A sterile wire loop was used to slightly touch a colony of each isolate and emulsified in a drop of water on a clean slide, to make a thin smear. This was heat fixed. The smear was flooded with 2% crystal violet stain and left for 30 seconds after which it was rinsed off under a gently running tap. Two drops of Gram's iodine solution was then added to act as a mordant. This was left for 30 seconds and washed off under a gently running tap. 95% ethanol was used to decolorize the smear for 10 seconds until it appeared free of crystal violet stain. It was then rinsed under a running tap. Counter staining was done with 2 drops safranin for 30 seconds and then rinsed off under a running tap. The smear was blotted dry and left to air-dry before viewing microscopically under oil immersion objective lens.

Catalase Test

The method of Seelay and Van Demark (1972) was used. A sterile loop was used to touch a colony of 18 hour old isolates and transferred onto a clean glass slide. A drop of 3% hydrogen peroxide was then added and a reaction was observed. Evolution of gas bubbles as

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white froth indicates a catalase positive reaction while the absence of the troth indicates a negative reaction.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Oxidase Test

Using a spot-plate technique, a Whatman number 1 filter paper was impregnated with oxidase reagent (1% aqueous tetramethyl-p-phenylene diamine hydrochloride). With the aid of a wire loop, spots of test isolates were transferred onto the wet filter paper. Formation of a deep purple coloration between 10 – 15 seconds indicates an oxidase positive reaction (Seelay and Van Demark, 1972).

Methyl Red Voges-Proskauer Test

MRVP broth was prepared as described by Harrigan and McCance (1966). 10ml of the broth was dispensed into screw cap bottles and sterilized. Inoculation with test, isolates was done and incubated at 35^{o}C for 2 – 5 days. After incubation, the content of the screw cap bottle was divided into two portions labeled M and V respectively. Five drops of methyl-red solution was added to the M-labeled portion. A red colouration indicates a positive reaction while yellow colouration indicates a negative reaction. To the V-labeled portion, 0.5ml of α-naphthal solution and 0.5ml of KOH solution was added. Development of a red colour after thorough shaking within five minutes indicates a positive reaction.

Indole Production

Half of gram (0.5g) of sodium chloride was added to 100ml of peptone broth (2%w/v). 10ml of this was dispensed into screw cap bottles and sterilized. The bottles were inoculated with test isolates and incubated at 30^{o}C for 48 hours. Thereafter 5 to 6 drops of Kovac's

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reagent was added and the solution properly mixed by rotating between the palms of both hands. Formation of an alcoholic layer with red colouration indicates indole production.

Growth in 4% NaCl Broth

Four grams (4 grammes) of NaCl was dissolved in 100ml of MRS broth respectively, dispensed into screw cap bottles and sterilized. The bottles were inoculated and incubated at 35^{o}C for 48 hours. Turbidity of the medium indicates growth, while uninoculated bottles served as control.

Starch Hydrolysis

Equimolar amount of soluble starch was prepared and added to MRS agar without glucose or meat extract to give 1% soluble starch agar medium. The medium was sterilized, allowed to cool and poured into sterile petri-plates and left to set. Single streaks of the test isolates were made on the dried plates and incubated at 30^{o}C for 48 hours. The plates were then flooded with Gram's iodine. Unhydrolysed starch formed a blue-black colouration while clear zones around streaked-line indicate starch hydrolysis by the isolates.

Casein Hydrolysis

Skim milk agar was prepared by adding 1% (w/v) skim milk to MRS agar (Harrigan and McCance, 1966). The medium was sterilized by autoclaving at 110^{o}C for 10 minutes. It was dispensed into sterile petri-dishes and left to set. The plates were then streaked across once with the test isolates and incubated for 3 days at 35^{o}C. A clear zone around line of streak indicates casein hydrolysis. Uninoculated plates served as control.

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Gelatin Hydrolysis

Ten milliliters (10ml) of 10% gelatin broth (Harrigan and McCance, 1966) were dispensed into screw cap bottles and sterilized. The bottles were inoculated with the test isolates and incubated at 35^{o}C for 7 days. The bottles were then placed in the fridge for 1 hour, after which they were observed. Solidification of the broth indicates unhydrolysed gelatin while broth not changed after placing in the fridge indicates hydrolysed gelatin.

Nitrate Reduction Test

Five milliliters of nitrate peptone water medium was used, it was dispensed into screw cap tubes with inverted Durham tubes and sterilized. The tubes were then inoculated with test isolates and incubated at 35^{o}C for 4 days. 0.5ml of 1% sulphanillic acid in 5ml acetic acid following by 0.5ml of 0.6% dimethyl-napthylamine in 5ml acetic acid was added to each tube. The development of red coloration and production of gas in Durham tubes indicates the production of nitrogen (Payne, 1973).

Production of Ammonia from Arginine

A modified MRS broth (MRS-arginine broth) without glucose and meat extract, but containing 0.3% arginine and 0.2% sodium citrate instead of ammonium citrate was used. The MRS broth without arginine was used as control. 18-hour old cultures were inoculated into already autoclaved 10ml broth in screw cap bottles and incubated at 30^{o}C for 4 days. Test samples of culture medium after growth was placed on a spot plate to which Nessler's reagent was added drop-wise. Cultures producing a deep yellow to brown colour indicate the production of ammonia from arginine while a yellow coloration indicates a negative reaction (Doring, 1988).

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Homofermentative/Heterofermentative Test

The semi-solid medium of Gibson and Abdelmalek (1945) as modified by Stainer *et al.*, (1964) was used. Twenty milliliters of the medium was dispensed into screw cap bottles and sterilized. The test organisms were inoculated in replicates and a sterile agar seal was poured onto the medium in each bottle. This was incubated at 35^{o}C for 14 days. Observations were made daily. Production of gas bubbles or forcing of the agar seal up the tubes indicates the presence of heterofermenters while the absence of gas indicates the presence of homofermenters.

Oxidative/Fermentative Test

Fifteen milliliters of Hugh and Leifson (1953) medium was dispensed into screw cap tubes and sterilized before being inoculated with test isolates in duplicates. A set of the tubes was covered with sterile vaspar (paraffin + wax) and the other set was left without vaspar seal. This was incubated for 5 days at 35^{o}C. Acid production is shown by colour change from blue to yellow. Fermentative isolates will have produced acid in both tubes while oxidative isolates will produce acid only in tubes without vaspar seal.

Sugar Fermentation Test

Modified MRS medium from which meat extract and glucose had been omitted (Sharpe *et al.*, 1966), but containing 0.05% (w/v) bromocresol purple indicator as basal medium. Filter sterilized solutions of the carbohydrates were added to a final concentration of 2%. Twenty milliliters of the solution was dispensed into screw cap tubes with Durham tubes inverted into each and sterilized. The test isolates were inoculated into the tubes and incubated at 30^{o}C for 4 days. A change in colour from purple to yellow indicated a positive result and a displacement of solution by air or gas production in the Durham tubes.

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Growth at Different pH

MRS broth with its pH adjusted to 4.5 and 9.6 using 0.1N Hcl was used. 10ml of the medium was dispensed into screw cap bottles and sterilized. The test isolates were then inoculated into the cooled medium and incubated at 30^{o}C for 48 – 120 hours. Turbidity of the broth as compared with uninoculated bottles was used as indicator of growth by the isolates.

Growth at Different Temperature

MRS broth was sterilized and inoculated with the test isolates and incubated at 15^{o}C respectively for 4 days. Turbidity of the medium as compared with uninoculated control bottles served as indicator of growth by the isolates.

Haemolysis of Blood

20ml MRS agar was sterilized and allowed to cool down to 45^{o}C in screw cap bottles. 1 – 2ml of blood was aseptically added to the molten-agar and thoroughly mixed by gently rotating between the palms of both hands, before being poured into a sterile petri-plate and allowed to set. The test isolates were streaked onto each plate and incubated, anaerobically for 48 hours at 37^{o}C. Streptococcal colonies appeared with clear zones around them, which present, indicating haemolytic activity due to the presence of haemolysin, which causes lysis of red blood cells.

Identification of Isolates

This was done with the aid of Bergey's Manual of Systematic Bacteriology (Sneath, 1986) volume 2 by comparing morphological and biochemical characteristics obtained from each isolate.

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Screening for Protease Production

Selection of Test Organism

Three of the isolates namely – *Streptococcus pyogenes, Lactobacillus acidophilus* and *Lactococcus lactic* were chosen due to their high protease production.

Determination of Inoculum Size/Cell Count

This was done using pour-plate method. Each isolate was sub-cultured onto fresh slants and 1ml sterile distilled water was used to wash the slant surface in order to obtain cell suspension of the isolates. The 1ml cell suspension was then serially diluted in bottles containing sterile distilled water to a dilution factor of 10^6 for each isolate. The dilutions 10^5 and 10^6 were then plated out and incubated at 35^{o}C for 24 hours, after, which distinct colonies were counted and the figures rounded up to the nearest decimal points.

Medium Inoculation and Enzyme Production

200ml each of MRS broth was sterilized at 121^{o}C for 15 mins before being left to cool down. They were then inoculated with 1ml aliquot of inoculum each containing a load of 1.0 x 10^6 cfu/ml and incubated at 35^{o}C for 96 hours. After incubation, the growth (turbidity) defined as the Optical Density was determined using Pye-Unicam SP6 – 250 visible spectrophotometer at 540nm wavelength. It was then centrifuged at 10,000rpm at 4^{o}C for 20mins. The filtrate was then assayed for its protein concentration and proteolytic activity using visible spectrophotometer at 660nm wavelength.

Optimization of Cultural Conditions for the Production of Protease

This was done by varying different parameters that affect the production of protease to determine the optimum conditions for production.

Effect of Different Nitrogen Sources on Protease Production

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This was carried out by substituting the nitrogen source in the chemically – defined medium (CDM) with the different nitrogen sources. 10ml each of the mediums was dispensed into screw – capped tubes and sterilized. They were inoculated with the test organisms and incubated at 35^{o}C for 48 hours for enzyme production. At the end of incubation period, the broth cultures were centrifuged at 10,000 rpm for 15 minutes; the enzyme extracted and supernatant was used for enzyme assay.

Effect of Temperature and Incubation period on Protease Production

20ml of the CDM with the best nitrogen source was dispended into screw – capped tubes and sterilized. The tubes were allowed to cool, and each tube was inoculated with the test organisms. Incubation was done at 15^{o}C, 28^{o}C, 30^{o}C, 35^{o}C and 40^{o}C for 24 hours, 48 hours, 72 hours and 96 hours for enzyme production. The broth cultures were centrifuged, the enzyme extracted and the supernatants were used for enzyme assay.

Effect of pH and Inoculum Concentration on Protease Production

The CDM with the best nitrogen source used for the cultivation of isolates was prepared and adjusted to pH 4.0, 4.5, 5.0, 5.5, and 6.0 by use of 0.1N HCI and 0.1M NaOH. 10ml of the different CDM was dispensed into screw – capped tubes and sterilized. After cooling, the tubes were all inoculated with different inoculum concentrations of 0.10ml, 0.15ml, 0.20ml and 0.25ml. They were all incubated at the best temperatures and for the best incubation periods of each of the organisms. At the end of incubation period, the broth cultures were centrifuged, the enzyme extracted and the supernatant used for enzyme assay.

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Characterization of Produced Protease Enzyme

Effect of Substrate Concentration and Protease Activity of Isolate

This was done by varying the concentration of casein which was the substrate using the method of Keay *et al.*, (1970). 1%, 2%, 3%, 4% and 5% (w/v) casein solution were prepared in 0.2M-citrate phosphate buffer (pH5.5). The casein solutions were heat – denatured at 100^{o}C for 15 minutes in a water bath. These were then used to carry out protease activity.

Effect of Enzyme Concentration on Protease Activity of Isolate

The effect of enzyme concentration on protease activity was done using the Keay *et al.*, (1970) method. 1% casein solution was prepared in 0.2M-citrate phosphate buffer (pH5.5). The casein concentrations of the enzyme preparation were used, which were 0.5ml, 1ml, 1.5ml, 2.0ml and 2.5ml. Protease assay was carried out using the different concentrations of enzymes extracts.

Effect of amino Acid Concentration on Protease Activity of the Isolate

The effect of amino acid concentration on protease activity of the isolate was determined using Keay *et al.*, (1970) method. The amino acids used were Lysine, Serine, Leucine, Tyrosine, Tryptophan, Panthetonic acid, Guanine, Methionine and Glutamic acid. Protease assay was carried out by adding o.1ml, 0.2ml, 0.3ml, 0.4ml and 0.5ml of the amino acids to the reaction mixture of 0.5ml enzyme preparation and 1ml of 1% (W/v) casein solution.

Effect of Metal Ions on Protease Activity of the Isolate

This was done by using various concentrations of cations at concentrations of 0.1M, 0.2M, 0.3M, 0.4M and 0.5M. Protease assay was carried out by adding 0.1ml of each cation

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to the reaction mixture of 0.5ml enzyme preparation and 1ml of 1% (w/v) casein. Solution followed by the main procedure as earlier described.

Effect of Temperature on Protease Activity of the Isolate

This was determined by using Keay et al., (1970) method. 1% (W/v) casein solution was prepared in 0.2M-citrate phosphate buffer (pH 5.5). Protease assay was carried out by using 0.5ml of the enzyme preparation with 1ml of 1% (W/v) casein solution and the reaction mixture were incubated at 15^{o}C, 28^{o}C, 30^{o}C, 35^{o}C, 37^{o}C, 40^{o}C for 1 hour.

Effect of pH on Protease Activity of Isolate

1% casein solution were prepared in 0.2M citrate phosphate buffer at different pH of 3, 3.5, 4, 4.5, 5.5 and 6.0 obtained by the addition of 0.1N HCI or 0.1M NaOH as appropriate. The casein solutions were then heat – denatured at 1000^{o}C for 15 minutes in a water bath and allowed to cool. Protease assay as carried out using the casein solutions of different pH as the substrate.

Effect of Inhibitors on the Protease Activity

This was determined by using various concentrations of the inhibitors. The inhibitors used were Nitrophenol, urea and Ethylene Diamine Tetracetic acid (EDTA) at concentrations of 0.1M, 0.2M, 0.3M, 0.4M and 0.5M. Protease assay was carried out by adding 0.1ml of each inhibitor to the reaction mixture of 0.5ml enzyme preparation and 1ml of 1% (w/v) casein solution.

Protease Assay

Protease activity was measured using casein (BDH) as substrate in 0.1M citrate phosphate buffer (pH 5.5) (Kunitz, 1946). 1% (w/v) casein solution was prepared using the

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buffer heat-denatured at 100^{o}C for 15 minutes in a water bath and allowed to cool. 1ml of the substrate was then thoroughly mixed with 0.5ml of the enzyme extract and incubated for 1 hour at 35^{o}C after which the reaction was terminated by adding 3ml of 2^{o}C cold 10% Trichloroacetic acid (TCA). The bottles were then allowed to stand for one hour at 2^{o}C to allow undigested protein to precipitate. The mixture was then centrifuged at 10,000rpm at 4^{o}C for 5 minutes. Optical Density visible spectrophotometer at 660m wavelength against a blank containing the control. Control bottles contained 1ml of substrate (incubated at 35^{o}C for 1 hour) and 3ml of 2^{o}C cold 10% TCA subjected to the same conditions as the reaction bottles. One protease unit is defined as the amount of enzyme, which catalyzes the release of 1µmol casein per minute.

Protein Estimation

With respect to the modified Lowry Folin-Ciocalteau's method (Lowry *et al.*, 1951), Reagent A containing 2grains of anhydrous sodium carbonate (Na_2CO_3) dissolved in 100ml of 0.1M sodium hydroxide (NaOH) was mixed with Reagent B containing 1 gram of sodium tartarate and 0.5gram of cupric sulphate ($CuSO_4$) dissolved in 100ml distilled water, to obtain Reagent C. While Reagent D contained Folin's reagent. To 0.1ml of enzyme filtrate was added 0.5ml sterile distilled water, 3ml of Reagent C and 0.3ml of Reagent D, the mixture was thoroughly mixed and incubated at room temperature for 30 minutes. Optical Density readings of the resulting coloured solution were measured with a Pye-Unicam SP6-250 spectrophotometer at 660m against a blank control containing distilled water.

Production of Sodom Apple (Calotropis) Extracts

Freshly harvested leaves of Sodom apple *Calotropis procera* were obtained from residential areas around Akobo (a residential area of Ibadan) and within the University of

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Ibadan campus. Four different extraction methods were employed to produce crude extract, ethanolic extract, methanolic extract and water-soluble extract.

Crude Extract

A traditional enzyme extraction method (Akinloye *et al.*, 2002) was used to obtain the crude extract. One hundred grams of the leaves of Sodom Apple was obtained and rinsed thoroughly under a running tap. It was then dried and macerated before being transferred into a beaker containing 300ml of distilled water and boiled for 20 minutes. After boiling, it was allowed to cool and then filtered using Whatman's No. 1 Filter paper. A yellowish filtrate with a vegetable smell was obtained.

Production of Alcoholic Extracts

Ethanolic and Methanolic extracts were obtained using the methods which employs a Soxhlet extraction unit comprising the Soxhlet extractor, condenser, steam bath and a quick fit round-bottom flask (Arul *et al.*, 2004; Arya and Kumar, 2005). The solvents used in each case were ethanol and methanol.

1kg each of Sodom apple leaves were washed and dried in an oven at 50^0C for 48 hours. It was then blended till it appeared finely chopped and packed into the extractor, which was set up with the condenser attached to the tap and the quick-fit round-bottomed flask attached underneath. The unit has two side arms; one for the passage of vapour to the condenser and the other, the siphon, connects the extractor to the flask below it.

The solvent was poured into the extractor packed with the leaves so that it covered the top of the leaves up to the level of the side arm connecting the extractor to the round-bottomed flask, which was placed on the steam bath for heating. The solvent seeped through the leaves into the flask from where it vaporizes through the side arm of the extracts to the condensers.

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The condenser was connected to a water tank that cools the vapour causing it to condense and drop onto the sample. It leached out all the soluble materials and siphons down into the extraction flask where the non-volatile leaf extracts accumulates and the volatile solvent vapourizes. The solvent was used continuously until all the solvent-soluble components of the leaves got leached and accumulated in the round-bottomed flask with the solvent.

The mixture (solvent and plant extract) is distilled to obtain the pure extracts of the leaves and also to recover the solvent. The ethanolic extract is dark-green in colour while the methanolic extract is greenish yellow in colour.

Purification of microbial protease and Plant Extracts

The protease enzyme and Sodom apple extracts partially purified using Ammonium sulphate precipitation and Acetone precipitation methods.

Ammonium Sulphate Precipitation

Protease enzyme was partially purified by treating the enzyme extract with 24.3g, 28.5g and 15.7g of ammonium sulphate to 0-40%, 40-80% and 80-100% saturation respectively (Dixon and Webb, 1971). On ice, weighed amount of ammonium sulphate was stirred into the enzyme extract to avoid local areas of high salt concentration. It was stirred continuously for 20 minutes and allowed to sit at 4^{o}C while mixing occasionally until the salt was dissolved. The mixture was left to stand at 4^{o}C for 24 hours; it was then centrifuged at 10,000 rpm for 15 minutes. The supernatant was further treated to the next step of saturation until the final step; was reached. The final precipitate was dissolved in 100ml of 0.1m citrate phosphate buffer pH 5.5. it was then dialyzed with the buffer for 18 hours at 4^{o}C (Olutiola and Akintunde, 1979).

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Acetone Precipitation

Four volumes of cold acetone was added to one volume of enzyme extract, it was mixed thoroughly and left to stand at 20^{o}C for at least 90 minutes, after which it was centrifuged at 15,000 rpm for 15 minutes at 4^{o}C. The final precipitate was then dissolved in 100ml of 0.1M citrate phosphate buffer pH 5.5 (Wessel and Flugge, 1984).

Preparation of Sephadex

Two different grades of Sephadex were used. These are G-100, and C – 50. The column of sephadex was prepared according to the method described by Olutiola and Cole (1980) and was surrounded by a water jacket at 4^{o}C. 10g of each grade of Sephadex were weighed with 0.97g, of sodium oxide (NaN_3) into a sterile container. 100ml of sterile distilled water and 200ml of 0.2M citrate phosphate buffer was added and stirred continuously. It was soaked and kept at 4^{o}C for 3 days. The soaked sephadex was loaded into the column using a funnel. It was allowed to compact and the supernatant (buffer) was removed using a pipette.

Fractionation on Sephadex G – 100

The column containing the compacted Sephadex G – 100 surrounded by water jacket at 4ºC was equilibrated with 0.1M citrate phosphate buffer (pH 5.5). 2ml of the enzyme concentrate was applied to the column and eluted with 0.1M citrate phosphate buffer. The eluted fractions were collected in a calibrated 5ml tubes. Each eluted fractions was analyzed for protease activity and protein content.

Fractionation on Sephadex C – 50

Fractions that showed appreciable protease activity after passing through sephadex G-100 were combined and applied to the column of sephadex C – 50. 2ml of each of the

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fractions were applied to the column and eluted with 0.1M citrate phosphate buffer. The eluted fractions were collected in calibrated 5ml tubes and analysed for protease activity and protein content.

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RESULTS AND DISSCUSSION

Seven (7) isolates of lactic acid bacteria were obtained from West African soft cheese "Wara" samples. The isolates were identified as *Streptococcus lactis, Streptococcus pyogenes, Lactobacillus acidophilus, Lactobacillus brevis, Leuconostoc mesenteroides, Lactococcus lactis* and *Streptococcus faecalis.* The isolates were subjected to various morphological and biochemical tests. Proper identification was done with reference to Bergey's Manual of Systematic Bacteriology (Sneath, 1986).

Cultural and Morphological Characterization

The cultural and morphological characteristics of the isolates are shown in Table 1 while the biochemical characteristics are shown in Table 2. Seven out of the ten isolates were gram-positive cocci while two were rods and only one was coccobacilli. All the isolates grew at 4% NaCl and hydrolysed casein, none hydrolysed starch except for *Lactobacillus acidophilus*, all produced ammonia from arginine except for *Lb. acidophilus and Leuconostoc mesenteroides*, all were negative to blood haemolysis except for *Streptococcus pyogenes* and *Streptococcus faecalis*. Only *Leuconostoc mesenteroides* produced gas from glucose while the rest produced acid.

Frequency of occurrence of the isolates is shown in Table 3. *Streptococcus pyogenes, Streptococcus faecalis* and *Lactococcus lactis* all had the same number of occurrence (20%) which was higher than the occurrence of the remaining isolates.

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Table 1. Cultural and Morphological Characteristics of Isolates

Sample Codes	Colour	Elevation	Shape	Edge	Surface	Opacity	Isolates
BD1	Cream	Raised	Circular	Entire	Moist	Opaque	*Streptococcus lactic*
BD2	Cream	Raised	Circular	Entire	Moist	Opaque	*Streptococcus faecalis*
BD3	Cream	Raised	Circular	Entire	Moist	Opaque	*Streptococcus faecalis*
BD4	Cream	Raised	Circular	Entire	Moist	Opaque	*Streptococcus pyogenes*
BD5	Cream	Raised	Circular	Entire	Moist	Opaque	*Lactococcus lactis*
OW1	Whitish	Raised	Circular	Entire	Moist	Opaque	*Streptococcus pyogenes*
OW2	Whitish	Raised	Circular	Entire	Moist	Opaque	*Lactobacillus acidophilus*
OW3	Cream	Raised	Irregular	Crenated	Moist	Opaque	*Lactobacillus brevis*
OW4	Whitish	Flat	Circular	Entire	Moist	Opaque	*Leuconostoc mesenteroides*
OW5	Whitish	Flat	Circular	Entire	Moist	Opaque	*Lactococcus lactis*

BD: "Wara" purchased from Bodija Market

OW: "Wara" purchased from Owode Township

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Table 2. Identification of Lactic Acid Bacteria Isolates from West African Soft Cheese "Wara" Samples

Isolates	Grams Reaction	Catalase	Oxidase	Methyl Red	Voges Proskaeur	Starch Hydrolysis	Gelatin Hydrolysis	Casein Hydrolysis	Ammonium from Arginine	Blood Haemolysis	Growth at					O/F Test	Homo/Hetero	Sugar Fermentation										Probable Organisms
											15°C	45°C	pH 4.5	pH 9.6	4% NaCl			Glucose	Lactose	Maltose	Mannitol	Sorbose	Sorbitor	Xylose	Meliboise	Sucrose	Inositol	
BD1	Cocci in pairs and chains	-	-	+	-	-	-	+	+	-	+	+	+	-	+	F	HM	+	+	+	+	+	+	+	+	+	+	*Streptococcus lactis*
BD2	Cocci in pairs and chains	-	-	+	-	-	-	+	+	+	+	+	+	+	+	F	HM	+	+G	+G	+G	+	+G	+	-	+	W+	*Streptococcus faecalis*
BD3	Cocci in pairs and chains	-	-	+	-	-	-	+	+	+	+	+	+	+	+	F	HM	+	+G	+G	+	-	+	W+	-	+G	W+	*Streptococcus faecalis*
BD4	Cocci in pairs and chains	-	-	+	-	-	-	+	+	+	+	-	+	-	+	F	HM	+	+G	+	-	-	-	W+	+	+G	W+	*Streptococcus pyogenes*
BD5	Cocci in pairs chains and cluster	-	-	+	-	-	-	+	+	-	-	+	-	-	+	F	HM	+	+G	+G	+	-	+	W+	W+	+	W+	*Lactococcus lactis*
OW1	Cocci in pairs and chains	-	-	+	-	-	-	+	+	+	+	-	+	-	+	F	HM	+	+	+	-	+	-	W+	+	+	+	*Streptococcus lactis*
OW2	Slender rods in chain	-	-	+	-	+	-	+	-	-	-	+	+	-	+	F	HM	+	+	+G	-	+	-	+	+	+	W+	*Lactobacillus acidophilus*
OW3	Slender rods	-	-	+	-	-	-	+	+	-	+	-	+	+	+	F	HE	+	+G	+	+	-	-	-	+	+	-	*Lactibacillus brevis*
OW4	Cocci with rod-like formation	-	-	+	-	-	-	+	-	-	+	-	+	+	+	F	HE	+G	+G	+	+	+	W+	W+	+	+	+	*Leuconostoc mesenteroides*
OW5	Cocci in pairs, chains and clusters	-	-	+	-	-	-	+	+	-	-	+	-	-	+	F	HM	+	+	+	+	-	+	W+	+	+	W+	*Lactococcus lactis*

+G: Positive with gas production
W+: Weakly positive

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Table 3. Frequency of Occurrence of Isolate

ISOLATES	OCCURRENCE	FREQUENCY(%)
Streptococcus lactis	1	10
Streptococcus pyogenes	2	20
Lactobacillus acidophilus	1	10
Lactobacillus brevis	1	10
Leuconostoc mesenteriodes	1	10
Streptococcus faecalis	2	20
Lactococcus lactis	2	20
Total	10	100

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Growth in Chemically Defined Medium (CDM)

Figure 1 shows the growth of the isolates in chemically defined medium. *Streptococcus lactis* had the highest growth (36.70 units/ml), *Lactobacillus brevis, Leuconostoc mesenteroides, Lactococcus lactis* and *Streptococcus faecalis* all the same growth value (36.50 units/ml), *Lactobacillus acidophilus* (36.30 units/ml) and *Streptococcus pyogenes* (36.10 units/ml).

Figure 2 shows the protein content of crude extract of all the isolates. *Lactococcus lactis* (0.28mg/ml) had the highest protein content, *Streptococcus pyogenes* (0.20mg/ml), *Streptococcus faecalis* and *Streptococcus lactis* (0.19mg/ml), *Lactobacillus acidophilus, Lactobacillus brevis* and *Leuconostoc mesenteroides* (0.18mg/ml).

All the isolates were found to produce protease. Figure 3 shows the protease activity of each isolate. *Streptococcus lactis* produced the highest protease (1.83 units/ml), *Lactobacillus acidophilus* (1.00 units/ml), *Lactococcus lactis* (0.91 units/ml), *Leuconostoc mesenteroides* (0.64, units/ml), *Lactobacillus brevis* (0.54 units/ml), *Streptococcus pyogenes* (0.41 units/ml) and *Streptococcus faecalis* had its highest level of production to be (0.06 units/ml).

Effects of Varying Nitrogen and Carbon Sources on Protease Production

Results of the study of the effect of different nitrogen sources in the presence of various forms of carbon on the growth of three of the ten isolates based on the level of protease production in chemically defined medium is shown in figures 4 to 8. All the nitrogen sources favored the growth of the three isolates. In figure 4 with yeast extract as nitrogen source, *Streptococcus lactis* had the highest protease production (5.02 units/ml) in the presence of glucose as carbon source.

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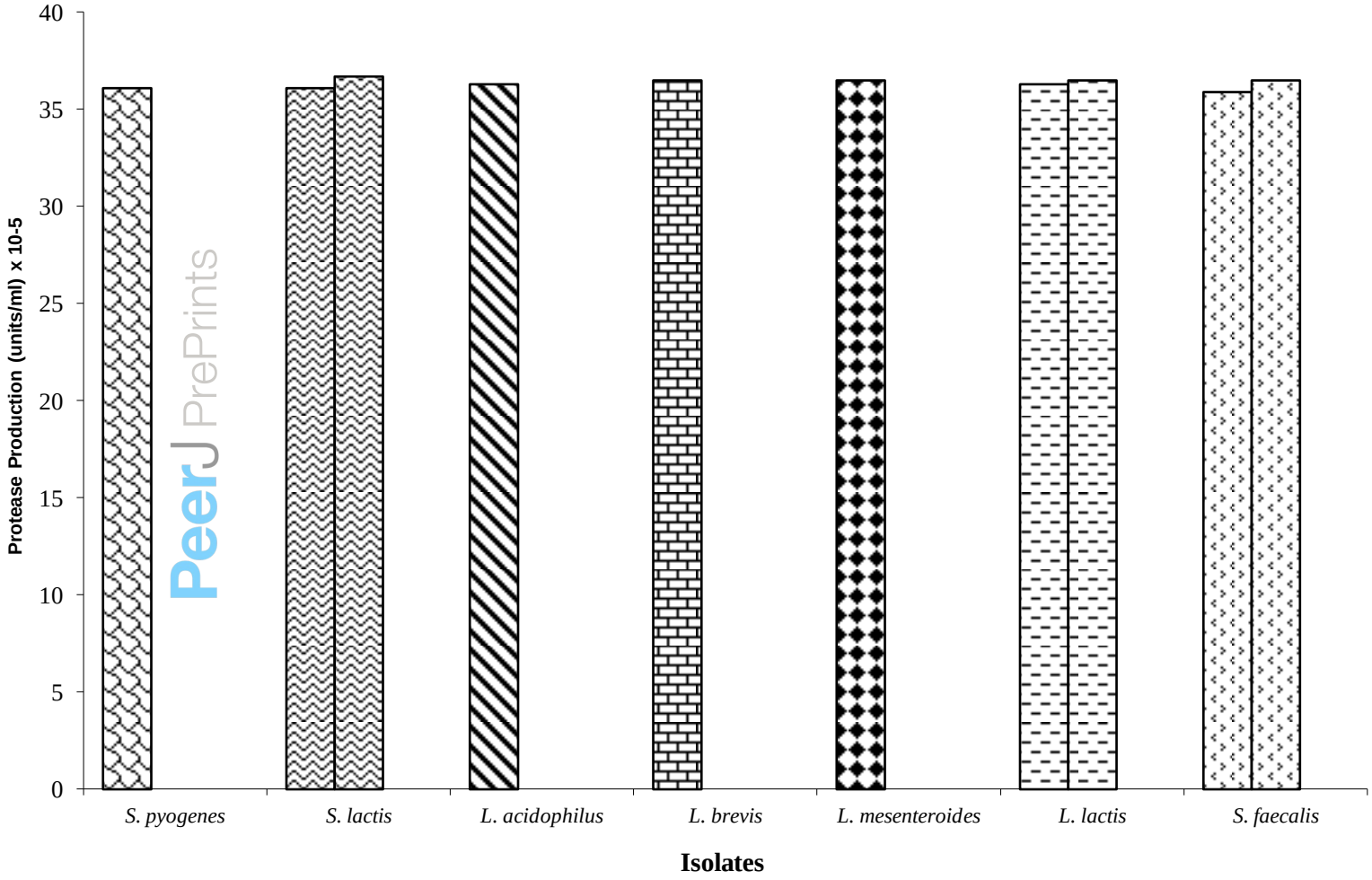

Fig. 1. Growth of Isolates in Chemically Defined Medium (CDM)

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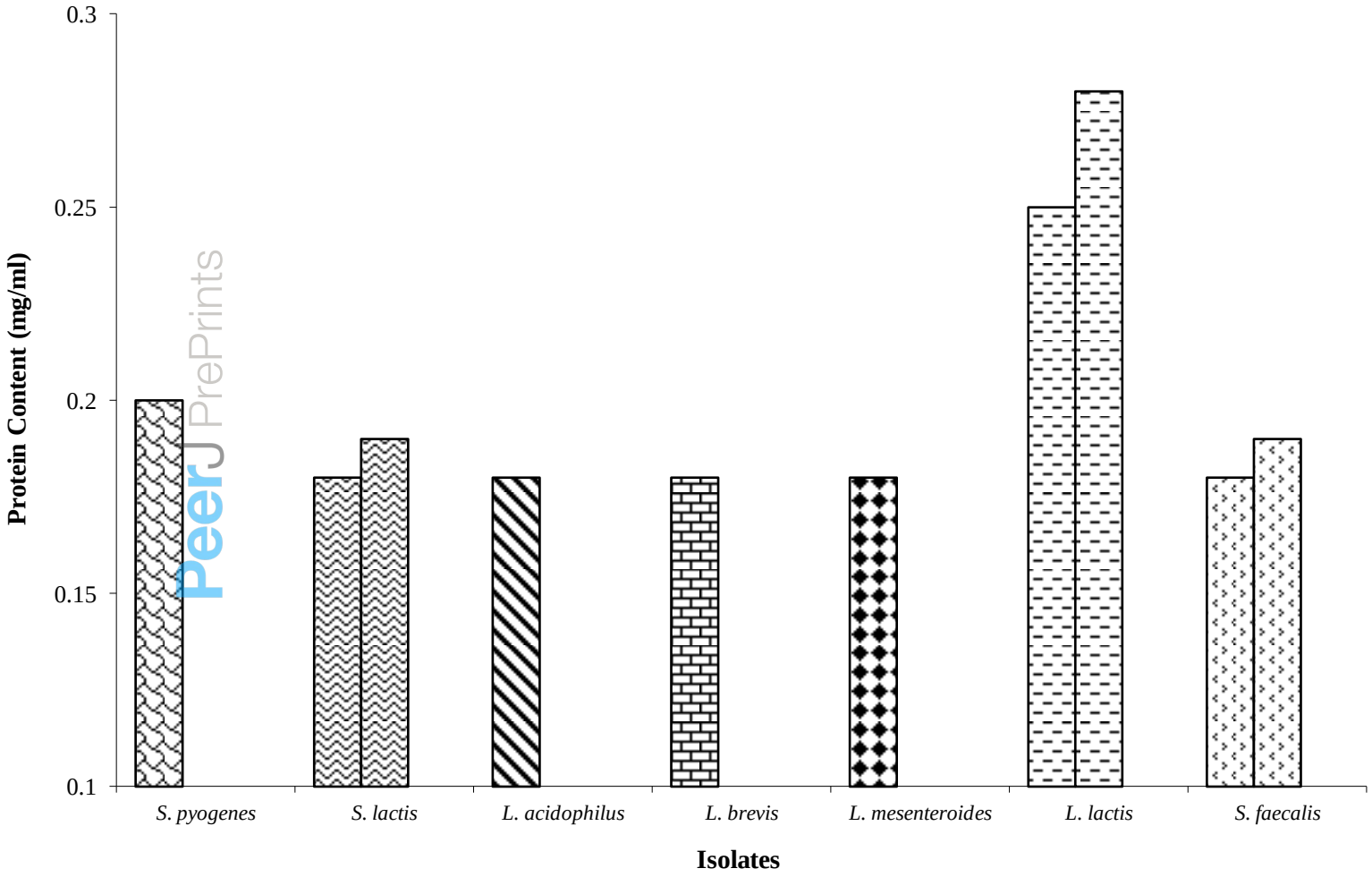

Fig. 2. Protein Content of Crude Extracts of Isolates in Chemically-defined Medium

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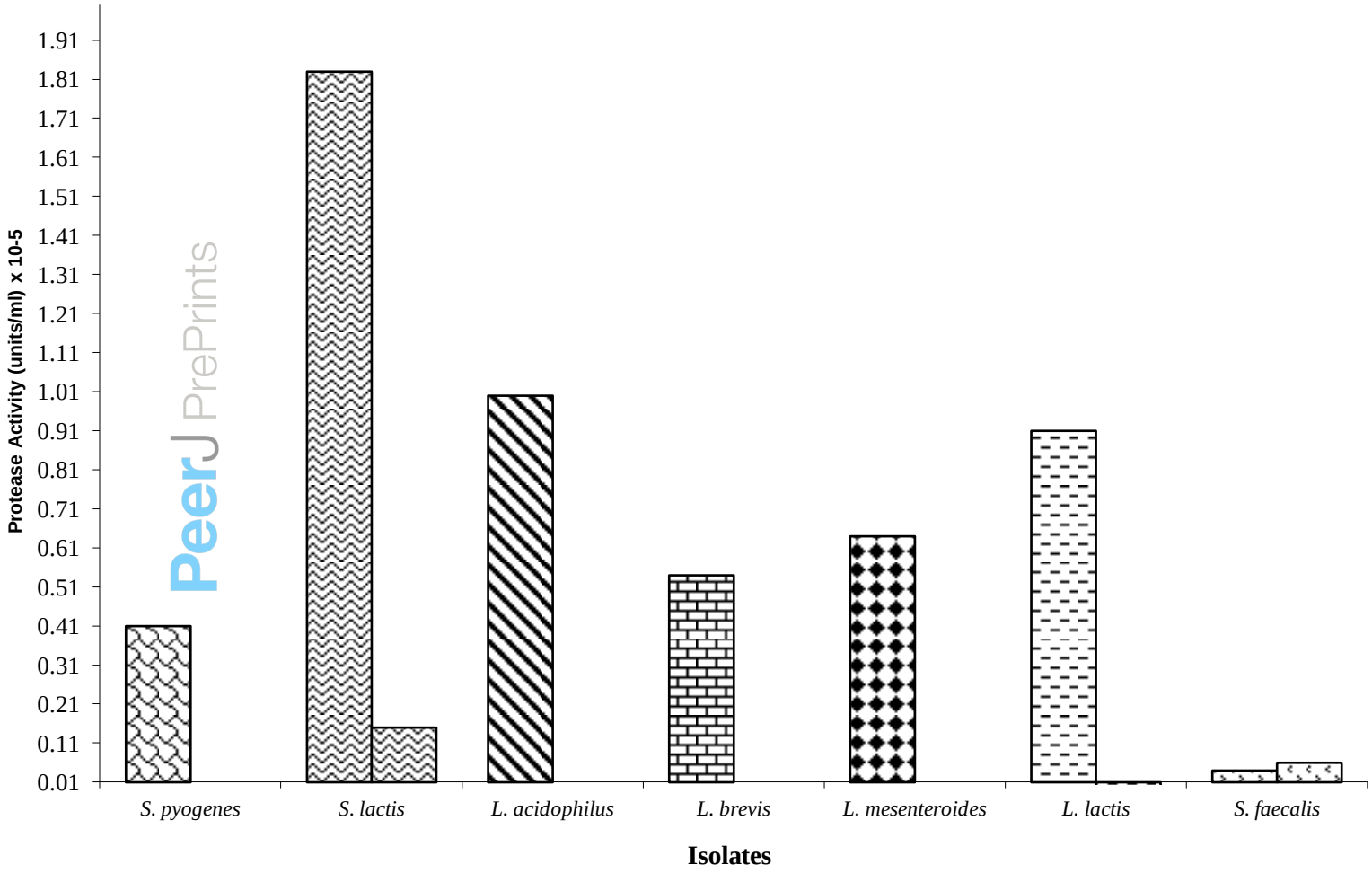

Fig. 3. Protease Activity of Isolates in Chemically-defined Medium

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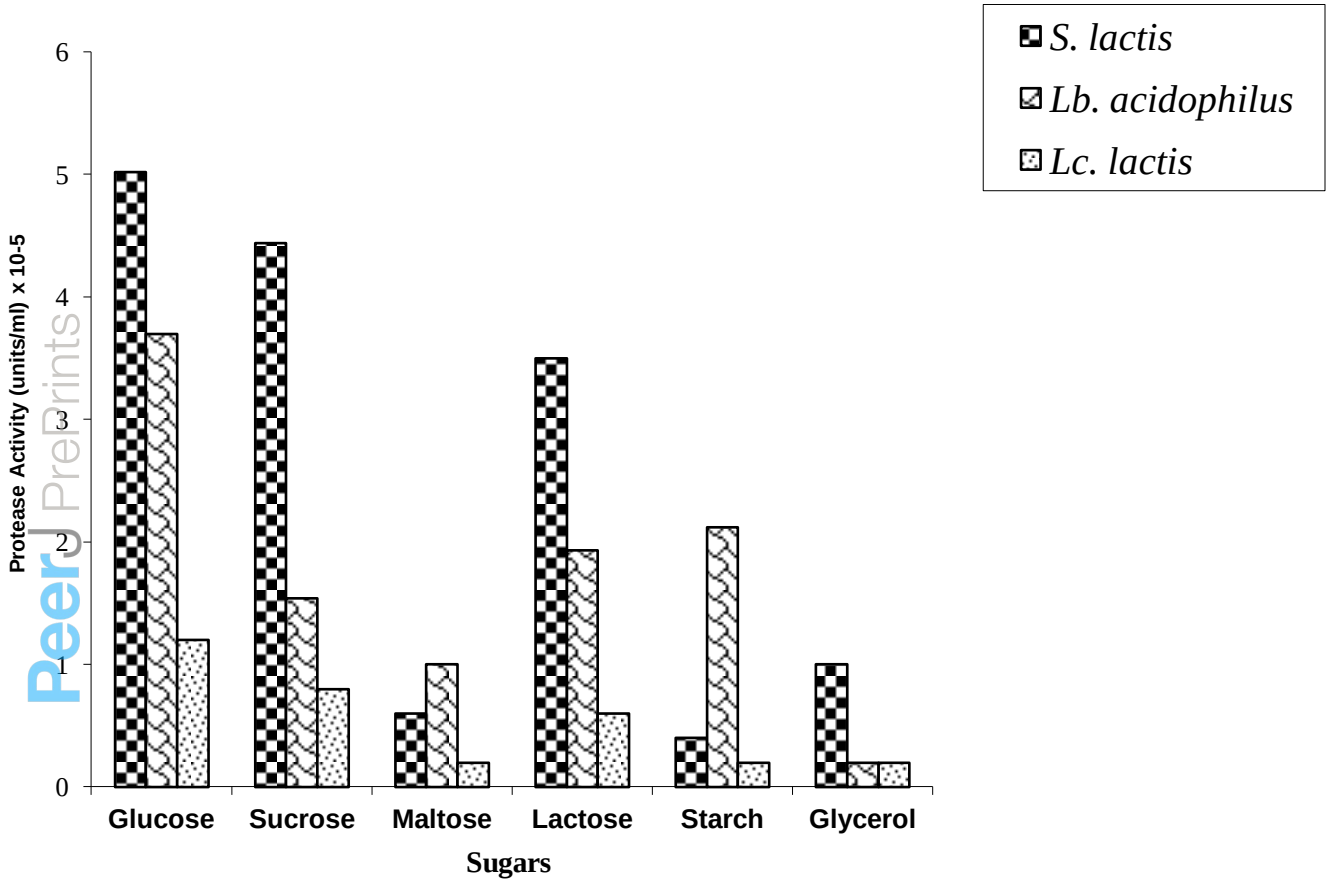

Fig. 4. Effect of Yeast Extract as Nitrogen Source on Protease Production in the Presence of various Carbon Sources by Three Isolates

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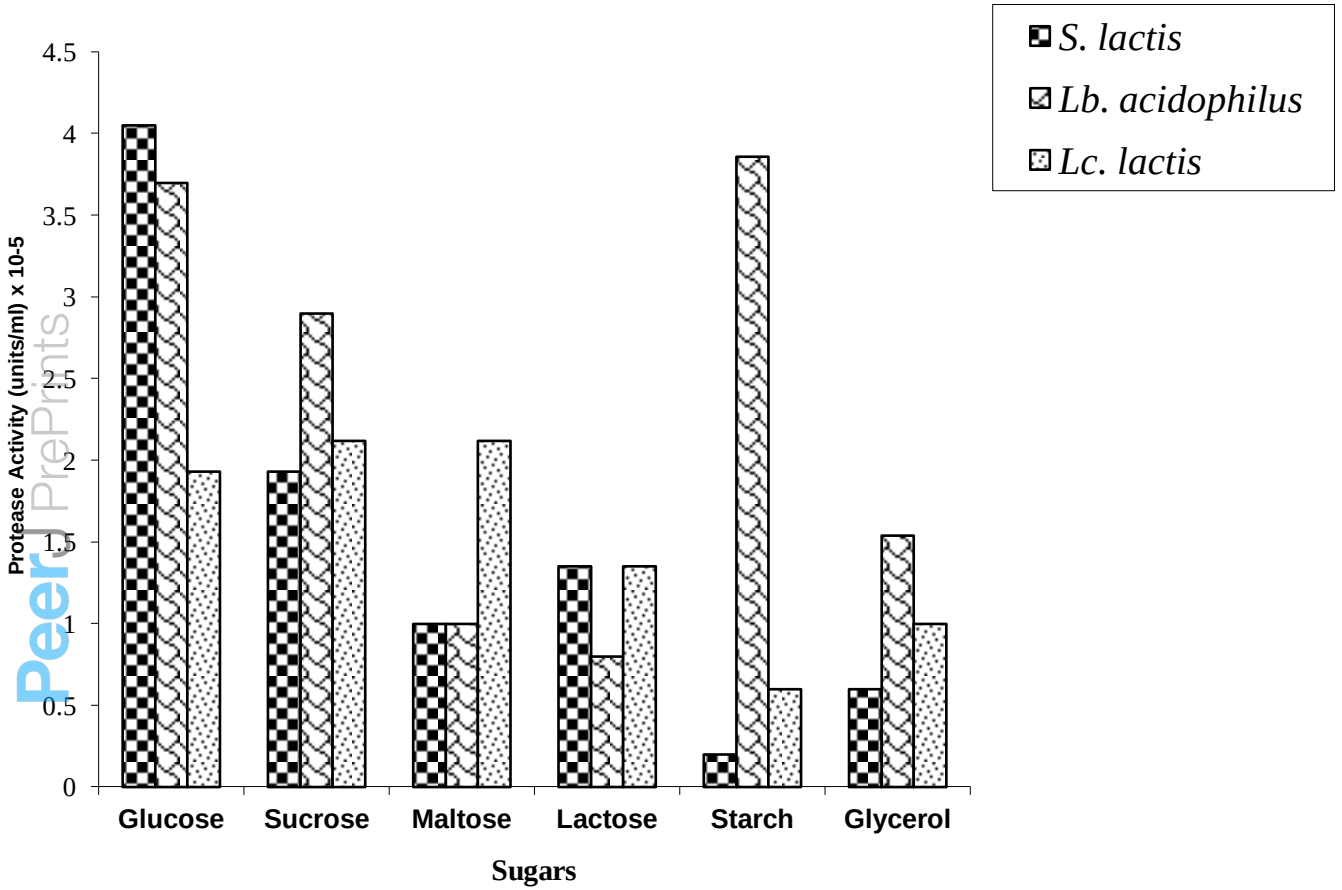

Fig. 5. Effect of Peptone as Nitrogen Source on Protease Production in the Presence of various Carbon Sources by Three Isolates

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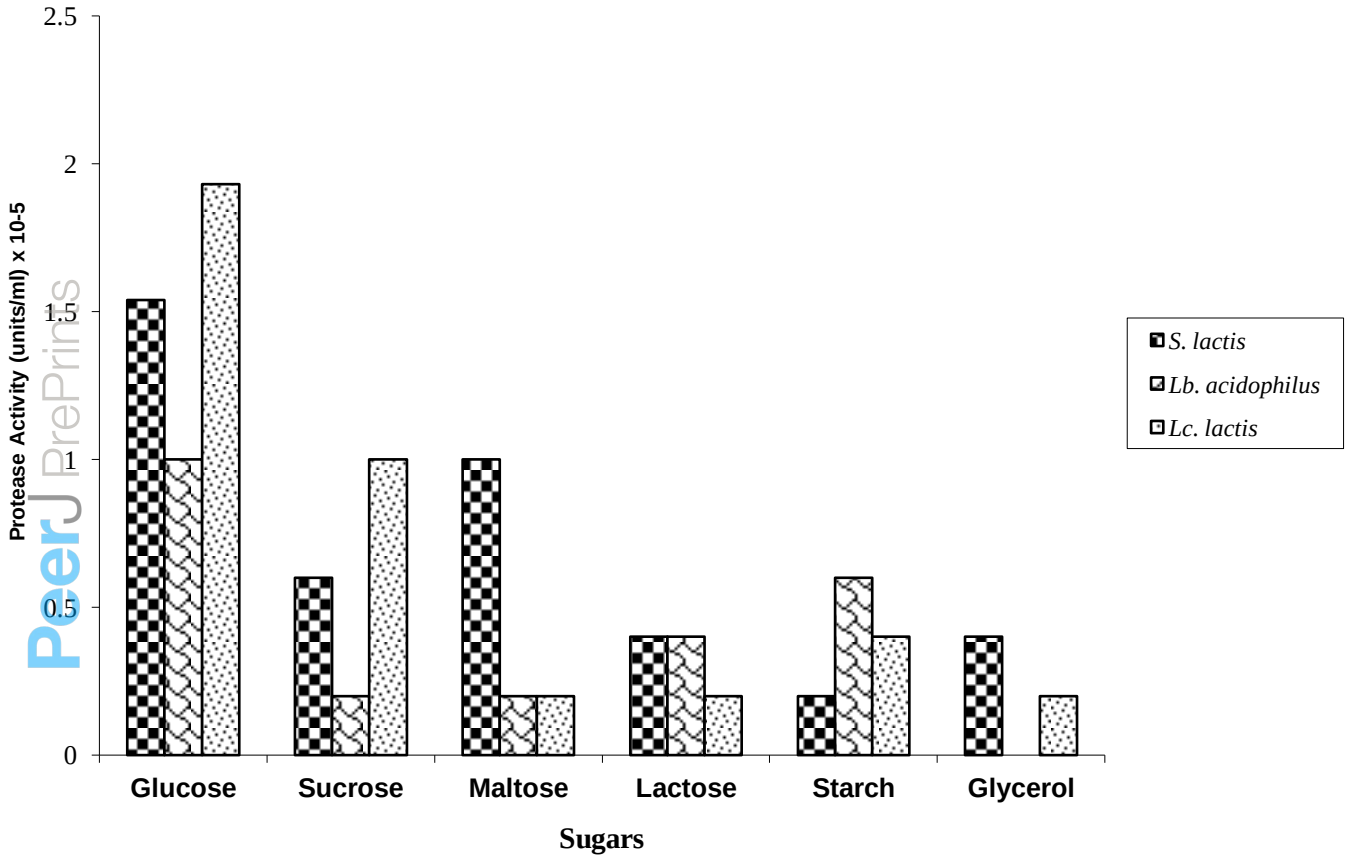

Fig. 6. Effect of Meat Extract as Nitrogen Source on Protease Product Ion in the Presence of various Carbon Sources by Three Isolates

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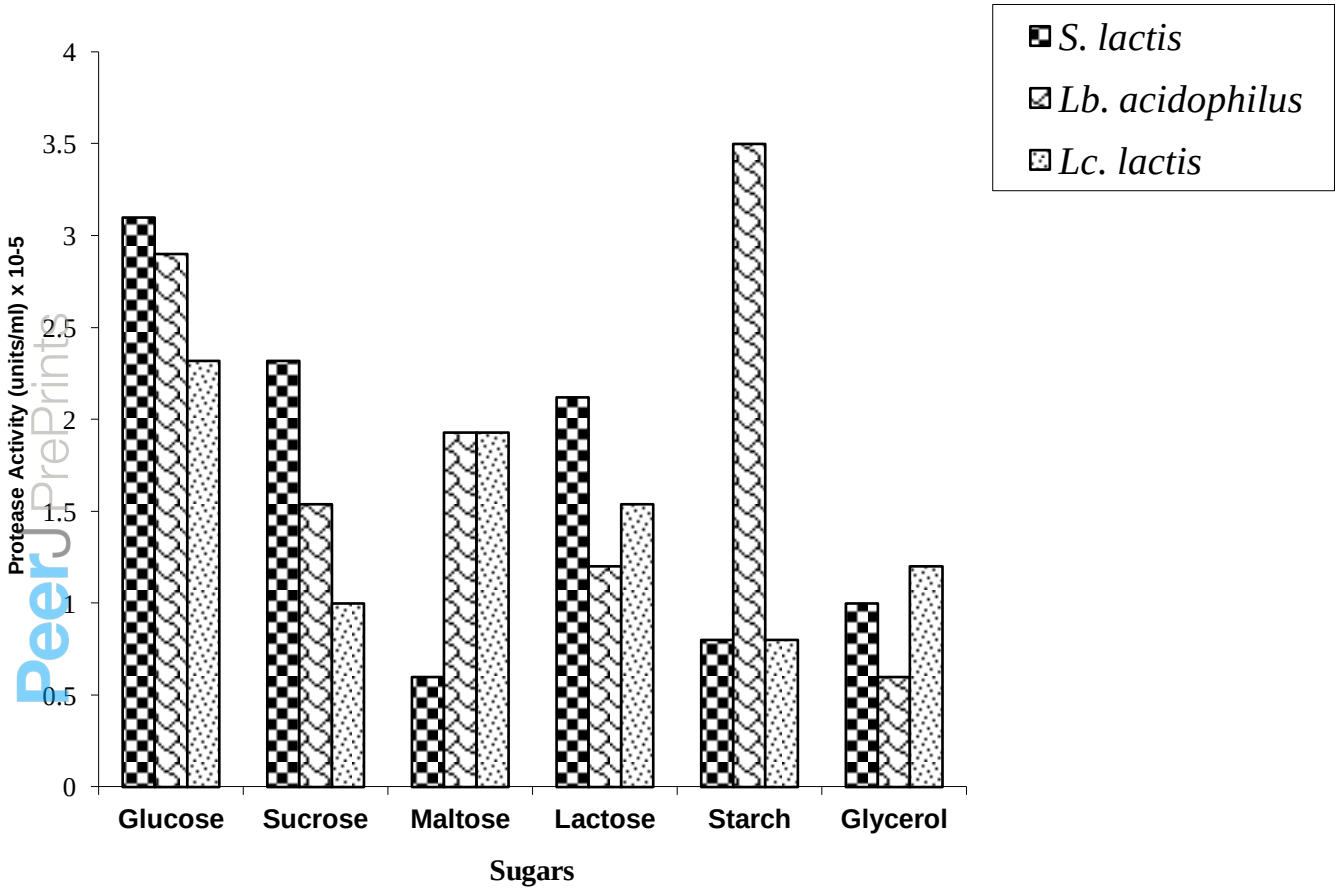

Fig. 7. Effect of Ammonium Citrate as Nitrogen Source on Protease Product Ion in the Presence of various Carbon Sources by Three Isolates

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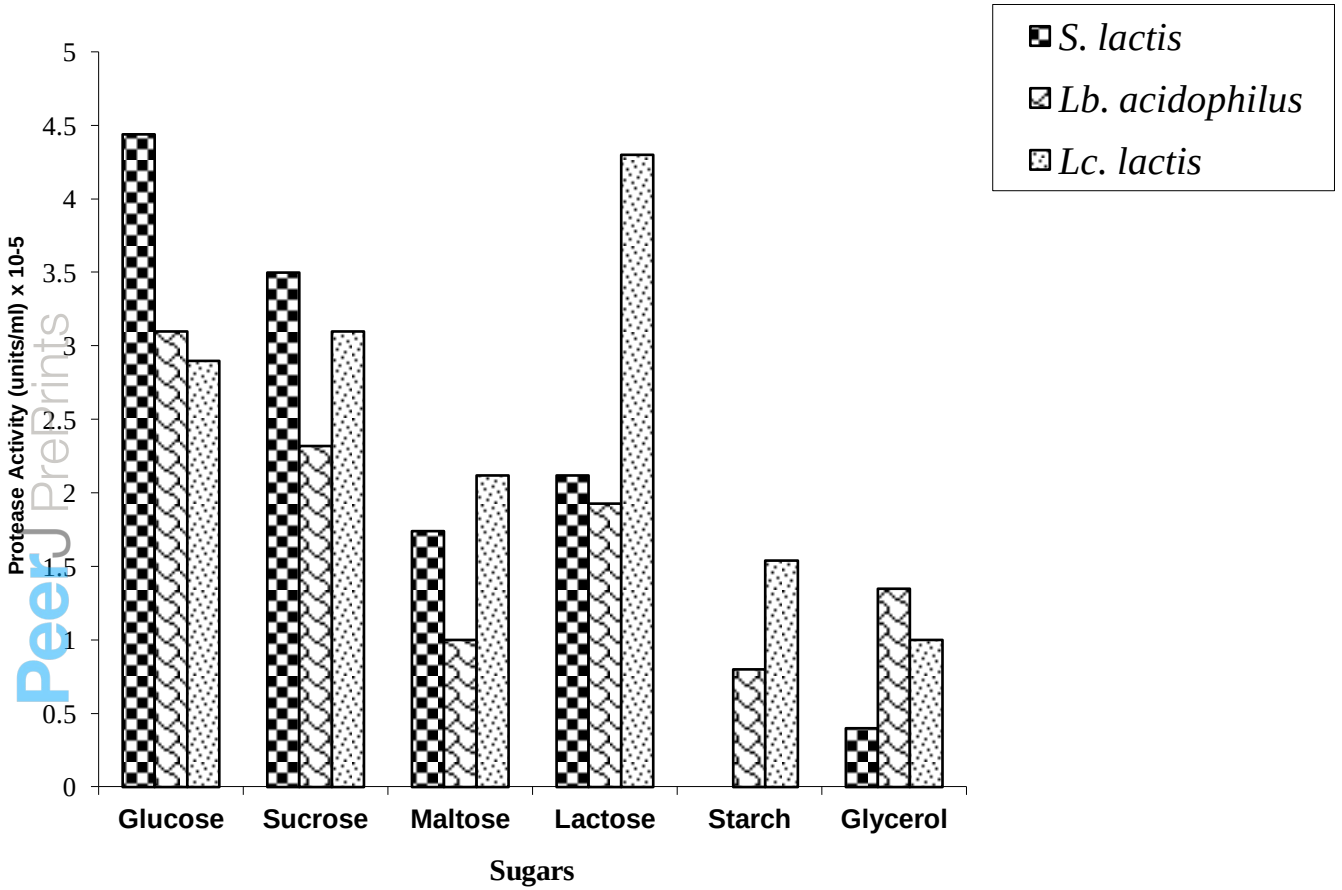

Fig. 8. Effect of Casein as Nitrogen Source on Protease Product Ion in the Presence of various Carbon Sources by Three Isolates

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In figure 5 with peptone as nitrogen source, *Streptococcus lactis* had the highest protease production (4.05 units/ml) in the presence of glucose as carbon source. In figure 6 with meat as nitrogen source, *Lactococcus lactis* had the highest protease production (1.93 units/ml) in the presence of Glucose as carbon source.

In figure 7 with ammonium citrate as nitrogen source, *Lactobacillus acidophilus* had the highest protease production (3.50 units/ml) in the presence of starch as carbon source. In figure 8 with casein as nitrogen source, *Streptococcus lactis* had the highest protease production (4.44 units/ml) in the presence of glucose as carbon source.

Effect of Varying Temperature and Incubation Period on Protease Production

In Figure 9 at 15^{o}C, the protease produced by three isolates each rose gradually between 24 hrs and 48 hrs incubation period and finally had their highest protease production at 72 hrs of incubation before decline started after 96 hrs of incubation. This same trend was noticed at 28^{o}C in figure 10 except that *Lactococcus lactis* and *Lactobacillus acidophilus* had their maximum protease production at 48 hrs incubation period before a decline started at 72 hrs of incubation, but protease production for *Lactobacillus acidophilus* leveled off at 96 hrs incubation period.

In figure 11 at 30^{o}C protease produced by the three isolates rose to a maximum at 48 hrs of incubation before dropping at 72 hrs of incubation. Protease production increased again for *Streptococcus lactis* at 96 hrs incubation while that of *Lactobacillus acidophilus* leveled off at the same hour. This same trend was also noticed in figure 12 at 35^{o}C except while protease production by *Lactococcus lactis* leveled off at the 96th hour that of *Lactobacillus acidophilus* dropped at the same hour.

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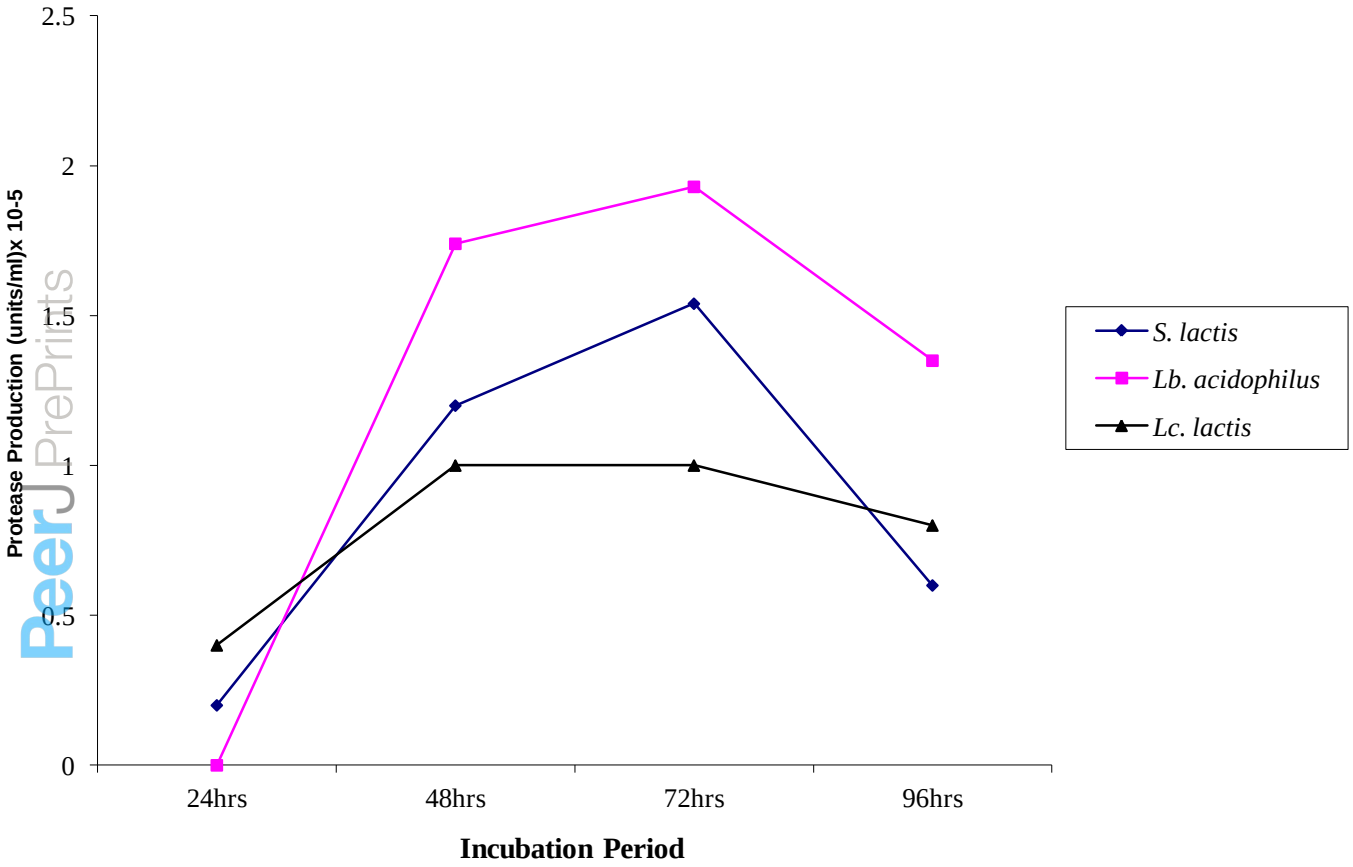

Fig. 9. Time course of Protease Production at 15°C by the three Isolates

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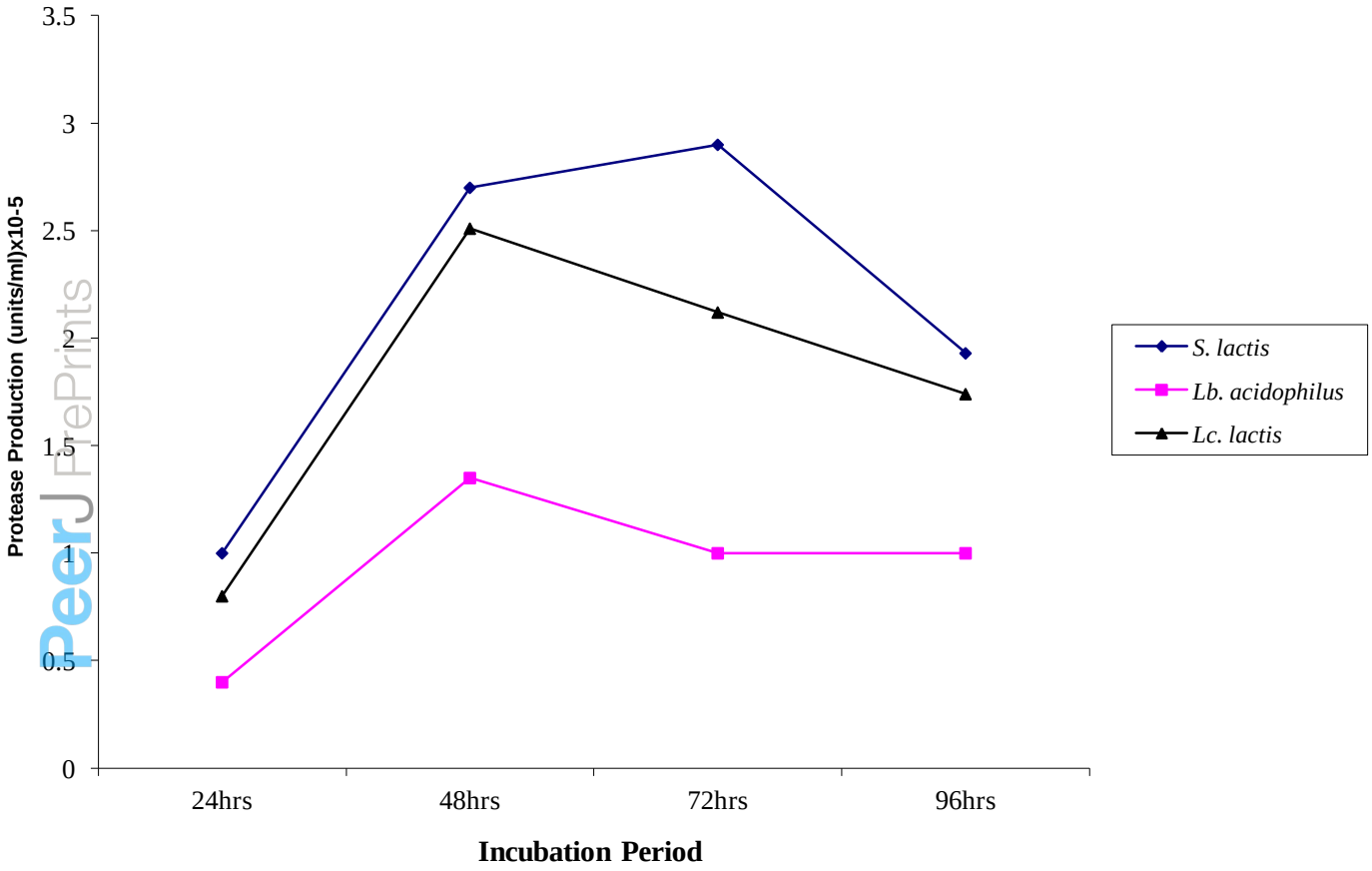

Fig. 10. Time course of Protease Production at 28°C by the three Isolates

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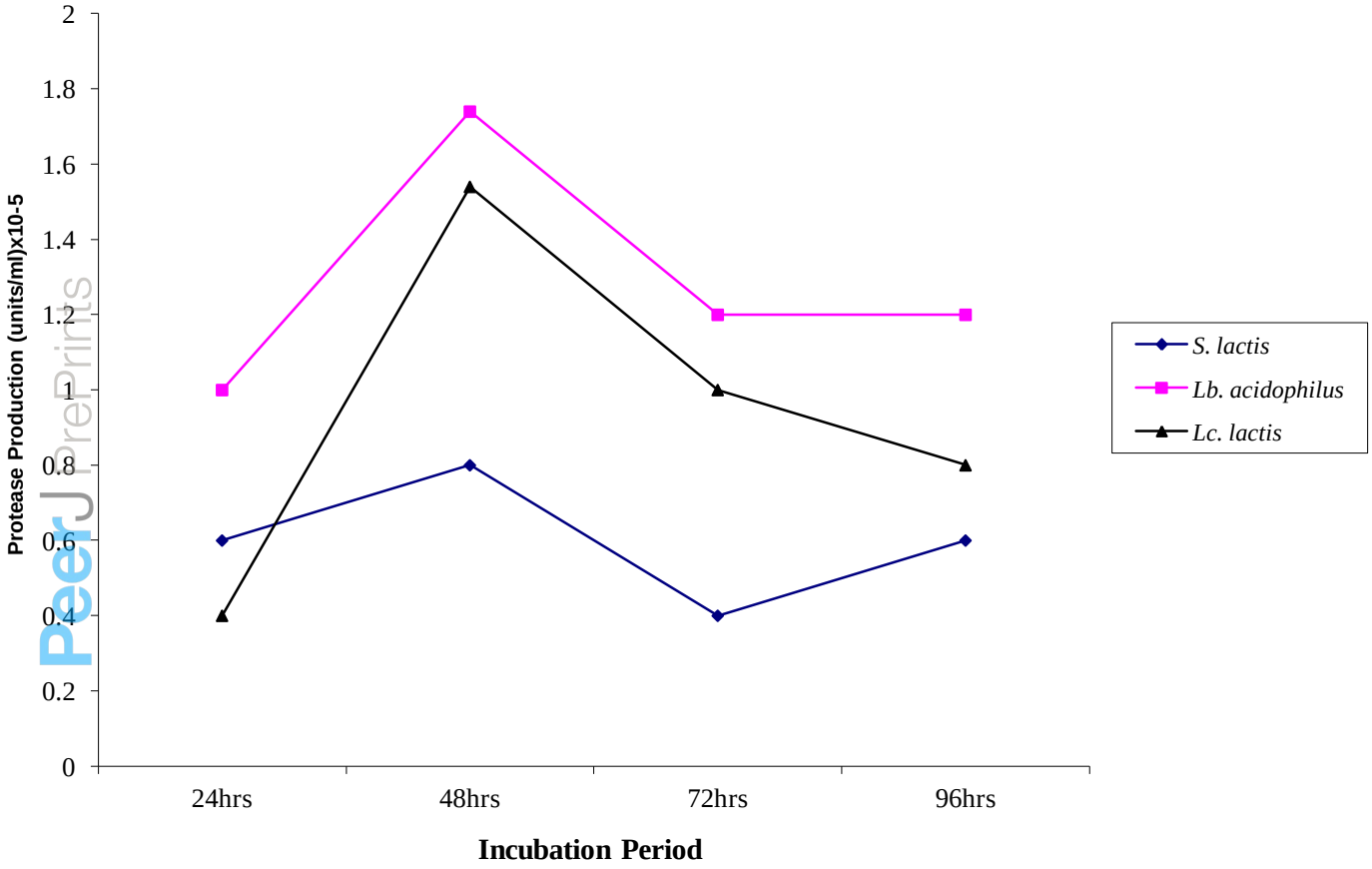

Fig. 11. Time course of Protease Production at 30°C by the three Isolates

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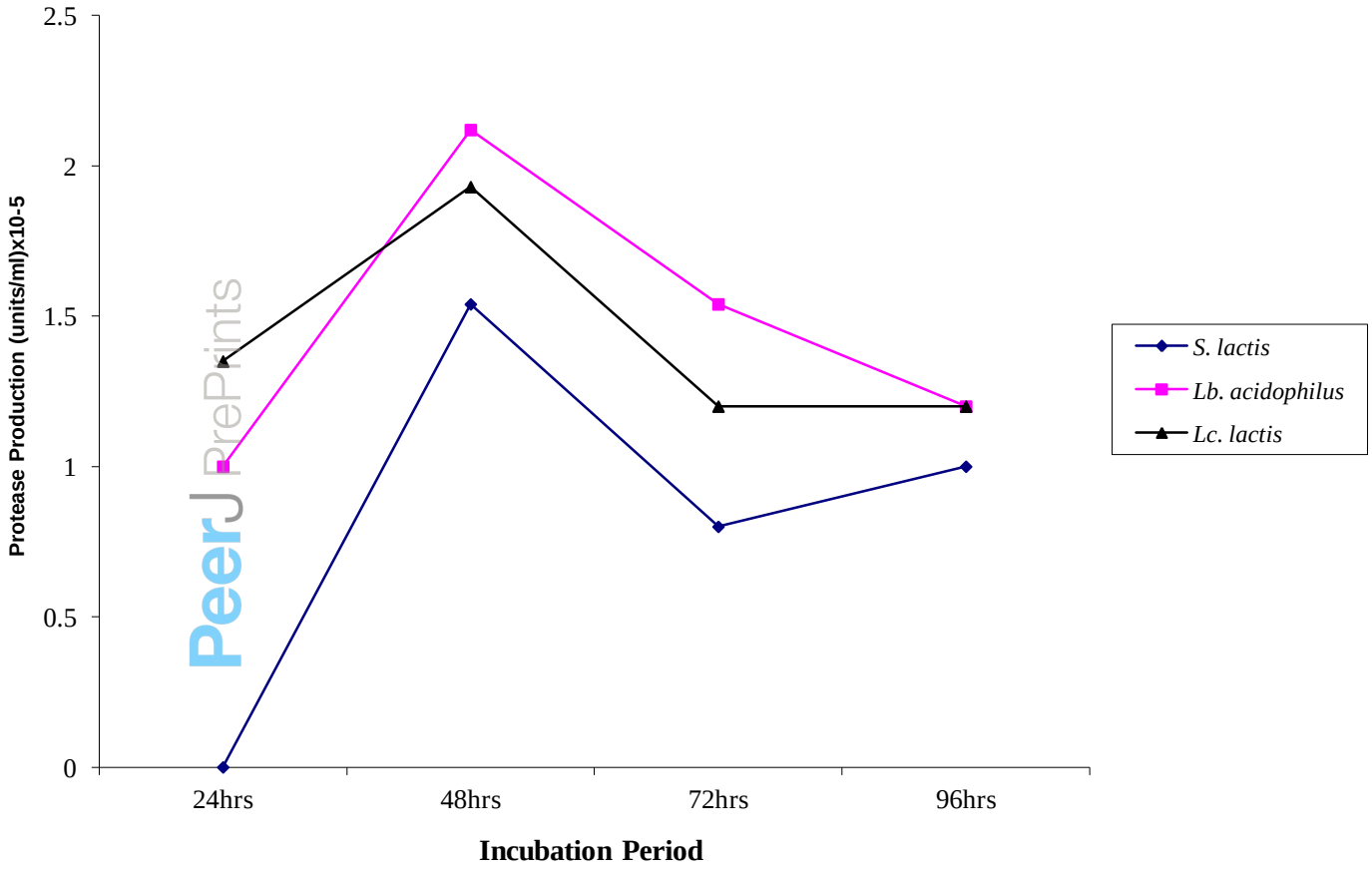

Fig. 12. Time course of Protease Production at 35°C by the three Isolates

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In figure 13 at 37^{o} protease production rose after 24 hrs to attain a maximum at 48 hours of incubation, a decline was noticed for protease produced by *Lactobacillus acidophilus* after 48 hrs, and it became stable between 72 and 96 hrs of incubation while for *Lactobacillus lactis* stability was noticed between 48 to 96 hrs incubation immediate after attaining it peak. Protease production by *Streptococcus lactis* declined after 72 hrs of incubation prior to being stable after 48 hrs incubation.

In figure 14 at 40^{o}C protease production by the three isolates rose to a maximum at 48 hrs and subsequently dropped for both *Lactobacillus acidophilus* and *Streptococcus lactis* at 72 hrs incubation. An increase was later noticed for *Lactobacillus acidophilus* while *Streptococcus lactis* leveled off, both at 96 hrs incubation. Protease production by *Lactobacillus lactis* became stable after 48 hrs but declined after 72 hrs.

Effect of Varying Medium pH and Inoculum Concentration on Protease Production

In figure 15 at 0.5ml inoculum concentration, there was a rapid increase in protease production by *Streptococcus lactis* and this was favoured by subsequent increase in pH till a peak was reached at pH 5.0 before a decline was noticed afterwards. For *Lactobacillus lactis* and *Lactobacillus acidophilus*, protease production decrease after starting off with a high value at pH 4.0 but for *Lactobacillus lactis* an increase began after pH 4.5 until a peak was attained at pH 6.0 while *Lactobacillus acidophilus* rose sharply to peak at pH 5.5.

In figure 16 at 1.0ml inoculum concentration, *Lactobacillus acidophilus* maintained its maximum production at pH 5.5 after a sharp increase between pH 4.0 and 4.5, likewise *Streptococcus lactis*, also maintaining its peak at pH 5.0 but *Lactobacillus lactis* reached its peak at pH 5.0 and maintained it.

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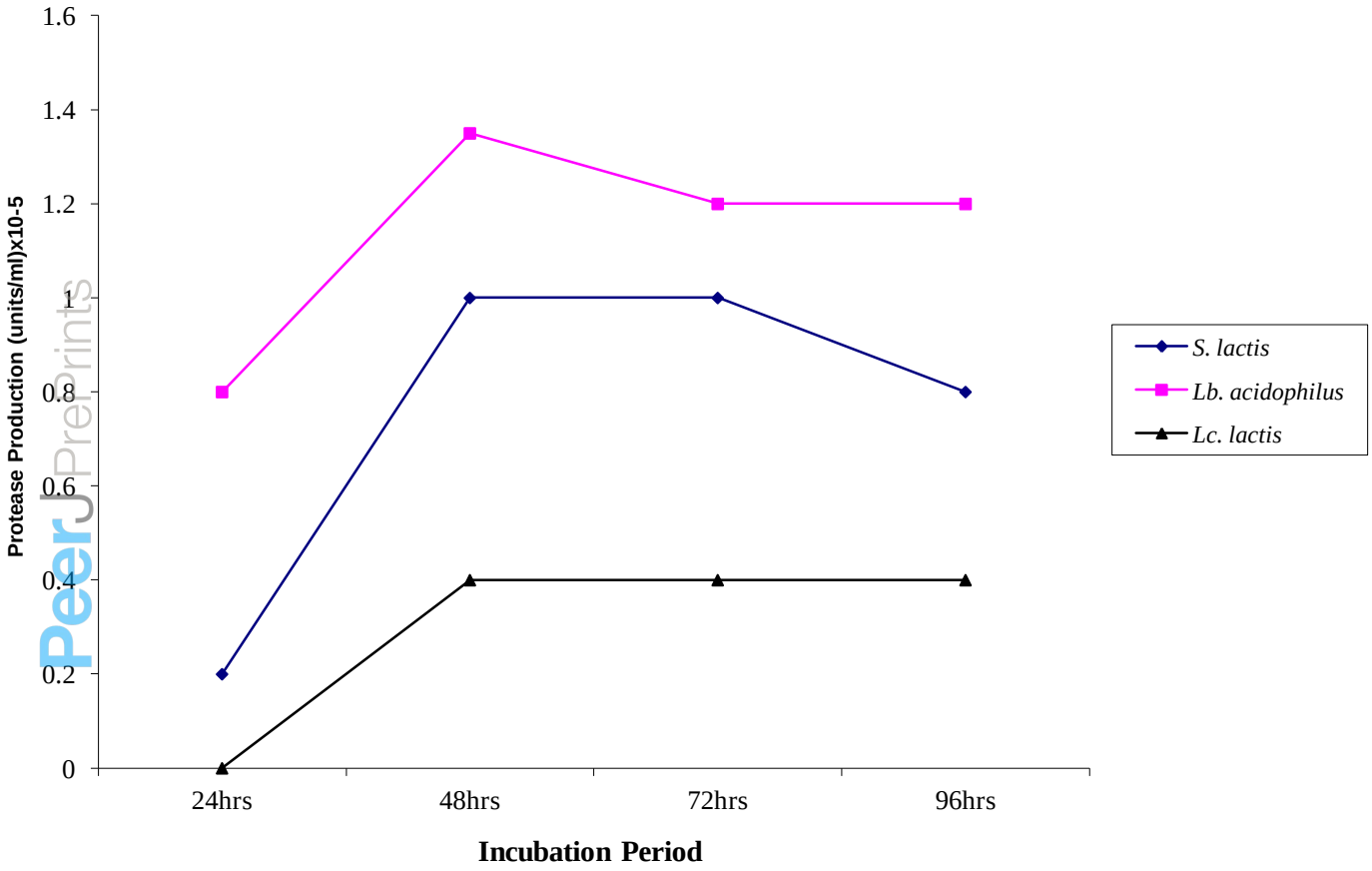

Fig. 13. Time course of Protease Production at 37°C by the three Isolates

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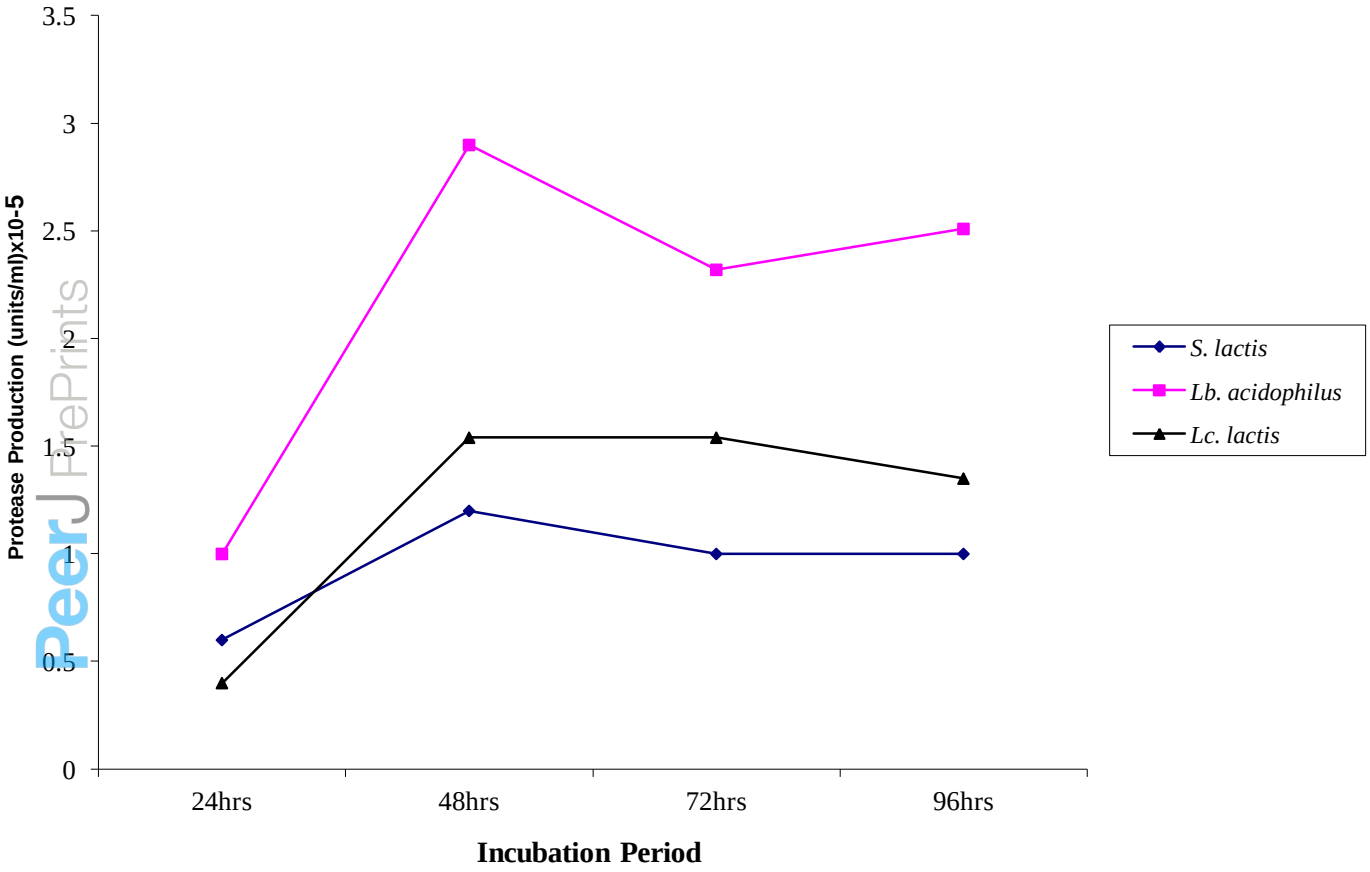

Fig. 14. Time course of Protease Production at 40°C by the three Isolates

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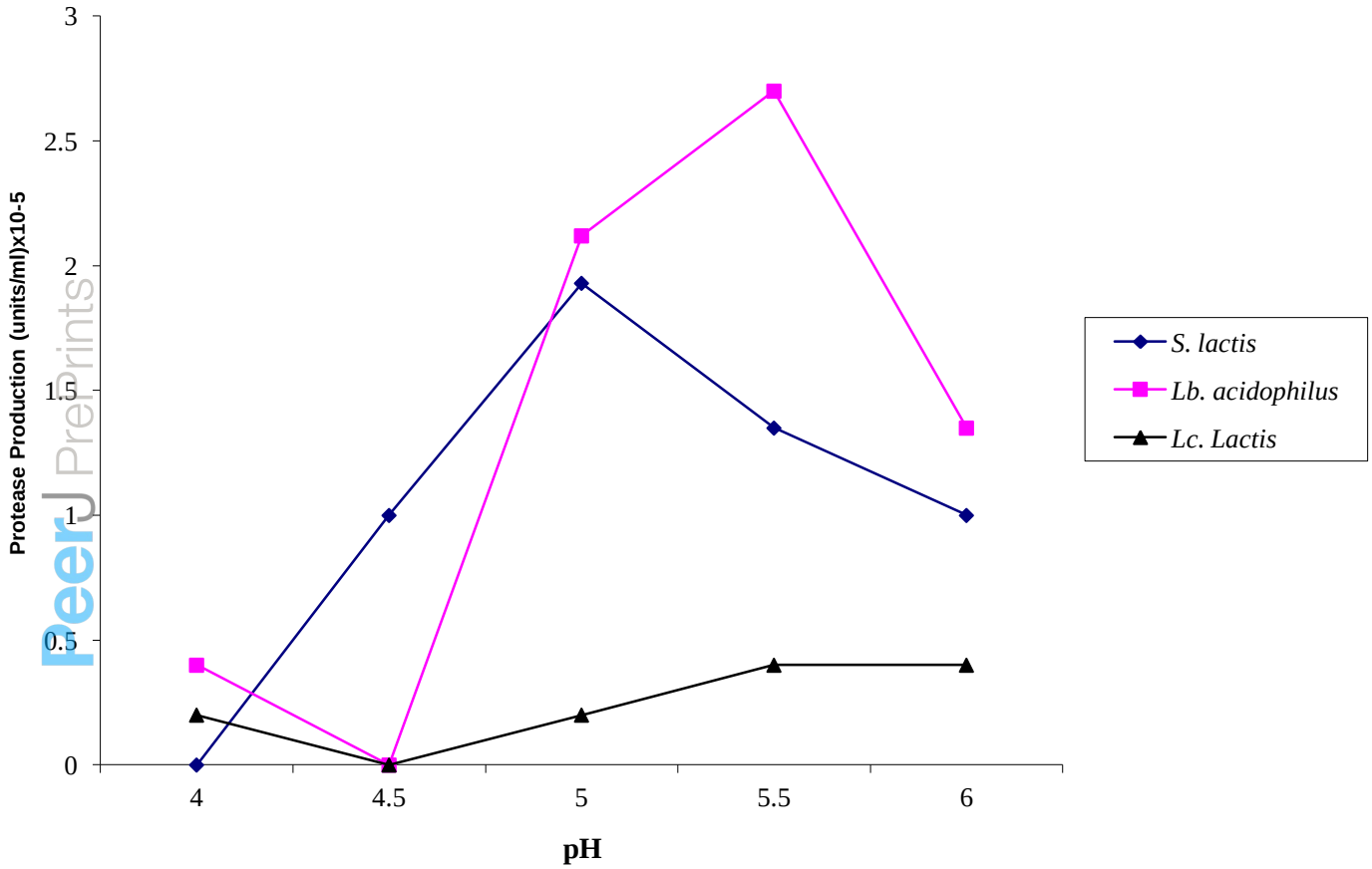

Fig. 15. Effect of varying medium pH at Inoculum Concentration of 0.5ml for the three isolates on Protease Production

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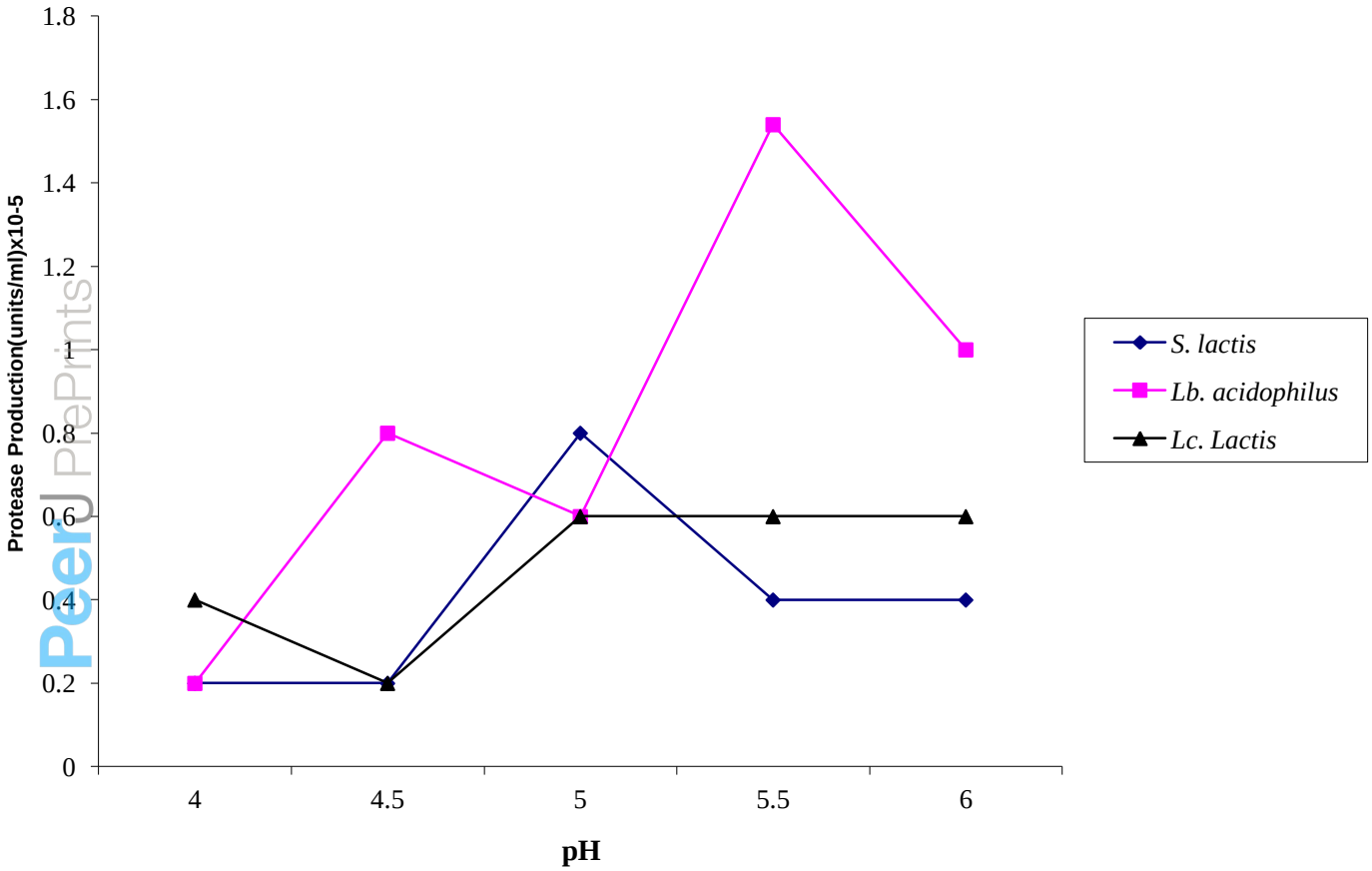

Fig. 16. Effect of varying medium pH at Inoculum Concentration of 1.0ml for the three isolates on Protease Production

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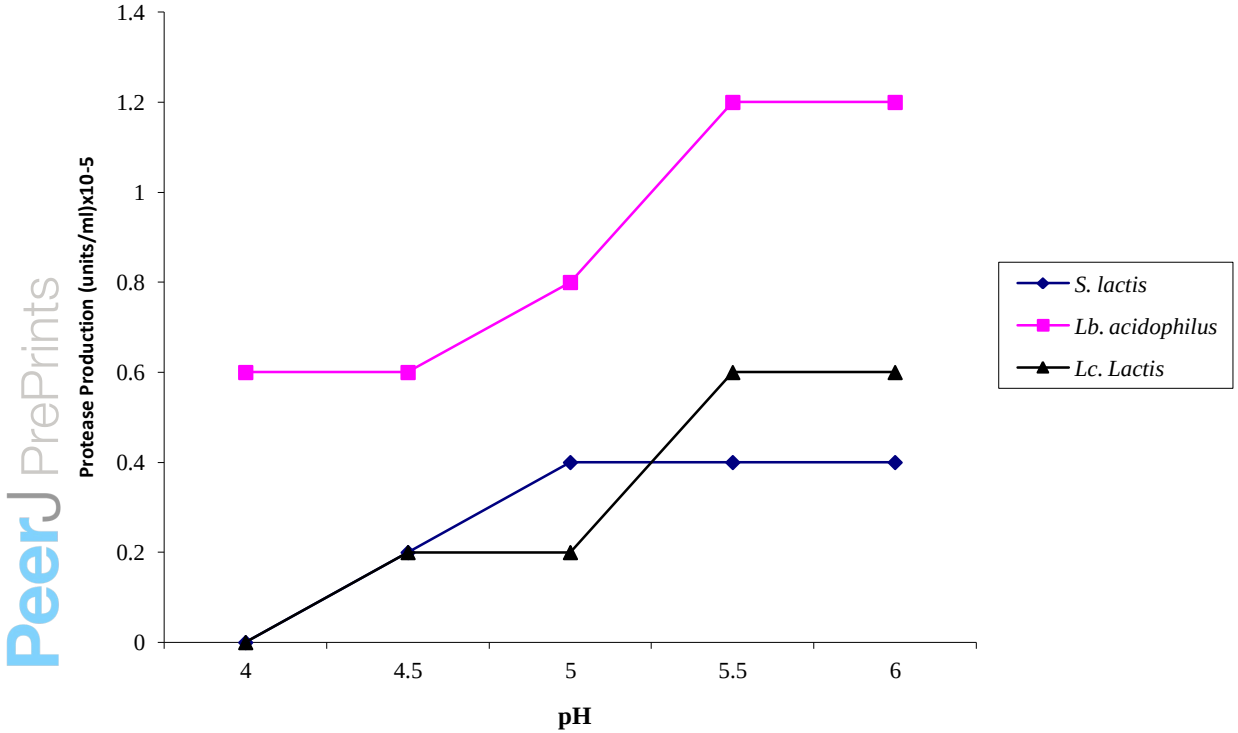

Fig. 17. Effect of varying medium pH at Inoculum Concentration of 1.5ml for the three isolates on Protease Production

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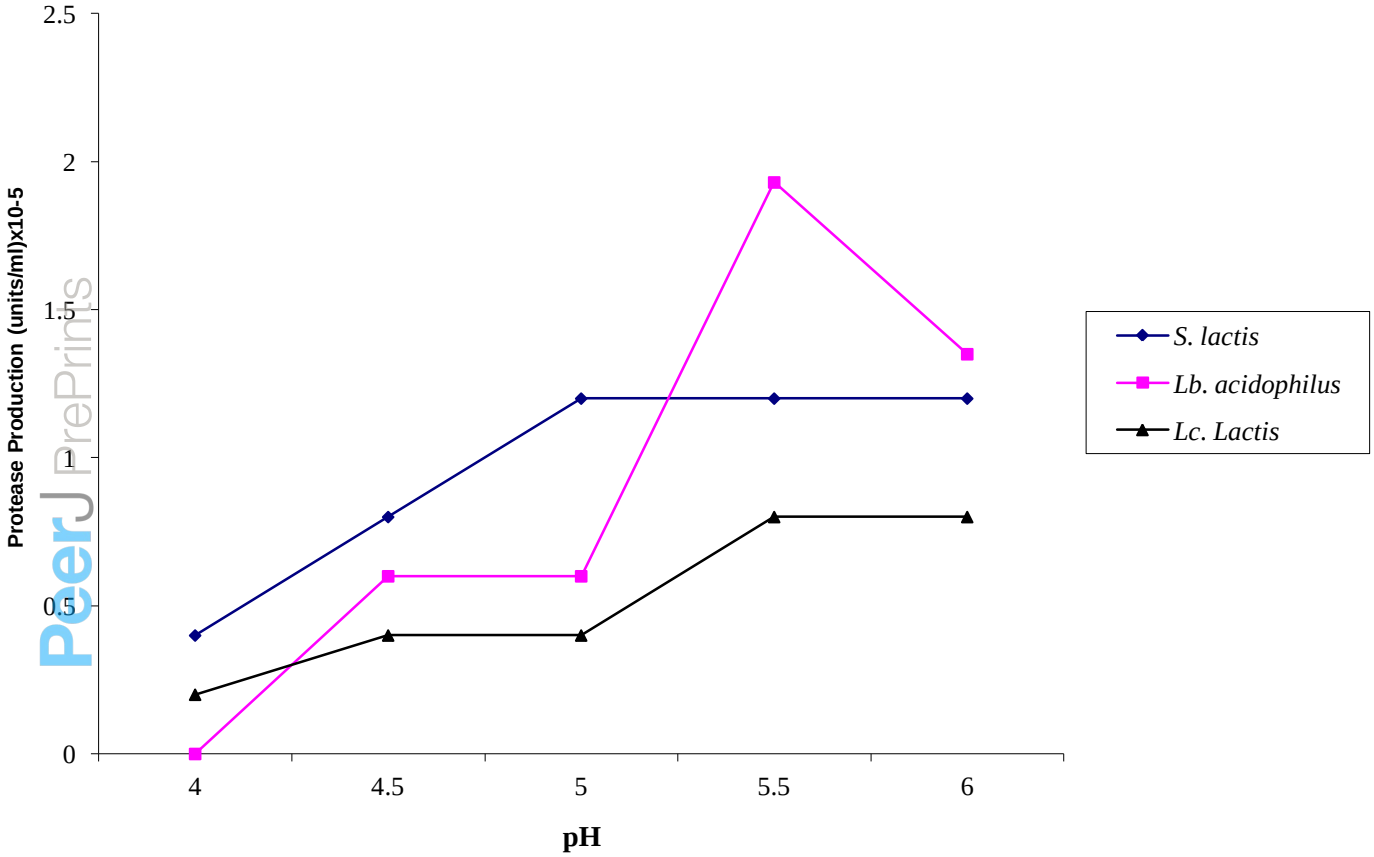

Fig. 18. Effect of varying medium pH at Inoculum Concentration of 2.0ml for the three isolates on Protease Production

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In figure 17 at an inoculum concentration of 1.5ml *Lactobacillus acidophilus* gradually increased till it attained and maintained its peak between pH 5.5 to 6.0. *Streptococcus lactis* and *Lactobacillus lactis* gradually rose to peak respectively at pH 5.0 and 5.5 to both become stable afterwards.

In figure 18 at 2.0ml inoculum concentration stability was noticed in protease production by *Lactobacillus acidophilus* between pH 4.5 and 5.0 before a sharp and rapid increase occurred between pH 5.0 and 5.5 where it peaked before dropping afterwards. Protease production for *Streptococcus lactis* was stable between pH 5.0 and 6.0 after a gradual increased that peaked at pH 5.0 while protease production for *Lactobacillus lactis* increased gradually till a peak at 5.5 was attained.

CHARACTERIZATION OF PRODUCED PROTEASE ENZYME

Effect of Substrate Concentration on Protease Activity

Six enzymic extracts from 3 lactic acid bacteria (*Streptococcus lactis, Lactobacillus acidophilus, Lactococcus lactis*) and Calotropis procera (crude extract, methanolic extract and ethanolic extract) were subjected to various parameters to determine the activity of the protease enzyme produced.

Figure 19 shows the protease activity in the presence of varying substrate concentration. *Streptococcus lactis* had its lowest activity (0.40) at 1% substrate concentration and it gradually increased till it attained its peak (6.20) at 4% substrate concentration. *Lactobacillus acidophilus* activity attained its peak (3.70) at 2% substrate concentration and while decreasing became stable (1.00) at both 4% and 5% substrate concentration *Lactococcus lactis* showed no activity (0.00) at 2% substrate concentration; it became stable (0.20) at 3% and 4% substrate concentration before attaining its peak (0.80) at 5% substrate concentration.

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The crude extract of *C. procera* attained its peak (1.20) at 1% substrate concentration before decreasing between 2% and 4% substrate concentration to begin ascending (0.8) at 5% substrate concentration. Ethanolic extract started with a high value (11.80) at 1% substrate concentration only to start a descend at 2% and attained its peak (12.60) at 5% substrate concentration. The methanolic extract readily attained its peak (2.12) and remained stable between 1% and 3% substrate concentration.

Figure 20 shows the Lineweaver-Burke plot for the hydrolysis of different concentration of casein by the partially purified protease from the LAB isolates and *C. procera* extracts. In figure 20a for *Streptococcus lactis*, substrate concentration had the affinity (km) of 0.42 with a maximum velocity (Vmax) of 2.0. Figure 20b shows substrate concentration of *Lactobacillus acidophilus* with an affinity (km) of 0.77 and a maximum velocity (Vmax) of 2.50 while in figure 20c that of *Lactococcus lactis* had an affinity (km) of 3.3 with a maximum velocity (Vmax) of 0.87. Figure 20d shows substrate concentration of the crude extract of *Calotropis procera* having an affinity (km) of 5.0 with a maximum velocity (Vmax) of 1.54. Figure 20e shows substrate concentration of the Ethanolic extract of *C. procera* having an affinity (km) of 2.44 with a maximum velocity (Vmax) of 8.77 while figure 20f shows substrate concentration of the methanolic extract of *C. procera* having an affinity (km) of 5.0 with a maximum velocity (Vmax) of 4.17.

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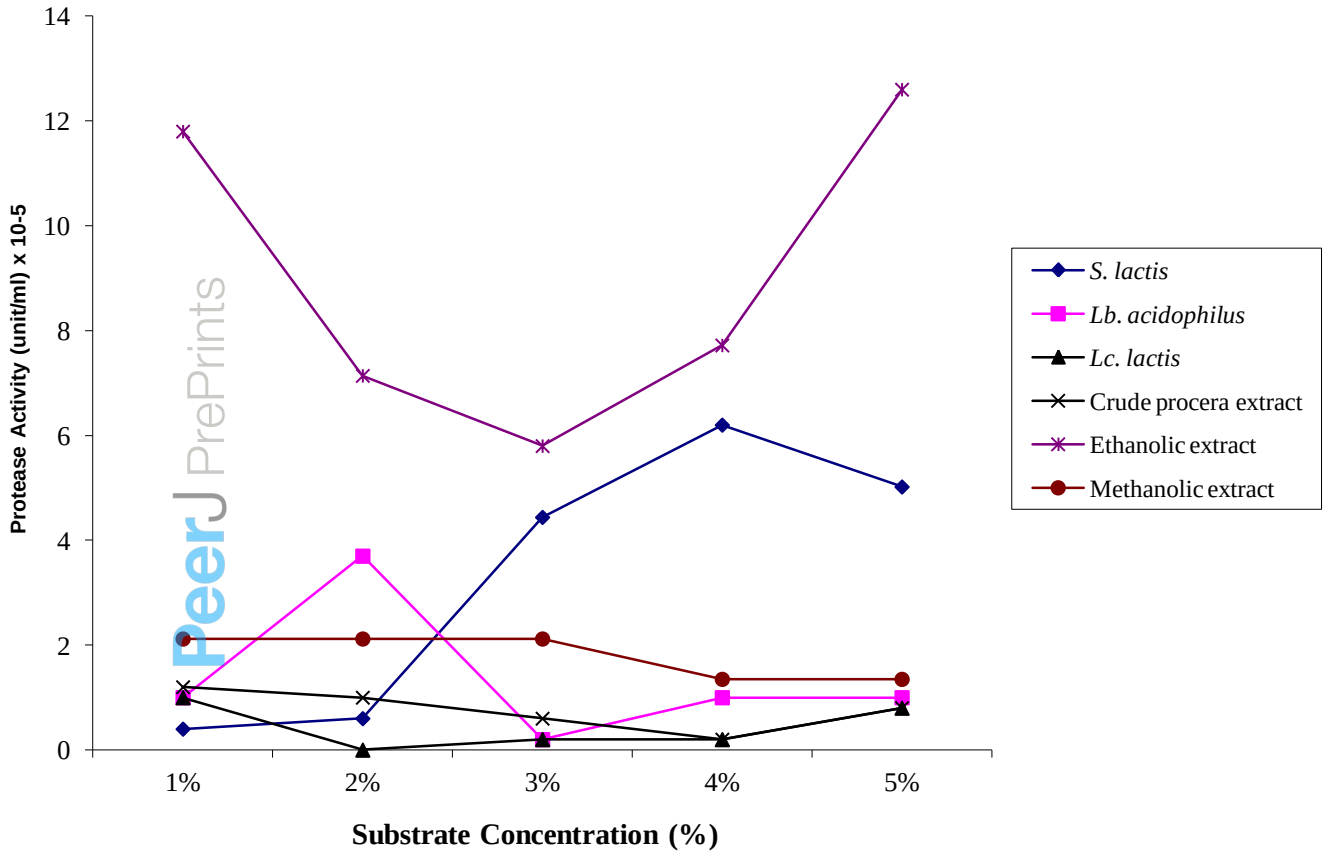

Fig. 19. Effect of Substrate Concentration on Protease Activity

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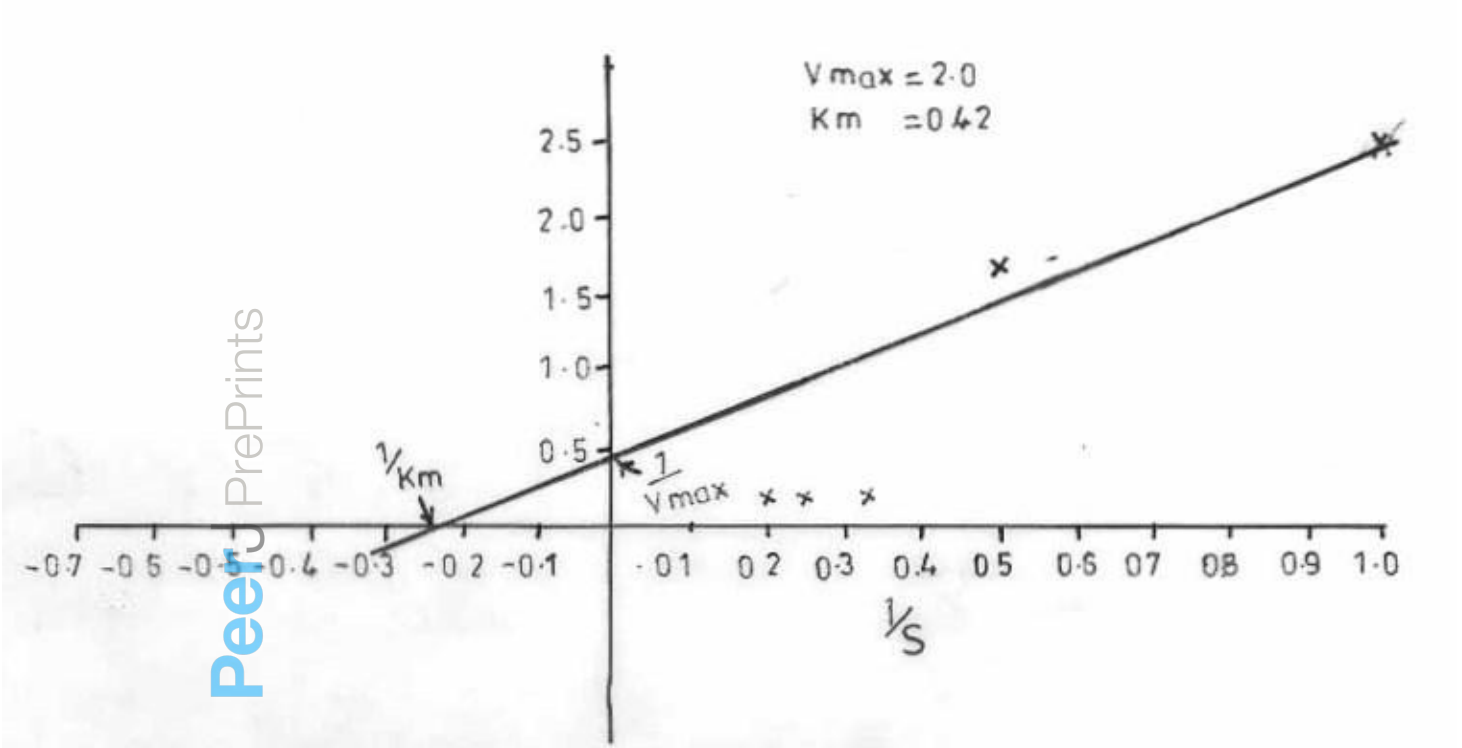

Figure 20a. Line weaver-Burke plot for the hydrolysis of different concentrations of casein by the partially purified protease from Streptococcus lactis isolated from 'wara'.

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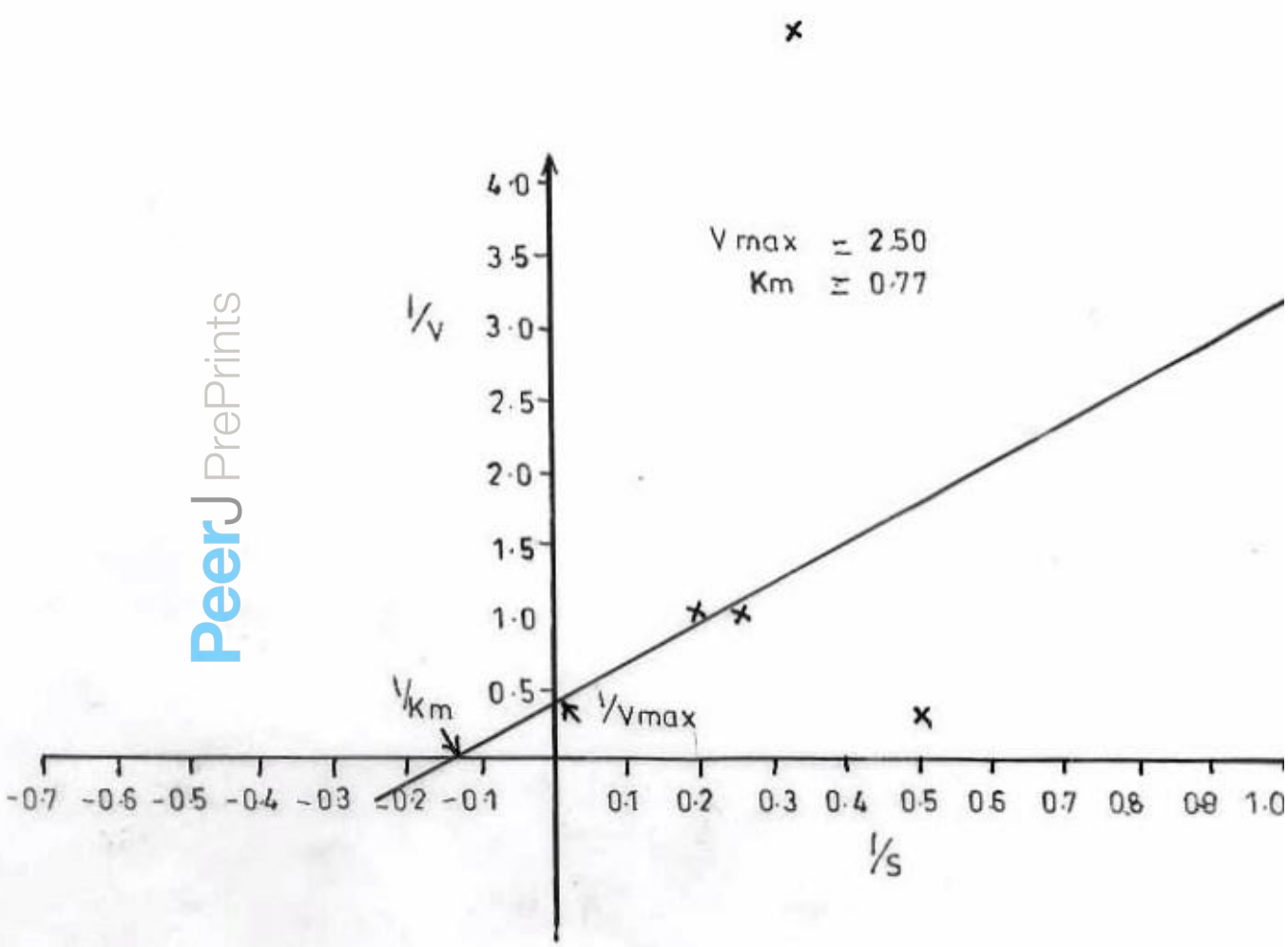

Figure 20b. Line weaver-Burke plot for the hydrolysis of different concentrationsof casein by partially purified protease from Lactobacillus acidophilus isolated from 'wara'

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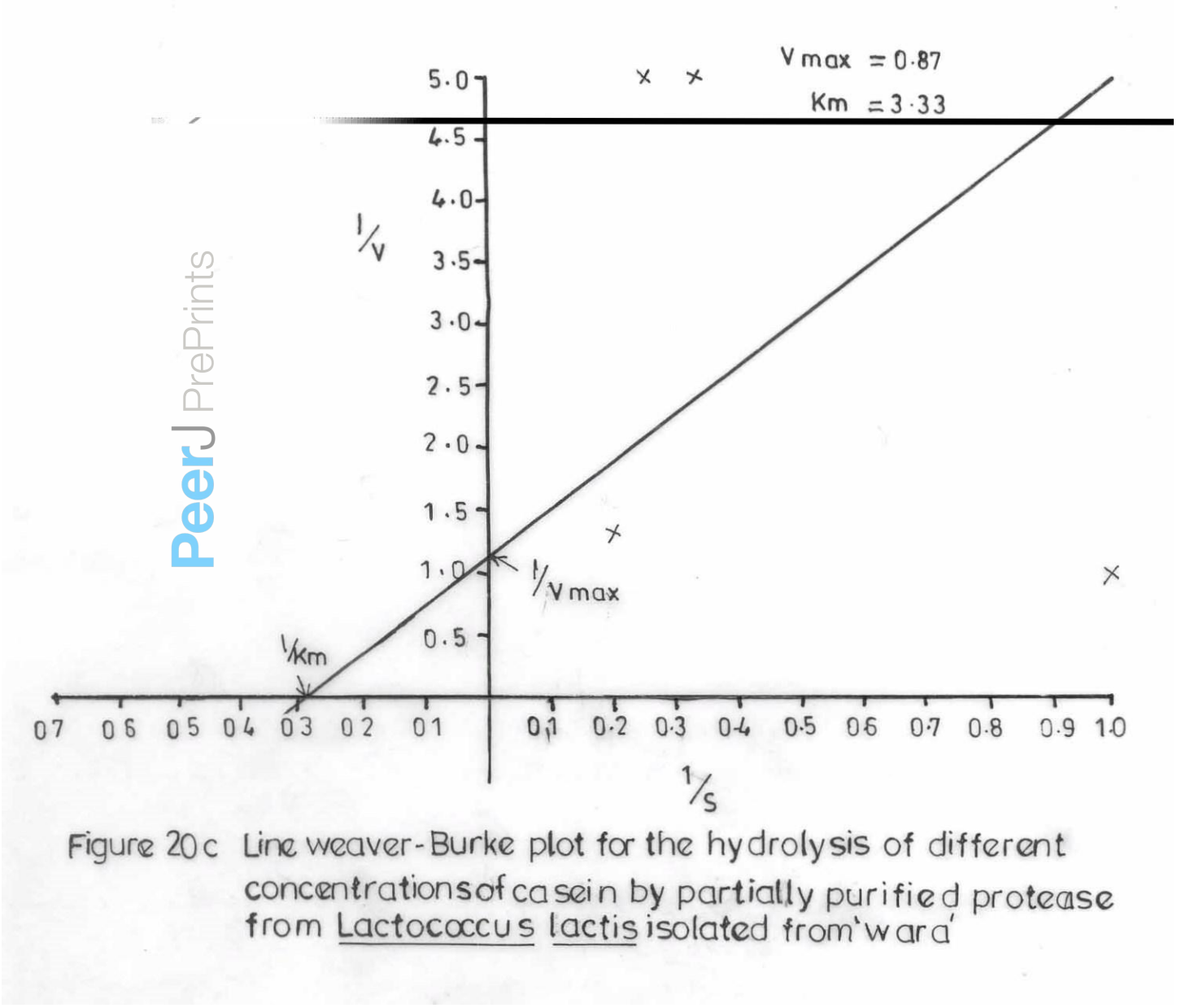

Figure 20c Line weaver-Burke plot for the hydrolysis of different concentrations of casein by partially purified protease from Lactococcus lactis isolated from 'wara'

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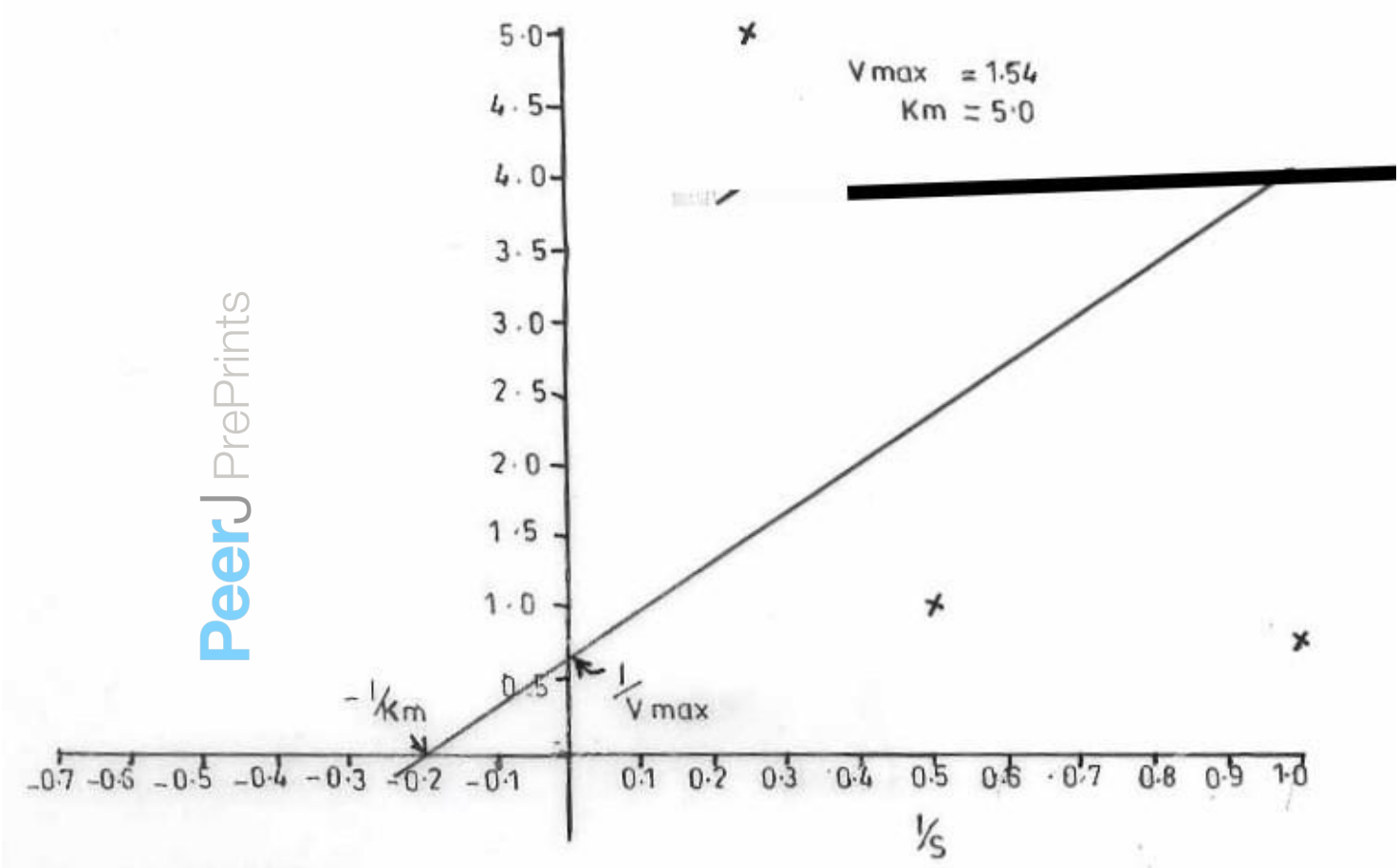

Figure 20d. Lineweaver-Burke plot for the hydrolysis of different concentration of casein by partially purified protease from the crude extract of Calotropis procera

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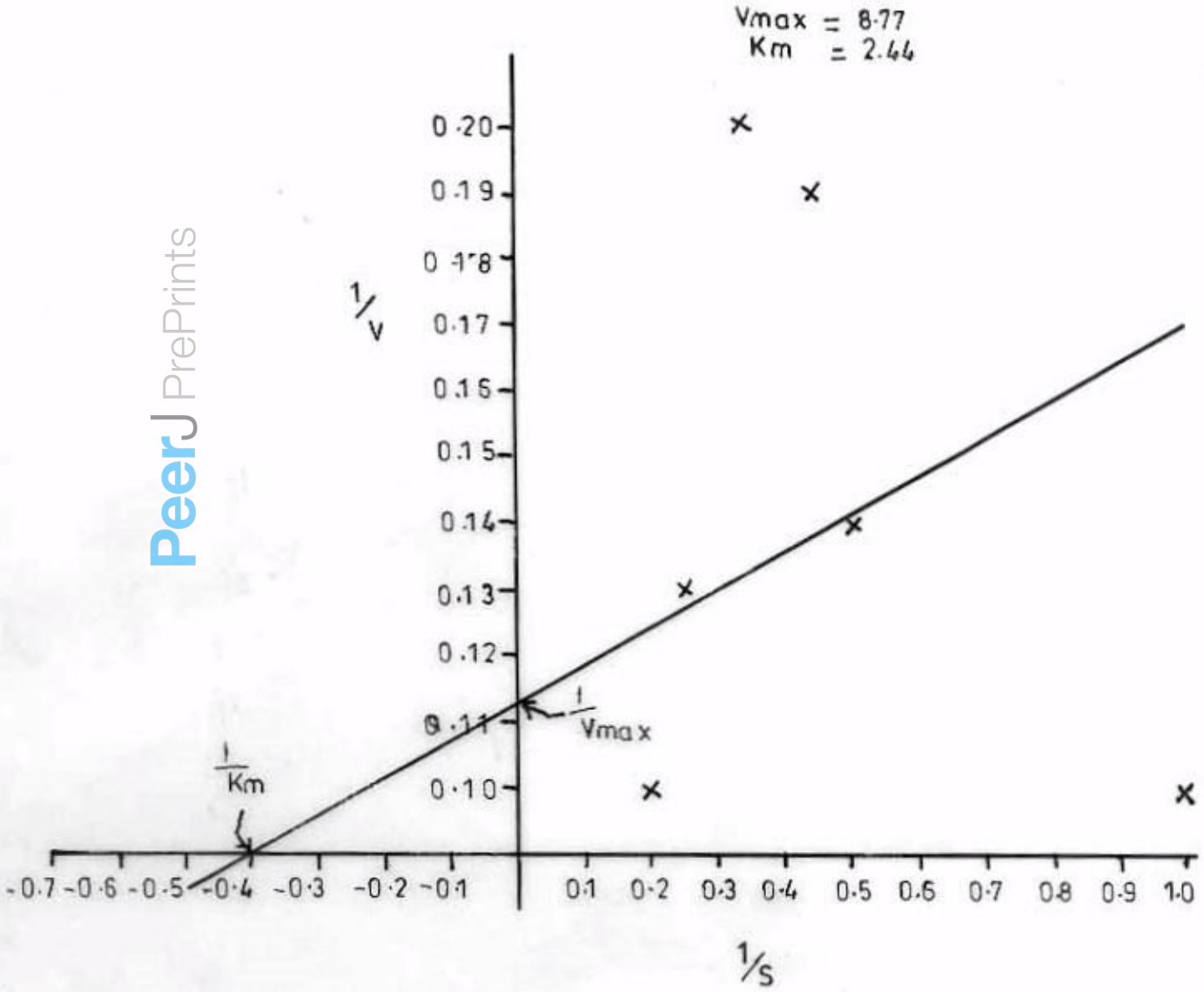

Figure 20e Lineweaver-Burke plot for the hydrolysis of different concentration of casein by partially purified protease from the Ethanolic extract of Calotropis procera

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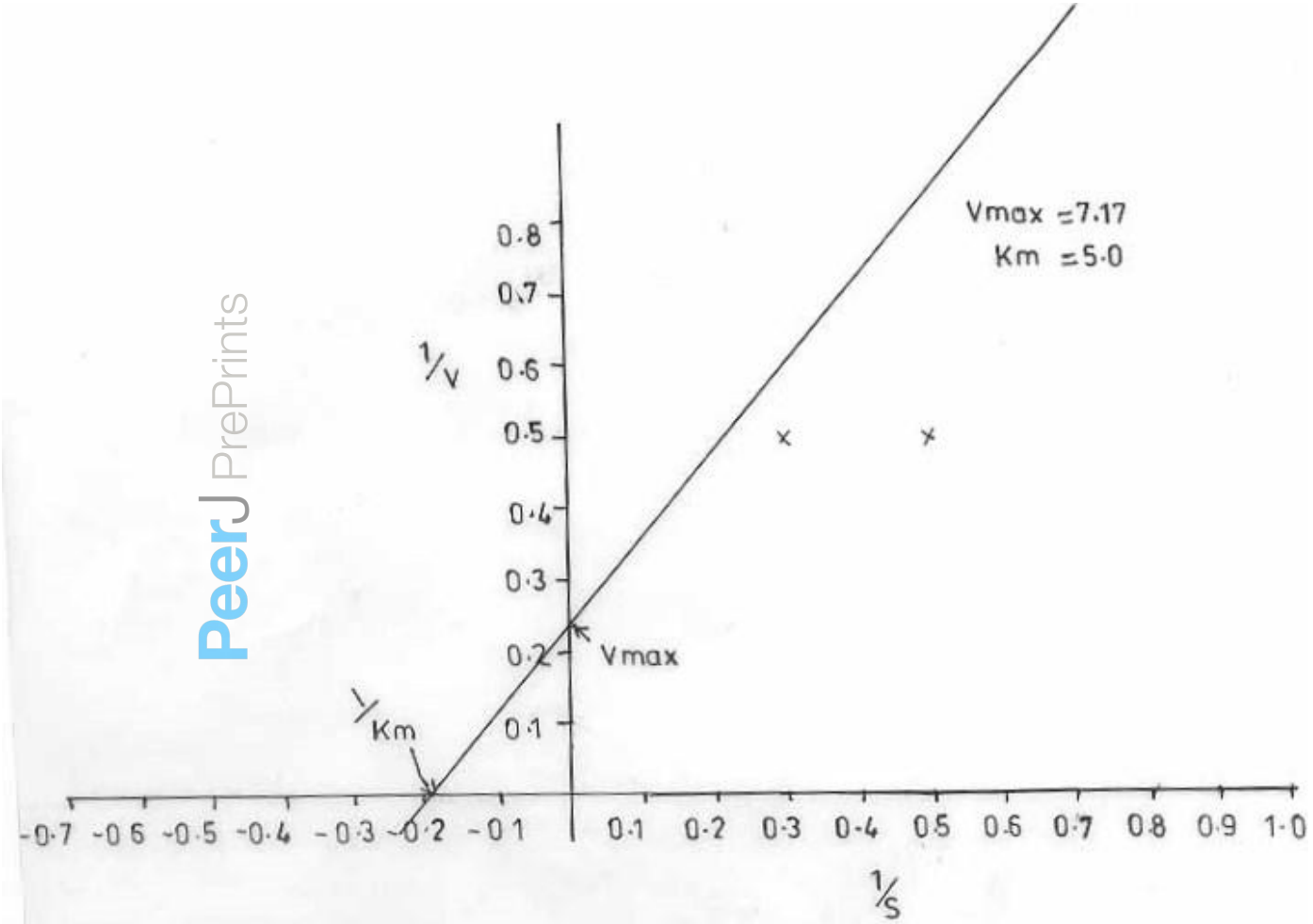

Figure 20f. Line weaver-Burke plot for the hydrolysis of casein by different concentrations partially purified protease from methanolic extract of Calotropis procera

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Effect of Enzyme concentration on Protease Activity

In figure 21, *Streptococcus lactis* activity was seen to attain a low activity of 0.20 at 1.5ml enzyme concentration after descending only to peak (1.35) at 2.5ml enzyme concentration. *Lactobacillus acidophilus* quickly attained its peak (1.35) at 0.5ml enzyme concentration and began a descend which stopped at 1.5ml enzyme concentration to ascend (1.00) at 2.0ml enzyme concentration. *Lactococcus lactis* began a steady rise with 0.20 at 0.5ml enzyme concentration until its peak (0.60) was reached and stabilized between 2.0ml and 2.5ml enzyme concentration. The crude extract of C. procera attained its peak (1.35) at 1.0ml enzyme concentration and descended to a low activity of 0.20 at 2.0ml enzyme concentration. The ethanolic extract attained a high activity (7.40) at 0.5ml enzyme concentration but decreased at 1.0ml enzyme concentration only to begin a rapid increase till its peak (26.10) was reached at 2.5ml enzyme concentration. The methanolic extract followed the same trend, after descending at 1.0ml enzyme concentration it began a rapid increase till its peak (10.62) was attained at 2.5ml enzyme concentration.

Figure 22 shows the Lineweaver-Burke plot for the hydrolysis of casein by different concentrations of partially purified protease from the LAB isolates and *C. procera* extracts.

Figure 22a shows the enzyme concentration of *Streptococcus lactis* having an affinity (km) of 1.82 with a maximum velocity (Vmax) of 1.12. Figure 22b shows the enzyme concentration of *Lactobacillus acidophilus* with an affinity (km) of 1.75 and a maximum velocity (Vmax) of 2.0. Figure 22c shows the enzyme concentration of *Lactobacillus lactis* having an affinity (km) of 1.22 with a maximum velocity (Vmax) of 0.98. Figure 22d shows the enzyme concentration of crude extract of *C. procera* with an affinity (km) of 2.13 and a maximum velocity (Vmax) of 2.0, the ethanolic extract in figure 22e shows enzyme

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concentration with affinity (km) of 2.63 and a maximum velocity (Vmax) of 1.45. In figure 22f, methanolic extract had an affinity (km) of 2.56 with a maximum velocity (Vmax) of 1.54.

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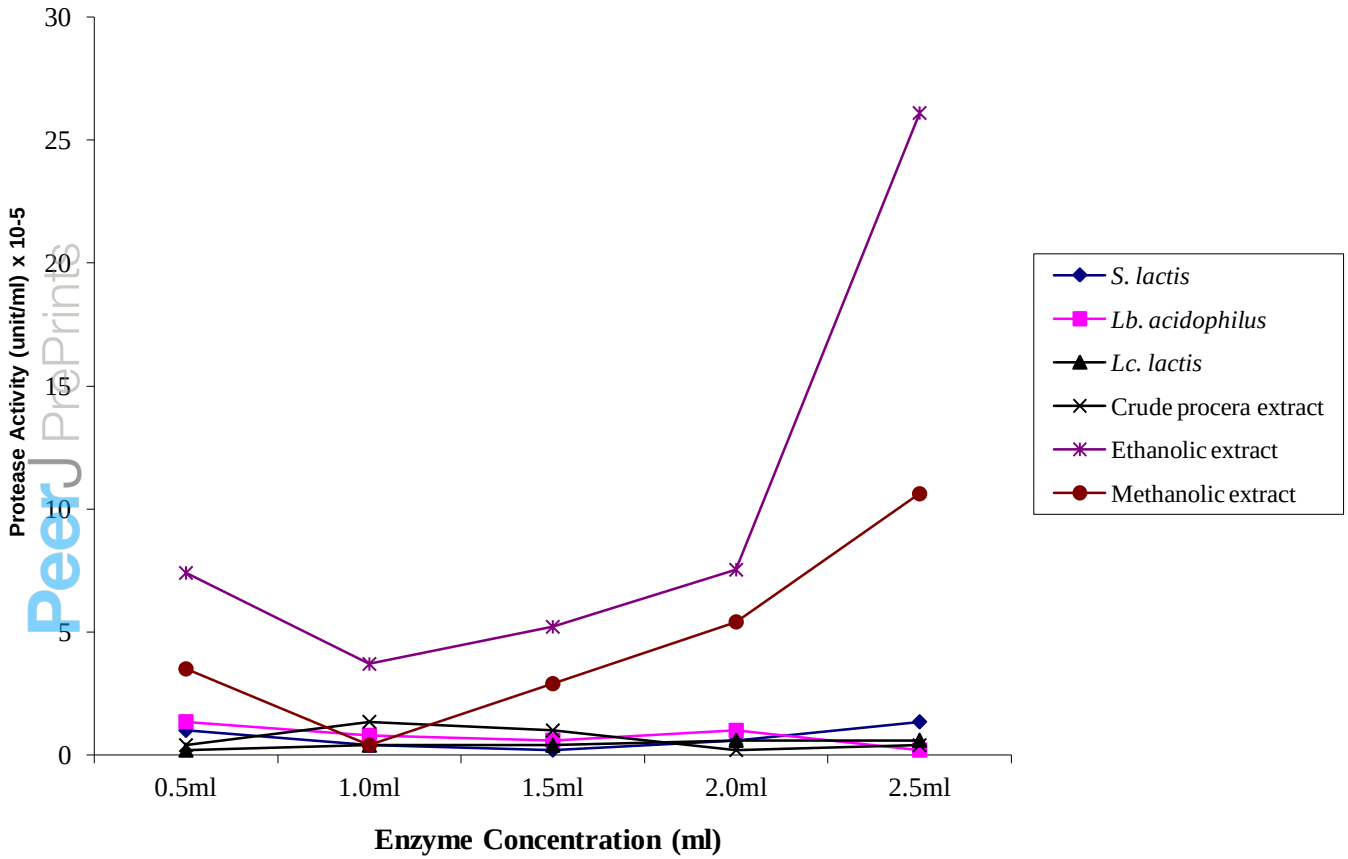

Fig. 21. Effect of Enzyme Concentration on Protease Activity

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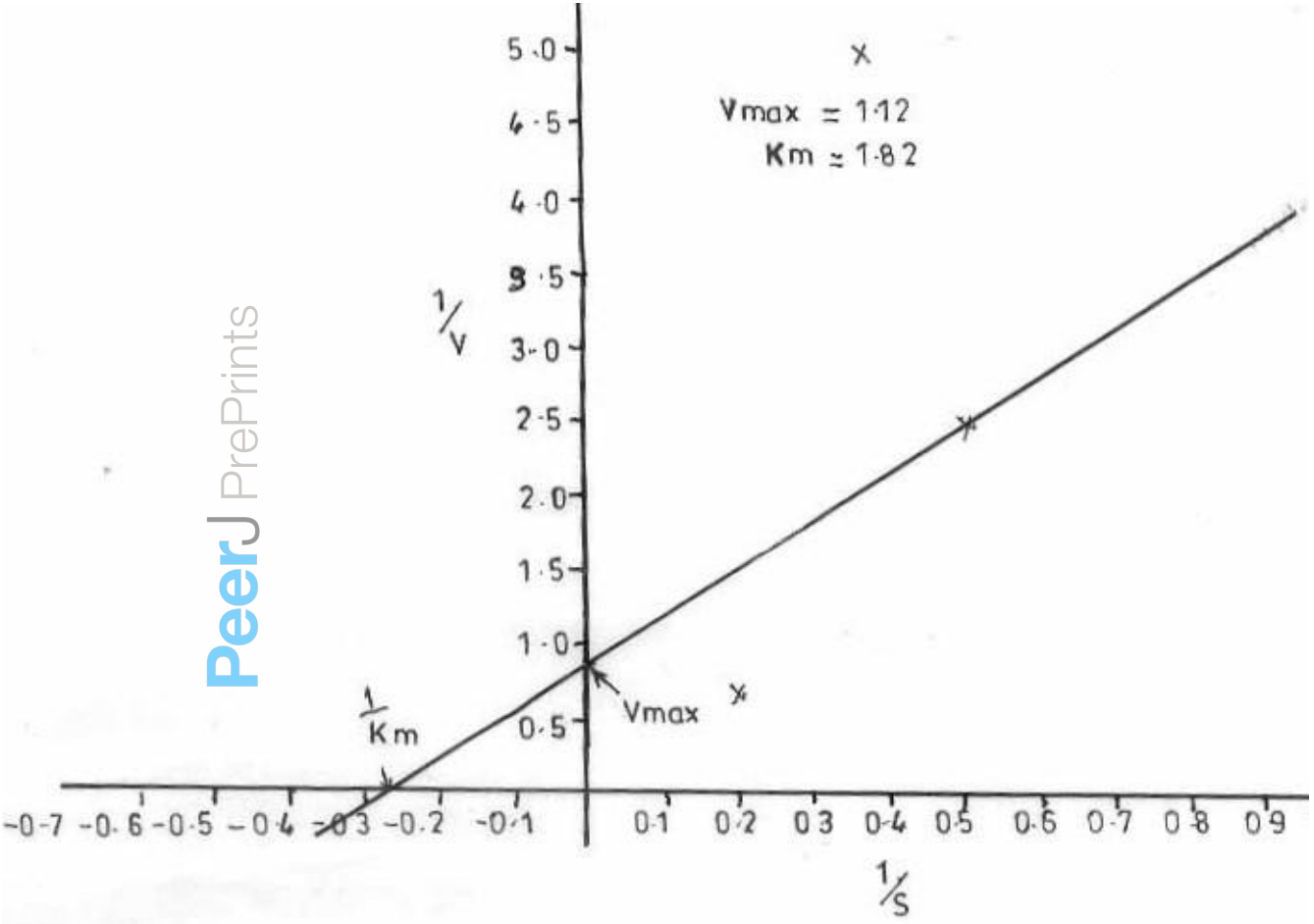

Figure 22a Line weaver Burke plot for the hydrolysis of different concentrations of casein by patially purified protea from Streptococcus lactis isolated from 'wara'

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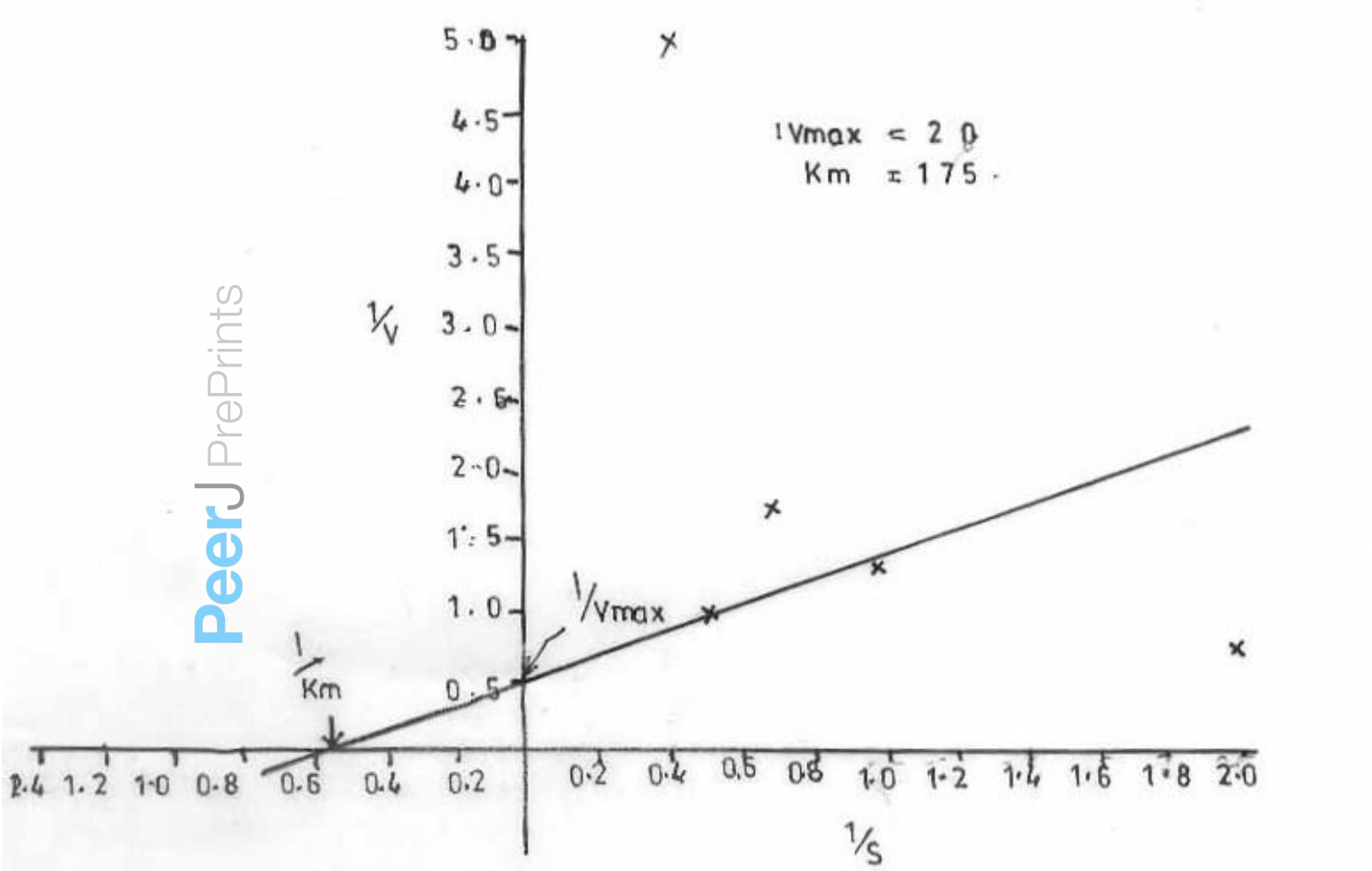

Figure 22b. Lineweaver-Burke plot for the hydrolysis of different concentrations of casein by partially purified protease from Lactobacillus acidophilus isolated from 'wara'

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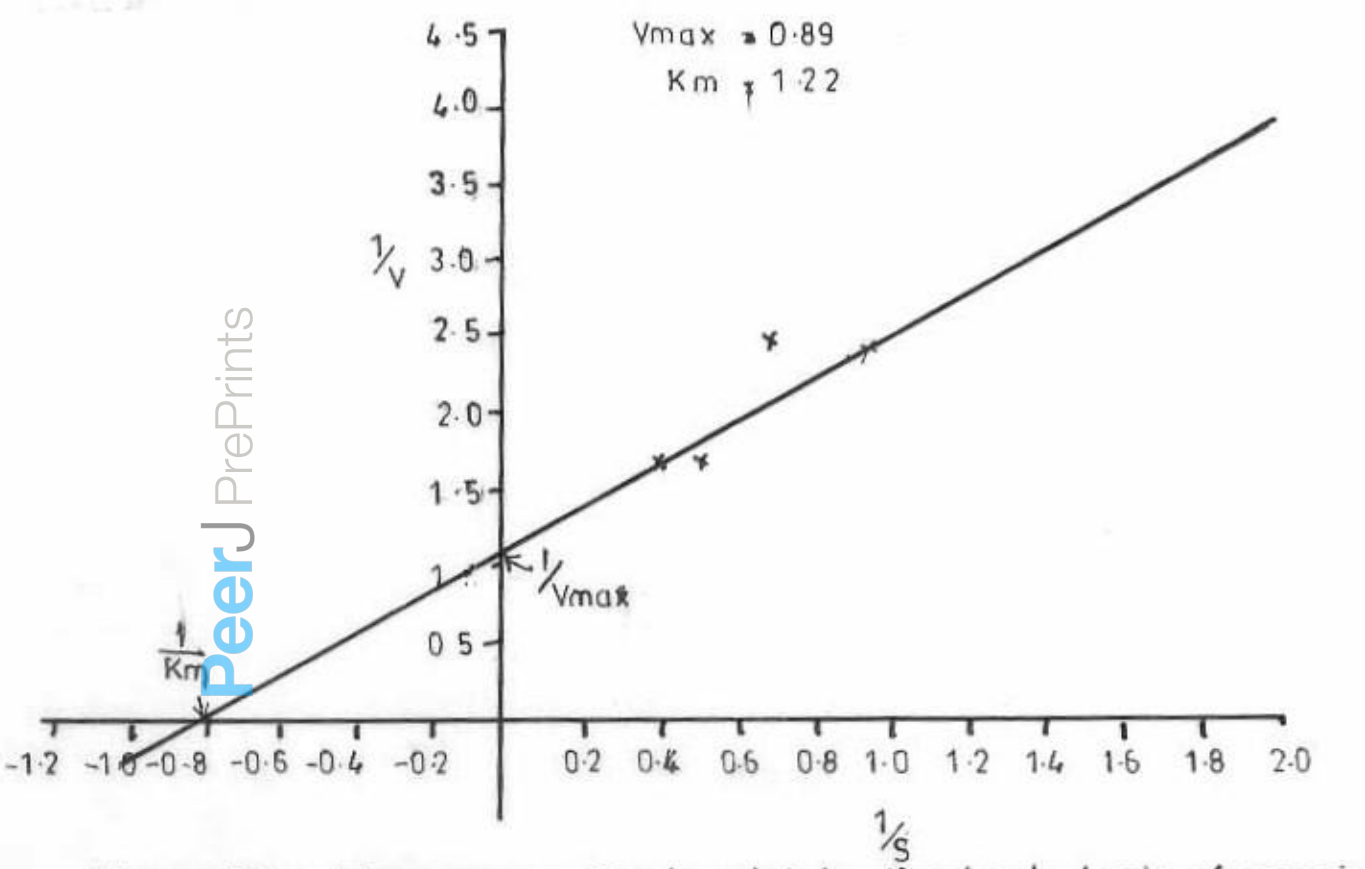

Figure 22c. Line weaver-Burke plot for the hydrolysis of casein by different concentrations of partially purified protease from Lactococcus lactis isolated from 'wara'

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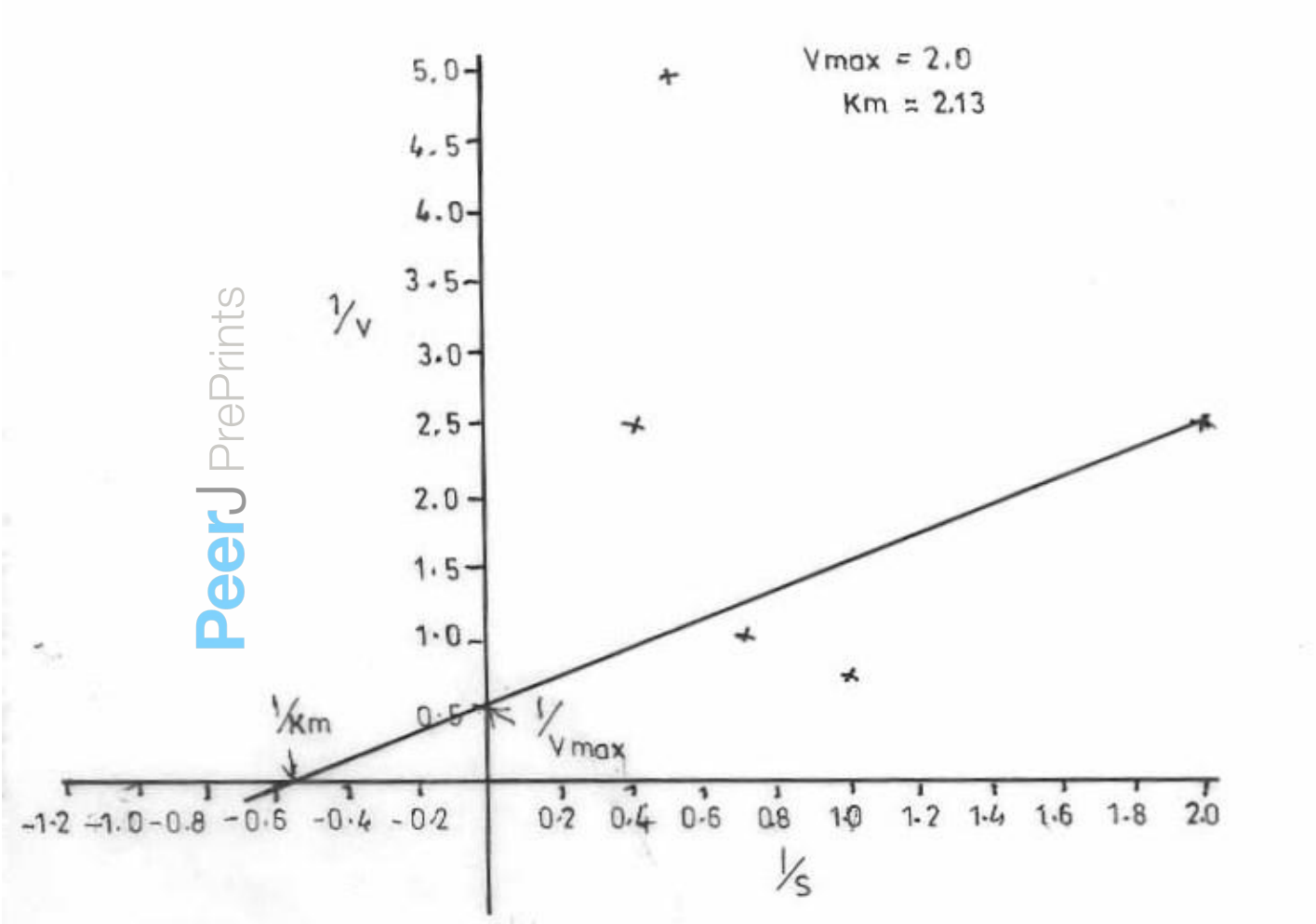

Figure 22d. Lineweaver-Burke plot for the hydrolysis of casein by different concentrations of partially purified protease from the crude extract of Calotropis procera

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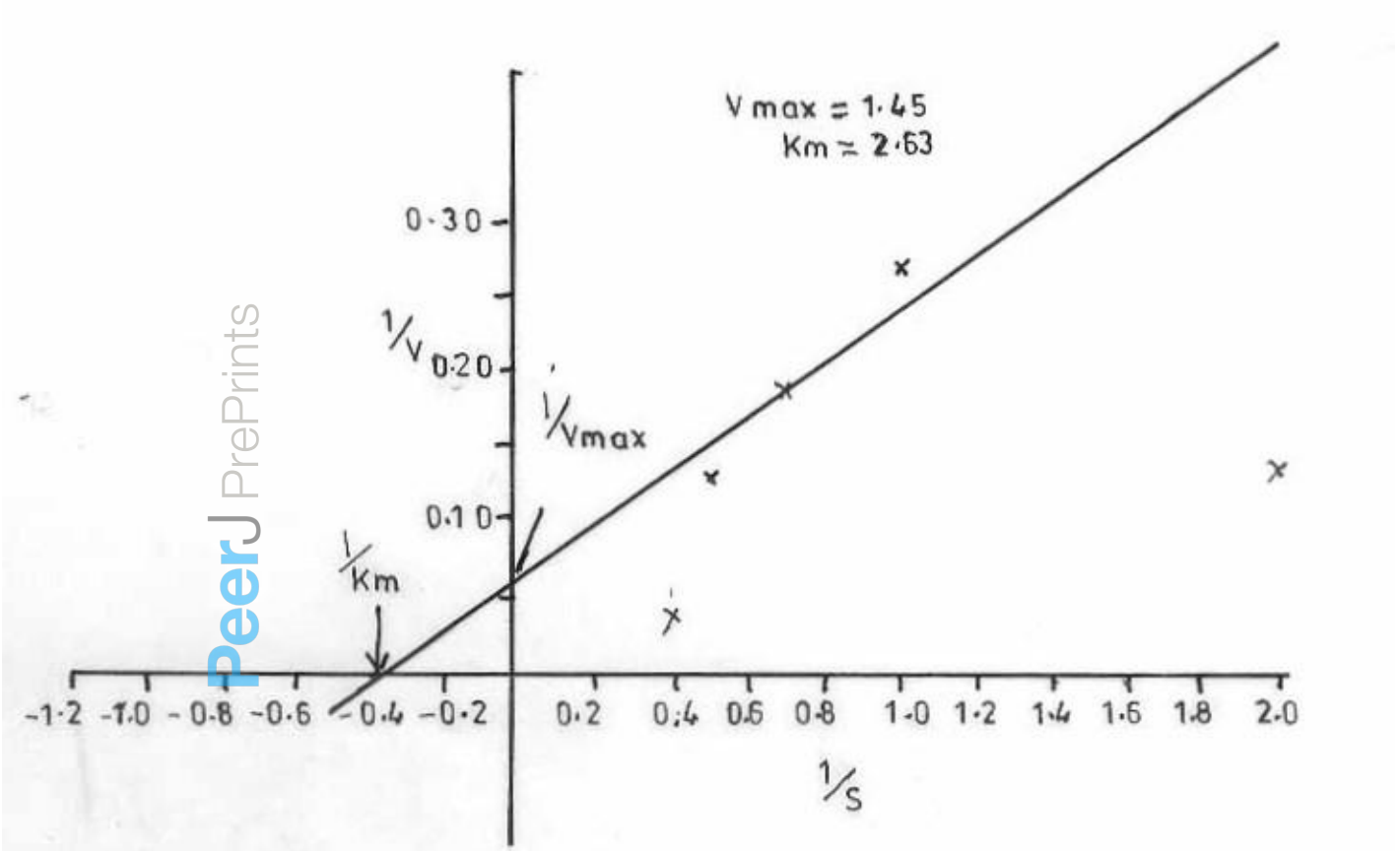

Figure 22e. Lineweaver-Burke plot for the hydrolysis of casein by different concentrations of partially purified protease from the Ethanolic extract of Calotropis proce

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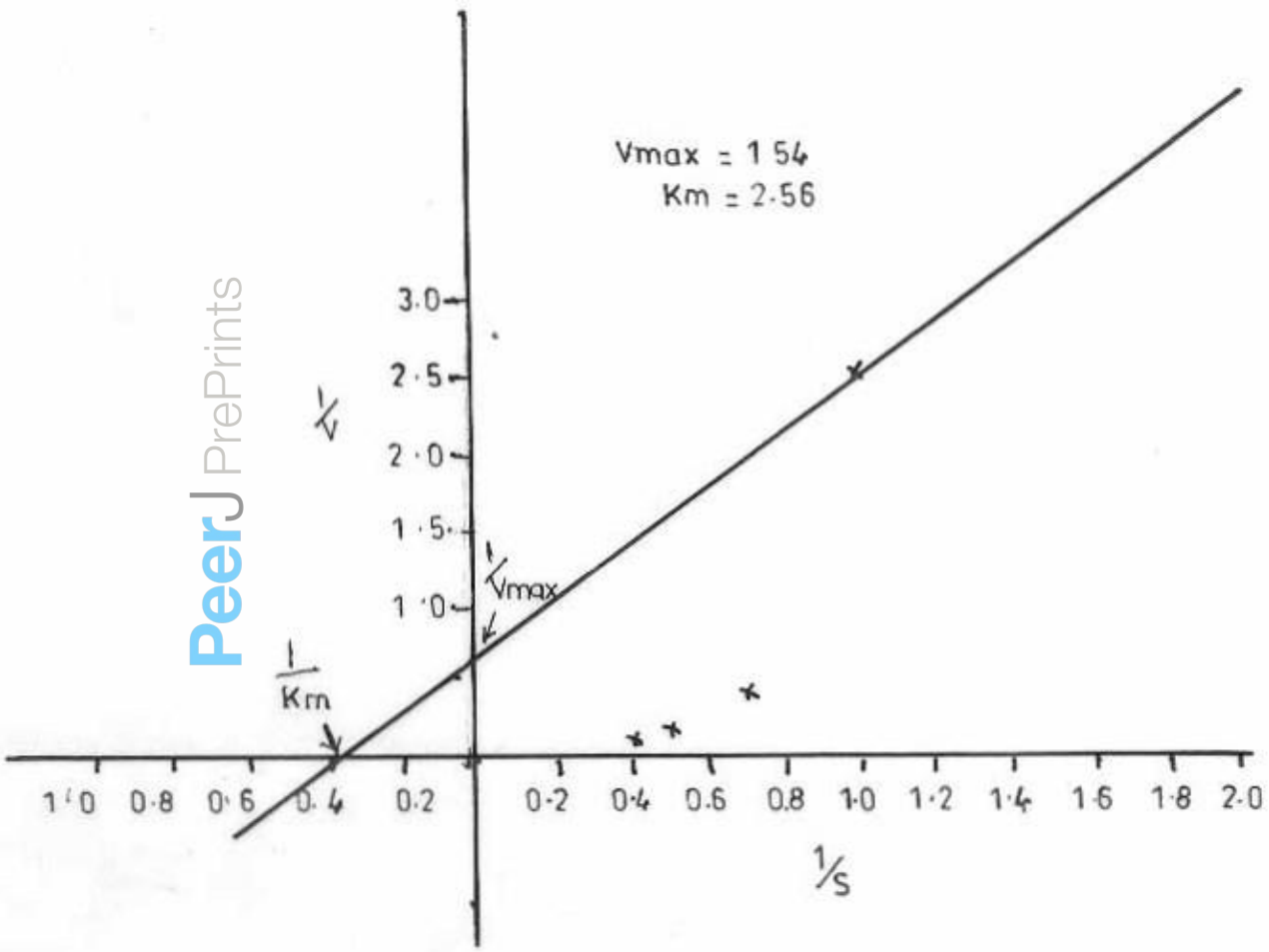

Figure 22 f Line weaver- Burke plot for the hydrolysis of casein by different concentrations partially purified protease from the methanolic extract of Calotropis procera

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Effect of Temperature on Protease Activity

In figure 23, protease activity of *Streptococcus lactis* attained its peak (2.32) at 28^{o}C before declining at 30 ^{o}C and re-ascended at 35 ^{o}C (1.35). *Lactobacillus acidophilus* followed the same pattern with its peak (2.32) at 40 ^{o}C thus corroborating its thermophilic nature. *Lactococcus lactis* reached its peak (2.32) at 28 ^{o}C, descended at 30 ^{o}C and re-ascended at 35 ^{o}C. The crude extract of *C. procera* gradually ascended till it reached a high activity (2.12) at 35 ^{o}C only to descend at 37 ^{o}C and finally reached its peak (2.90) at 40 ^{o}C. The ethanolic extract reached its peak (1.00) at both 28 ^{o}C and 35 ^{o}C while the methanolic extract climbed to its peak (1.00) at 28 ^{o}C and then declines at 35 ^{o}C downwards.

Effect of pH on Protease Activity

In figure 24, the activity of *Streptococcus lactis* protease did a series of descending and ascending before it finally reached its peak (2.32) at pH 5.0 and then descended again at pH 5.5 and 6.0, the same applies to that of *Lactobacillus acidophilus* though it became stable (1.74) at pH 4.0 and 4.5 before descending at pH 5.0 to reach its peak (2.90) at pH 5.5. *Lactococcus lactis* activity followed the same trend before stabilizing at its peak (2.90) both pH 5.5 and 6.0. The crude extract of *C. procera* quickly attained its peak (2.70) at pH 3.0 to be followed closely by an activity of 2.51 at both pH 5.0 and 6.0 respectively. The ethanolic extract started a downward trend between pH 3.0 and 4.0 before reaching its peak (1.20) at pH 5.0 and descended to absolutely no activity (0.00) at pH 6.0. The methanolic extract attained its peak (2.32) at pH 3.5 before starting a downward decline to 0.20 activity.

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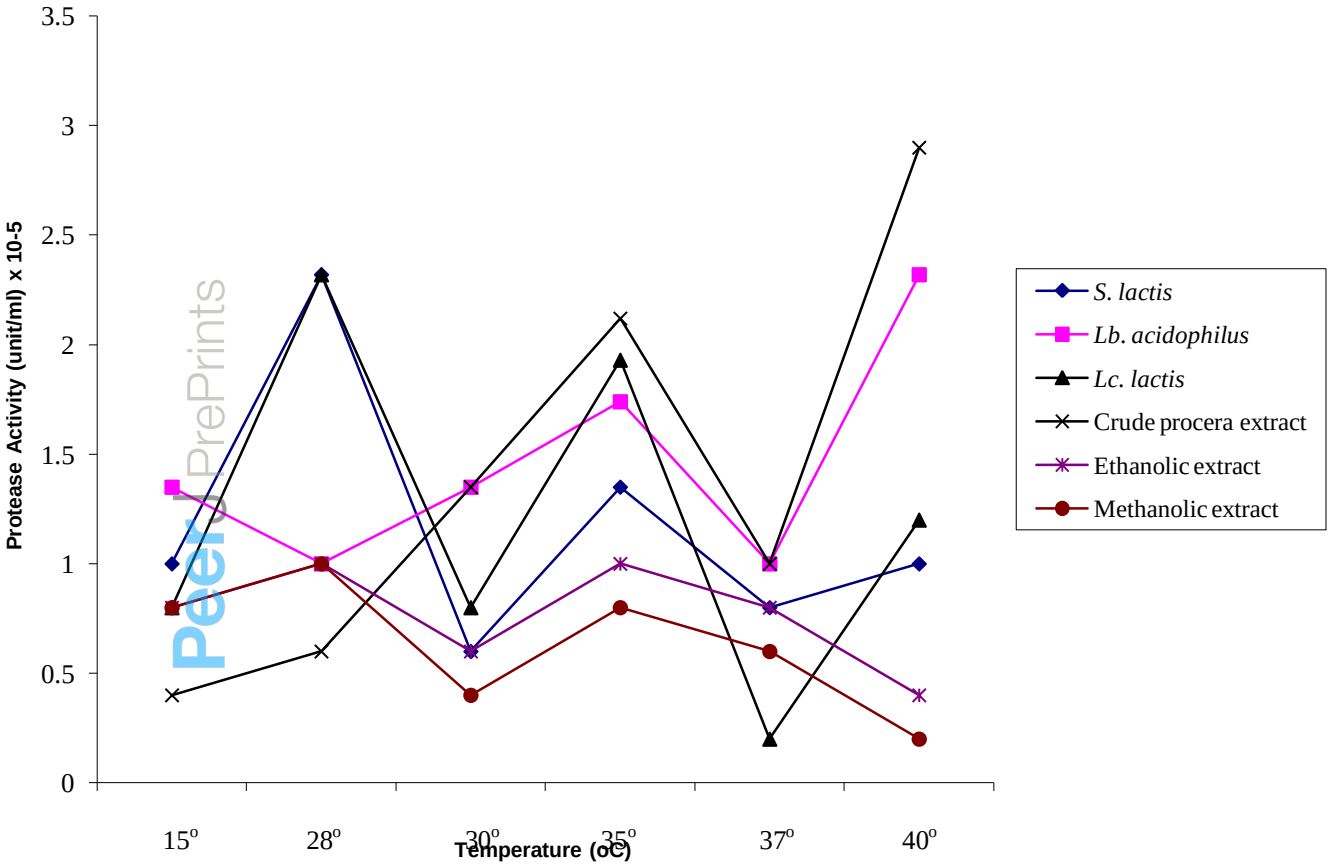

Fig. 23. Effect of Temperature on Protease Activity

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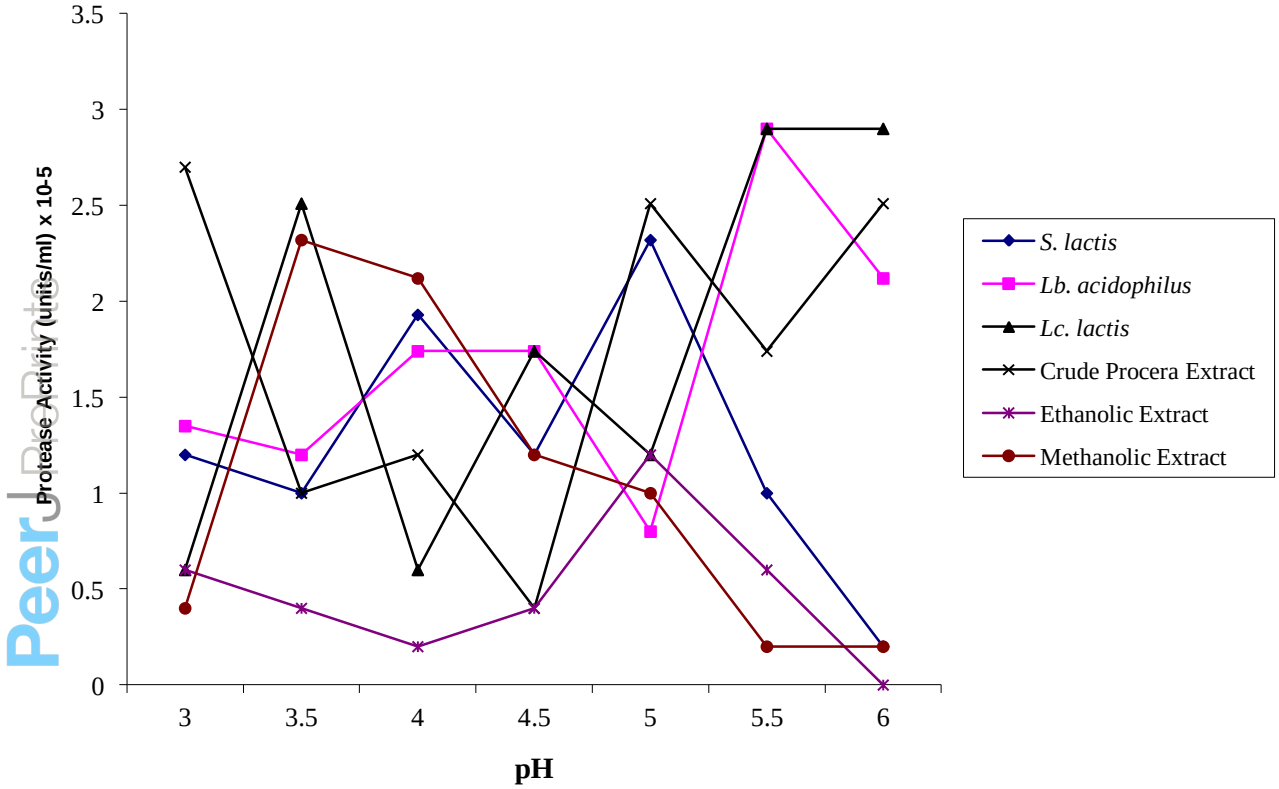

Fig. 24. Effect of pH on Protease Activity

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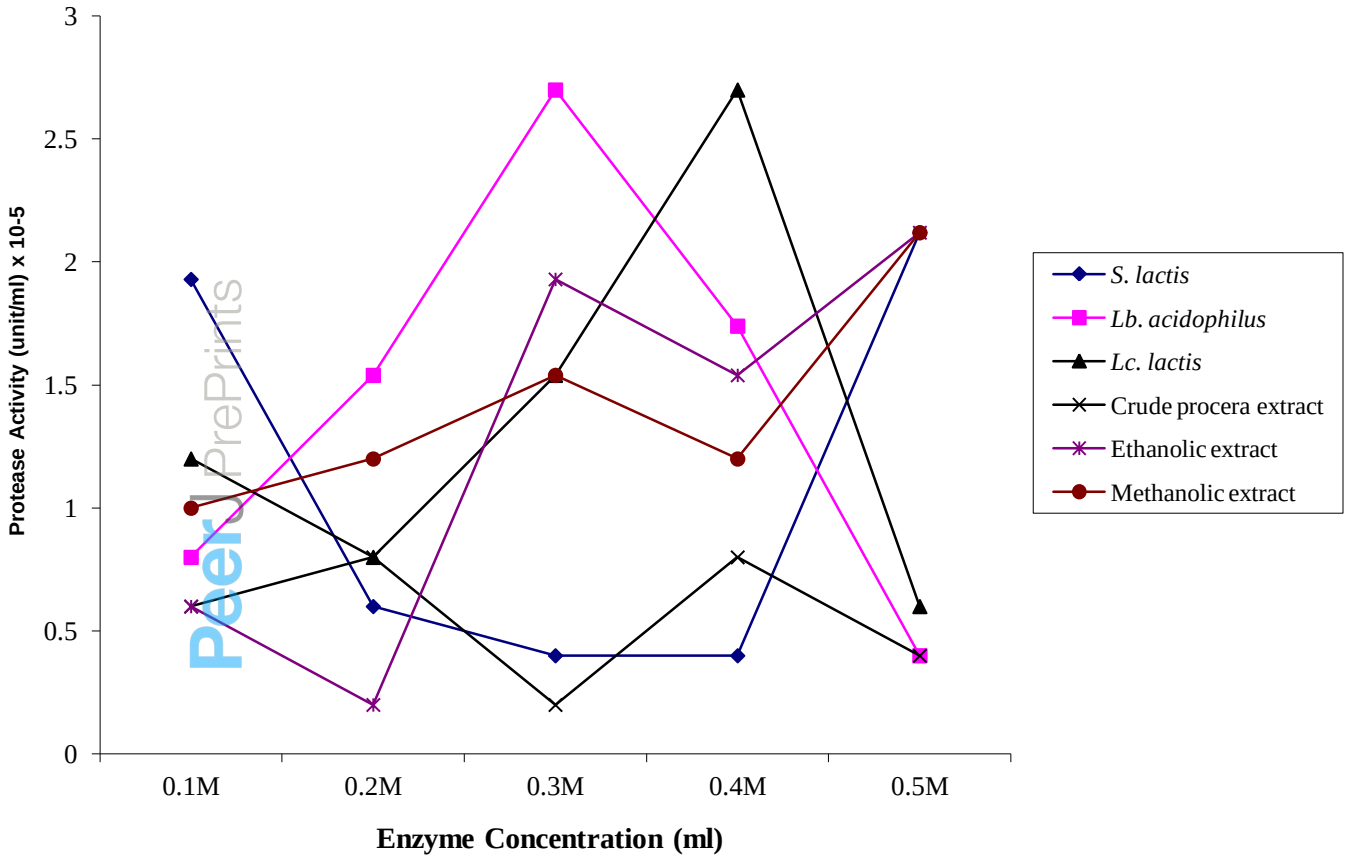

Fig. 25. Effect of Nitrophenol on Protease Activity of the isolates and *C. procera* extracts

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Effect of Inhibitors on Protease Activity

Figure 25 shows the effect of Nitrophenol inhibitor on protease activity of the six extracts. Protease activity of *Streptococcus lactis* was inhibited (0.40) at a concentration of 0.3M and 0.4M respectively while 0.1M and 0.5M concentration proved less inhibitive. For *Lactobacillus acidophilus* protease activity, the reverse was observed; inhibition was observed at 0.1M (0.80) and 0.5M (0.40) concentration while there was less inhibition at 0.3M (2.70) concentration. *Lactococcus lactis* protease activity was inhibited at 0.5M (0.60) and 0.2M (0.80) concentration while less inhibition was observed between 0.3M (1.54) and 0.4M (2.70) concentration. Inhibition was recorded for the crude extract of *C. procera* with a high at 0.3M (0.20) concentration while for the ethanolic extract inhibition was recorded at 0.2M (0.20) concentration and less inhibitive effect recorded at 0.5M concentration. The inhibitor had little effect o n the methanolic extract with no visible inhibition recorded, but a peak (2.12) was attained for its activity at 0.5M concentration after a decline at 0.4M concentration.

Figure 26 shows the inhibitive effect of EDTA on the extracts, at 0.3M and 0.2M concentrations, *Lactococcus lactis* and the crude extract of *C. procera* showed no activity (0.00) respectively. *Streptococcus lactis* activity increased gradually to attain a peak (2.90) at 0.3M concentration and then decreased afterwards. For *Lactobacillus acidophilus*, EDTA inhibited its protease activity (0.80) at 0.4M concentration after it peaked (6.00) at 0.2M concentration. *Lactococcus lactis* protease activity had its peak (4.83) at 0.5M concentration after being inhibited (0.00) at 0.3M concentration. Maximum activity (2.70) of the crude extract of *C. procera* was attained at 0.1M concentration while for the ethanolic extract maximum activity was recorded at 0.2M concentration after being inhibited at 0.1M concentration. Less inhibition was recorded for the methanolic extract as its activity rose

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gradually to a peak (3.86) at 0.5M concentration after stabilizing (1.74) at 0.3M and 0.4M concentration.

Figure 27 shows the inhibitive effect of urea on *Streptococcus lactis* and *Lactobacillus acidophilus* decreased as protease activity rose to its maximum 1.00 and 3.30 respectively at 0.3M concentration and began a decline afterwards. Inhibition (0.80) was observed for Lactococcus lactis at both 0.1M and 0.5M concentrations while protease activity peaked (3.10) at 0.4M concentration. Crude extract activity of *C. procera* was inhibited (0.60) at 0.4M concentration after a maximum activity (6.20) was attained at 0.3M concentration. After attaining a high activity (1.20) at 0.1M concentration for the ethanolic extract, a decline set in between 0.2M and 0.4M and the peak resurfaced at 0.5M concentration. The methanolic extract started off with a high activity (1.54) before being inhibited at both 0.2M and 0.4M concentrations, and finally attaining its peak (1.93) at 0.5M concentration.

Effect of Metal ions on Protease Activity

Figure 28 shows the effect of sodium nitrate metal ion on protease activity. The protease activity of *Streptococcus lactis* attained its peak (5.60) at 0.5M concentration after gradually increasing between 0.1M and 0.3M concentration. Activity for the crude extract of *C. procera* quickly attained its peak (8.11) at 0.2M concentration before falling to a low activity (0.40) at 0.5M concentration.

For *Lactococcus lactis*, there was an initial increase between 0.1M and 0.2M concentration before decreasing at 0.3M concentration and finally rising to peak at 0.5M, the same also applies to the activity of *Lactobacillus acidophilus* but its peak (3.86) was attained at 0.3M concentration. After series of increase and decrease the activity of Ethanolic extract of *C. procera* finally attained its peak (4.30) at 0.5M concentration. Maximum activity (5.60) was

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easily attained by the methanolic extract of *C. procera* at 0.1M concentration before decreasing to stability between 0.2M and 0.3M concentration.

Figure 29 shows the effect of ammonium chloride ion on protease activity. After easily attaining a peak (2.32) at 0.1m concentration, the activity of *Streptococcus lactis* plunged to absolutely no activity (0.00) at 0.3M concentration, but a steady increase was later noticed. This same pattern was noticed in the activity of the Ethanolic extract of *C. procera*, it peaked (4.30) at 0.1M concentration. *Lactococcus lactis* attained its peak (2.90) at 0.4M concentration after subsequent increase and decrease. *Lactobacillus acidophilus* activity decrease initially but rose to a peak (2.70) at 0.4M concentration before decreasing again at 0.5M concentration. After an initial increase at 0.1M concentration, both the crude and methanolic extract of *C. procera* activity decreased and rose to peak (2.51, 5.80) at 0.4M and 0.3M concentration respectively before decreasing again.

Figure 30 shows the effect of manganese sulphate ion on protease activity. After plunging to no activity at 0.2m concentration, both the activity of *Streptococcus lactis* and the crude extract of *C. procera* gradually rose to a peak (1.93, 1.20) at 0.4M and 0.5M concentrations respectively. The Ethanolic extract started with a decrease to no activity at 0.3M concentration before rising to a peak (1.00) at 0.4M concentration. The methanolic extract activity started off by peaking (2.2) at 0.1M concentration, it began to decrease until it increased again at 0.5M concentration. *Lactococcus lactis* activity rose to its maximum (2.32) at 0.2M concentration before a series of decrease and increase set in, while for *Lactobacillus acidophilus*, its activity gradually rose between 0.1M and 0.3M concentration before decreasing at 0.4M concentration and finally peaking (2.51) at 0.5M concentration.

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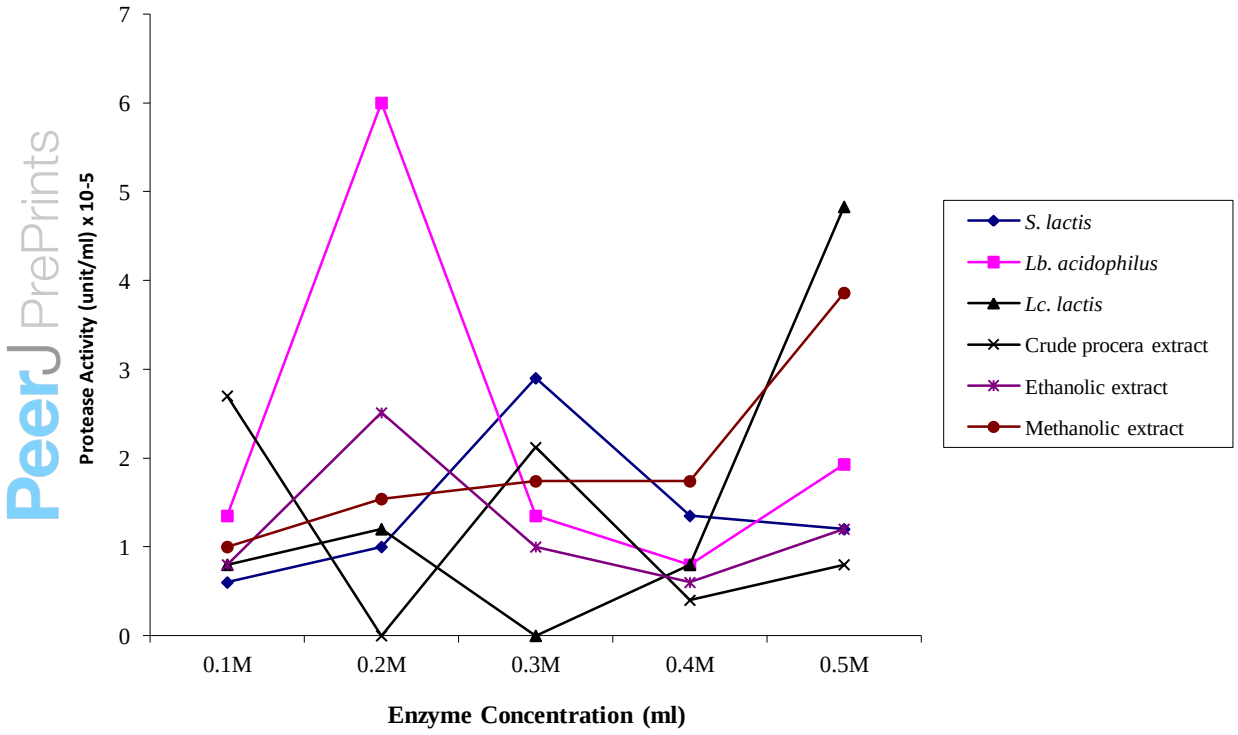

Fig. 26. Effect of EDTA on Protease Activity of the isolates and *C. procera* extracts

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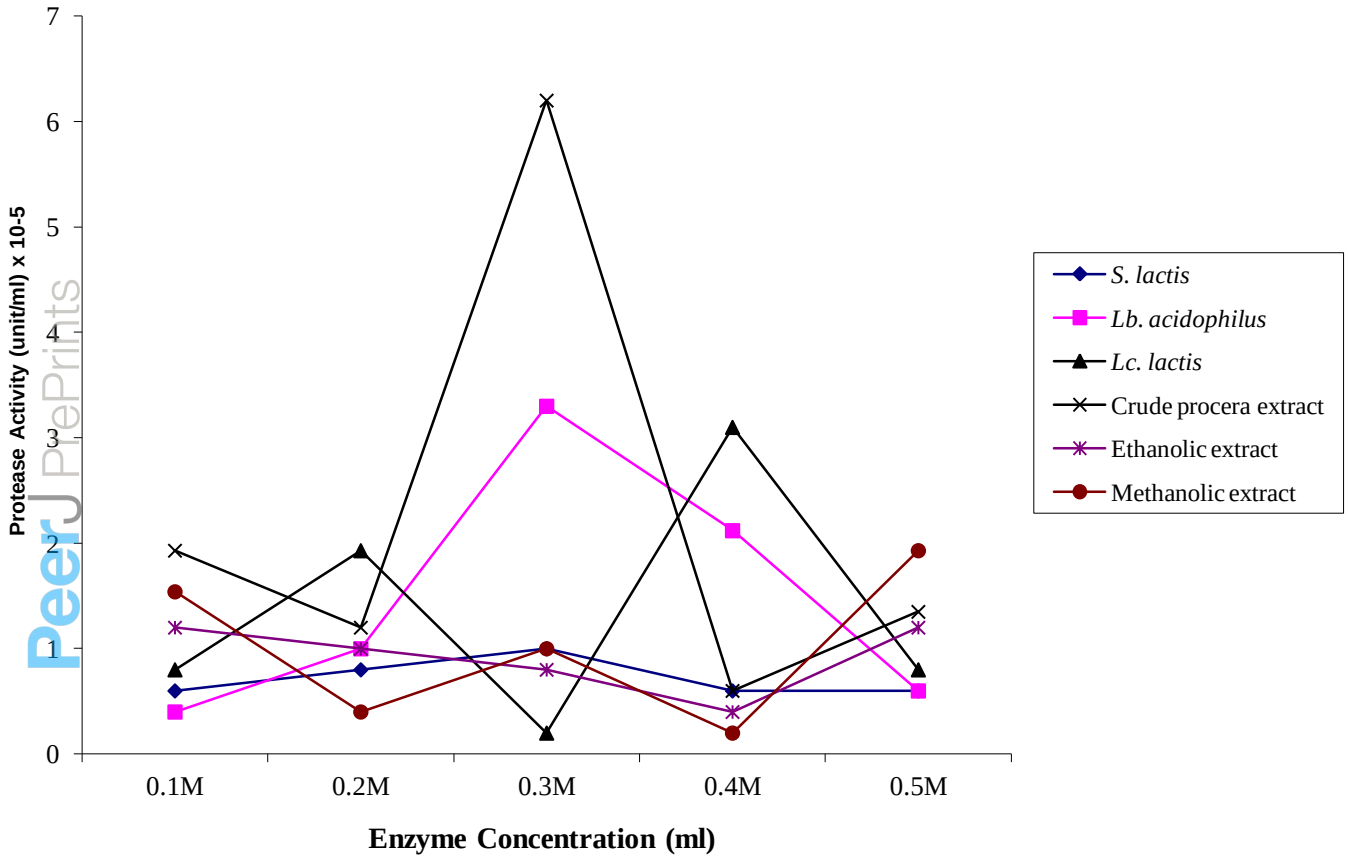

Fig. 27. Effect of Urea on Protease Activity of the isolates and *C. procera* extracts

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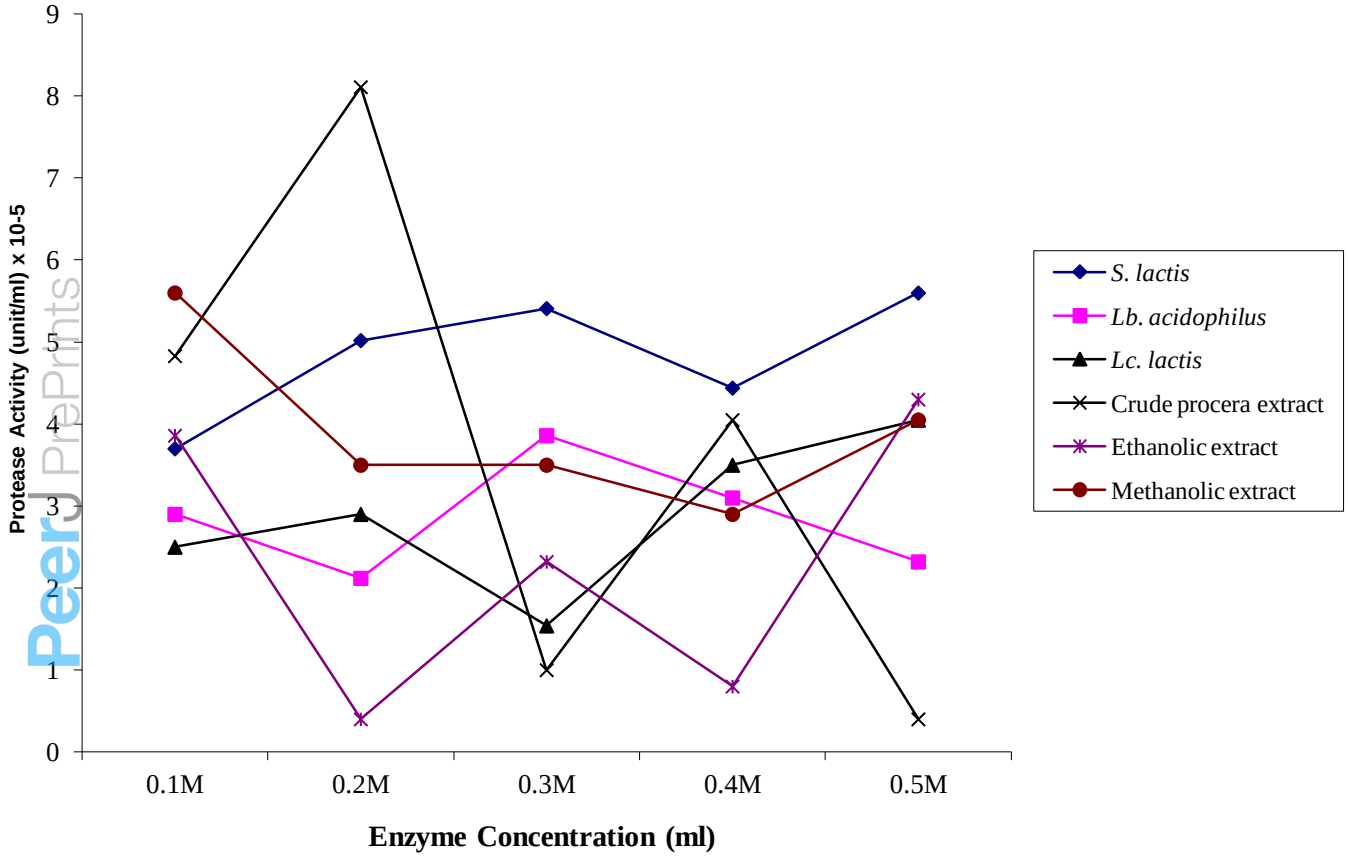

Fig. 28: Effect of $NaNO_3$ Metal ion on Protease Activity of the Six Extracts

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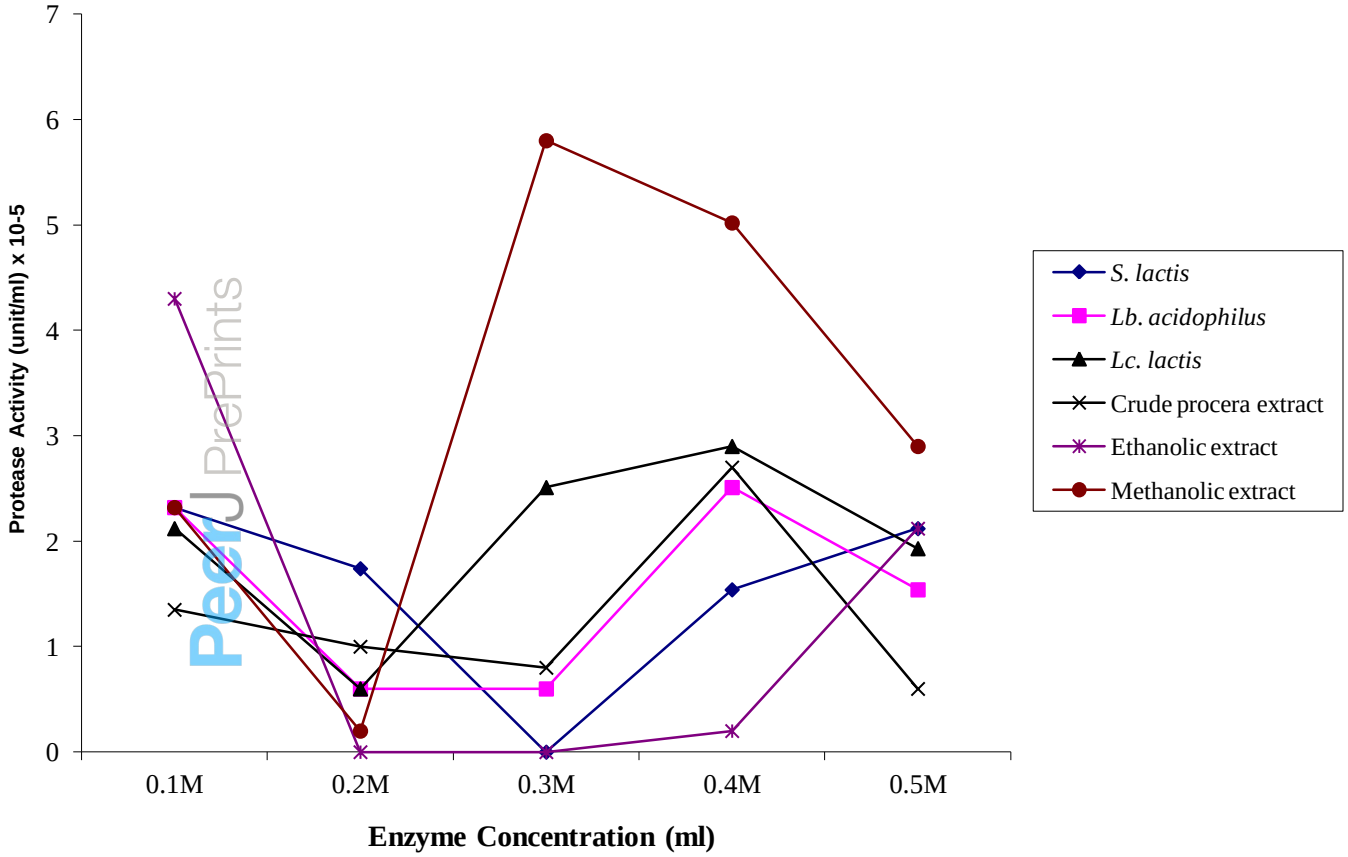

Fig. 29. Effect of NH_4Cl Metal Ion on Protease Activity

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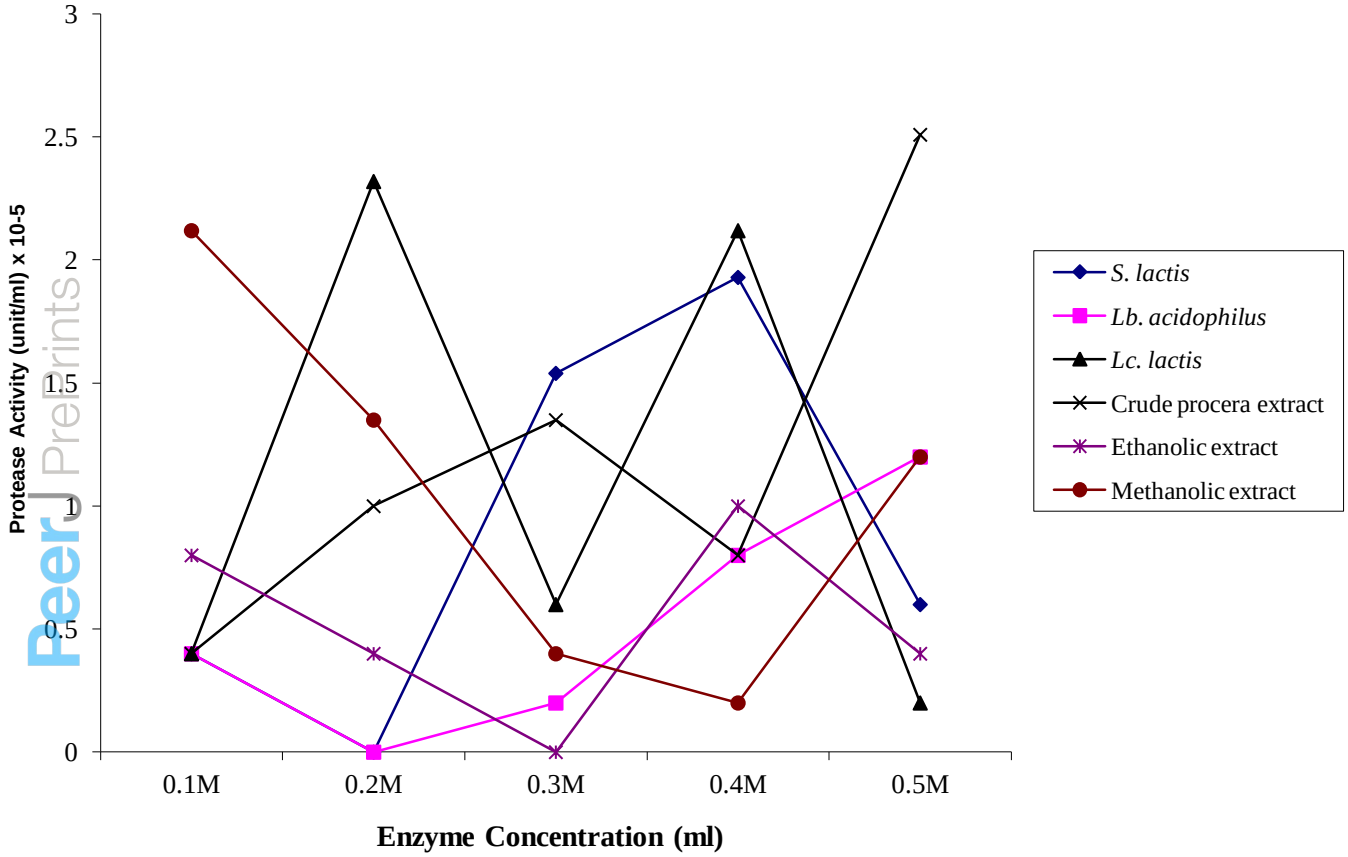

Fig. 30. Effect of $MnSO_4$ Metal Ion on Protease Activity

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Figure 31 shows the effect of potassium nitrate on protease activity. From no activity (0.00) at 0.1M concentration the crude extract of *C. procera* gradually rose to its maximum activity (2.90) at 0.3M concentration before falling to no activity again at 0.5M concentration. From a peak (2.12) at 0.1M concentration, the activity of *Streptococcus lactis* protease fell its lowest (0.80) at 0.4M concentration and suddenly peaked (2.12) again at 0.5M concentration. After a series of increase and decrease, the activity of *Lactococcus lactis* protease finally hit its maximum (2.90) at 0.5M concentration. From a high activity at 0.2M concentration the activity of *Lactobacillus acidophilus* fell at 0.3M to become stable at its maximum activity (2.51) between 0.4M and 0.5M concentration. The activity of the Ethanolic extract finally had its maximum (1.00) at 0.5M concentration after a series of increase and decrease while for the methanolic extract; a maximum activity (2.51) was maintained between 0.1M and 0.2M concentration before a decrease at 0.3M concentration.

Figure 32 shows the effect of calcium chloride ion on protease activity. From a high activity the activity of *Streptococcus lactis* protease fell between 0.2M and 0.4M concentrations and then rose to its maximum activity (2.51) at 0.5M concentration. After a decrease at 0.2M concentration the activity of the crude extract of *C. procera* hit its maximum (2.51) at 0.3M concentration while the activity of its Ethanolic extract gradually rose to its maximum (2.90) at 0.5M concentration. Likewise the methanolic extract, the activity rose to a stable maximum (2.51) at both 0.3M and 0.4M concentrations. After a decrease at 0.2M concentration the activity of *Lactococcus lactis* rose to become stable between 0.3M and 0.4M before hitting its maximum (2.51) at 0.5M concentration. *Lactobacillus acidophilus* activity finally peaked (2.70) at 0.4M concentration after a series of increase and decrease.

Figure 33 shows the effect of magnesium sulphate ion on protease activity. After attaining its maximum activity (3.50, 2.12) at 0.1M concentration, *Streptococcus lactis* and the crude

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extract of *C. procera* protease activity gradually decreased respectively while for both the activity of *Lactococcus lactis* and Ethanolic extract of *C. procera* a steady climb was noticed before attaining maximum activity (2.32, 1.74) at 0.3M and 0.4M concentration respectively. *Lactobacillus acidophilus* activity had its maximum (2.51) at 0.3M concentration after a decrease at 0.2M before another decrease was observed between 0.4M and 0.5M concentration. The activities of the methanolic extract peaked (2.12) at 0.2M and 0.4M concentration after a decrease at 0.3M concentration.

Figure 34 shows the effect of cupper sulphate on protease activity. From no activity at 0.1M concentration, maximum activity was noticed at 0.2M concentration for *Streptococcus lactis* protease while for *Lactobacillus acidophilus* a gradual decrease started after attaining its maximum activity (1.93) at 0.1M concentration. The crude extract of *C. procera* had its maximum activity (1.54) at 0.2M concentration before decreasing while for *Lactococcus lactis* maximum activity (2.51) was at 0.2M concentration before and after being stable at 0.1M and 0.3M concentration. After a decrease to stability between 0.3M and 0.4M concentration a maximum (2.12) was attained at 0.5M concentration for the ethanolic extract while a maximum (4.05) was easily attained at 0.1M concentration for the methanolic extract before decrease to its lowest activity at 0.2M concentration.

Figure 35 shows the effect of sodium chloride ion on protease activity. Starting with a decrease at 0.1M concentration, methanolic extract activity hit its maximum (2.51) at 0.2M concentration before decreasing again to retain its maximum activity at 0.5M concentration. The Ethanolic extract had a high activity at 0.1m concentration before gradually decreasing to no activity at 0.4M but rose to its maximum (3.70) at 0.5M concentration. *Lactobacillus acidophilus* maintained its maximum activity(3.30) at 0.1M and 0.5M concentration after a gradual decrease between 0.2M and 0.4M concentration. *Lactococcus lactis* maintained its

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maximum activity (3.10) at both 0.3M and 0.4M concentration after attaining it at 0.1M concentration with 0.5M concentration following close behind. The activity of the crude extract of *C. procera* gradually rose to become stable between 0.2M and 0.3M concentration before rising to its maximum (2.51) at 0.4M concentration. The activity of *Streptococcus lactis* peaked (2.70) at 0.2M concentration before decreasing between 0.3M and 0.4M to rise again at 0.5M concentration.

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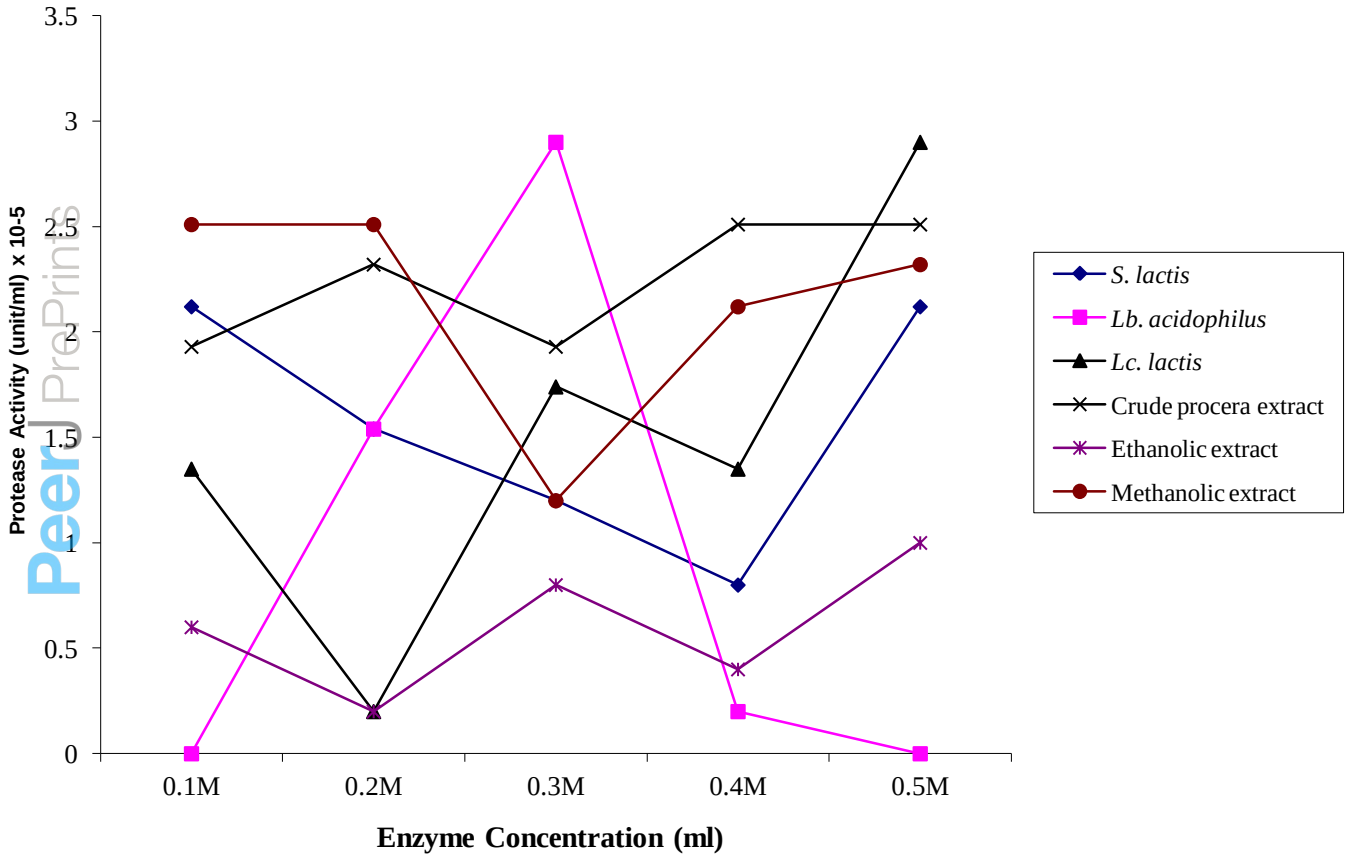

Fig. 31. Effect of KNO_3 Metal Ion on Protease Activity

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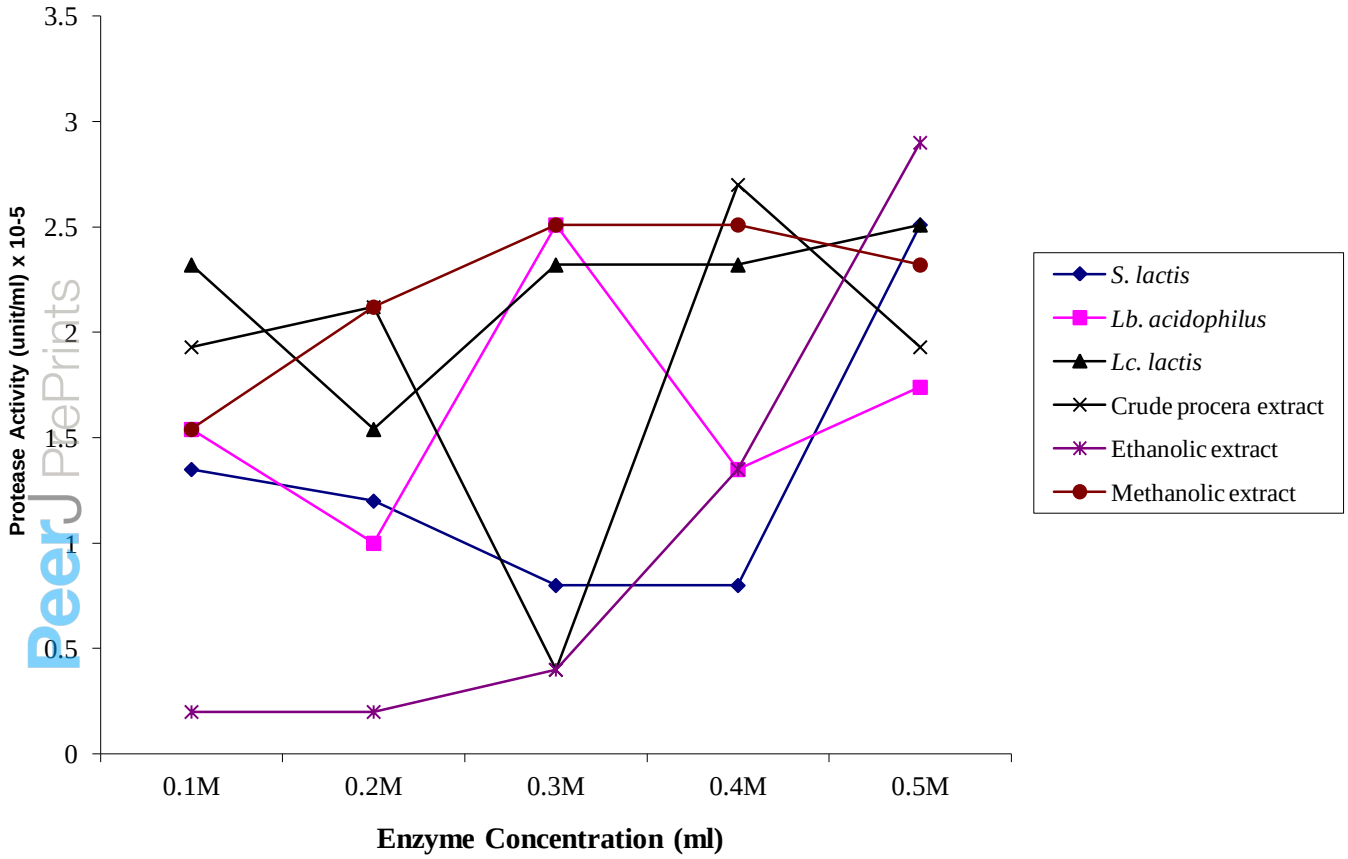

Fig. 32. Effect of $CaCl_2$ Metal Ion on Protease Activity

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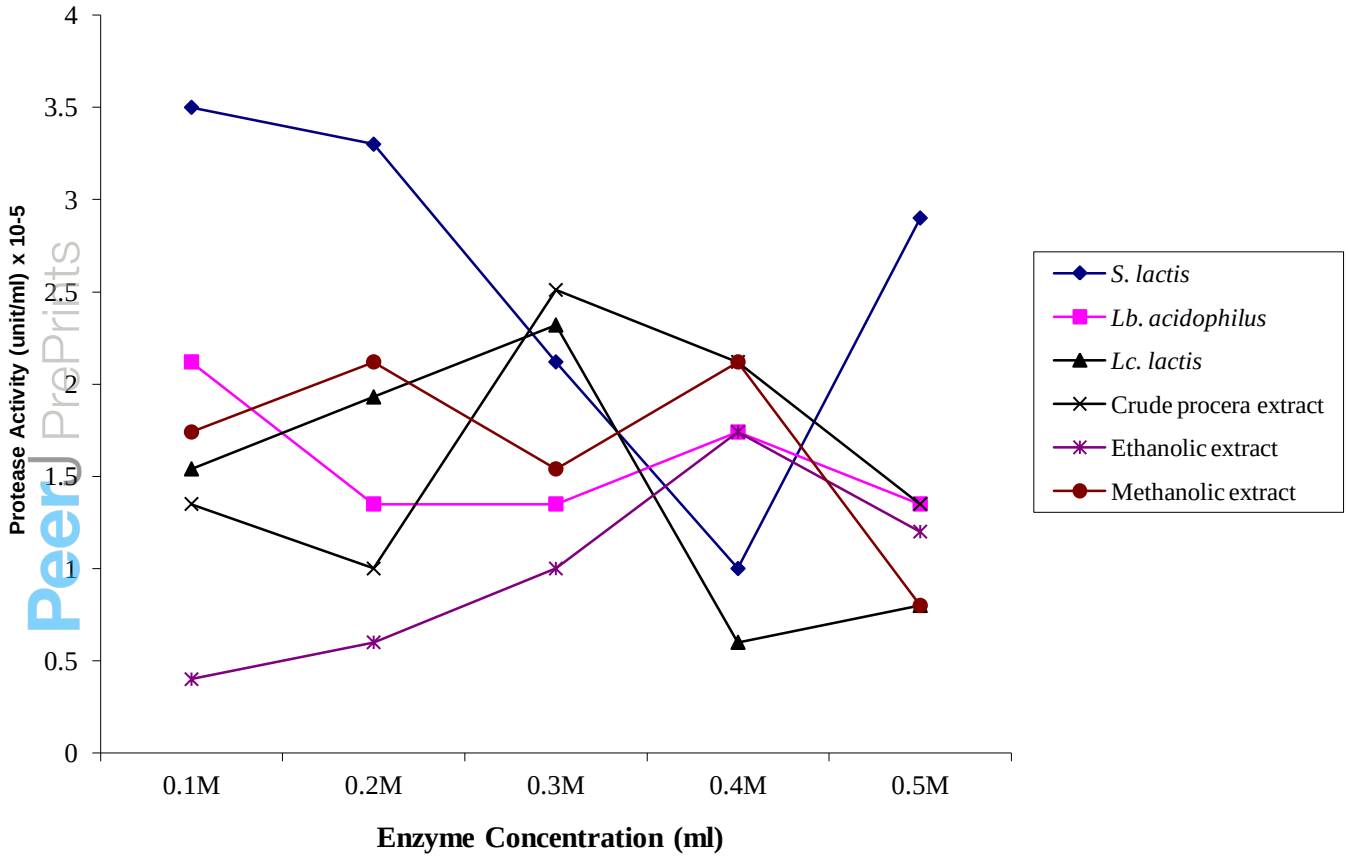

Fig. 33. Effect of $MgSO_4$ Metal Ion on Protease Activity

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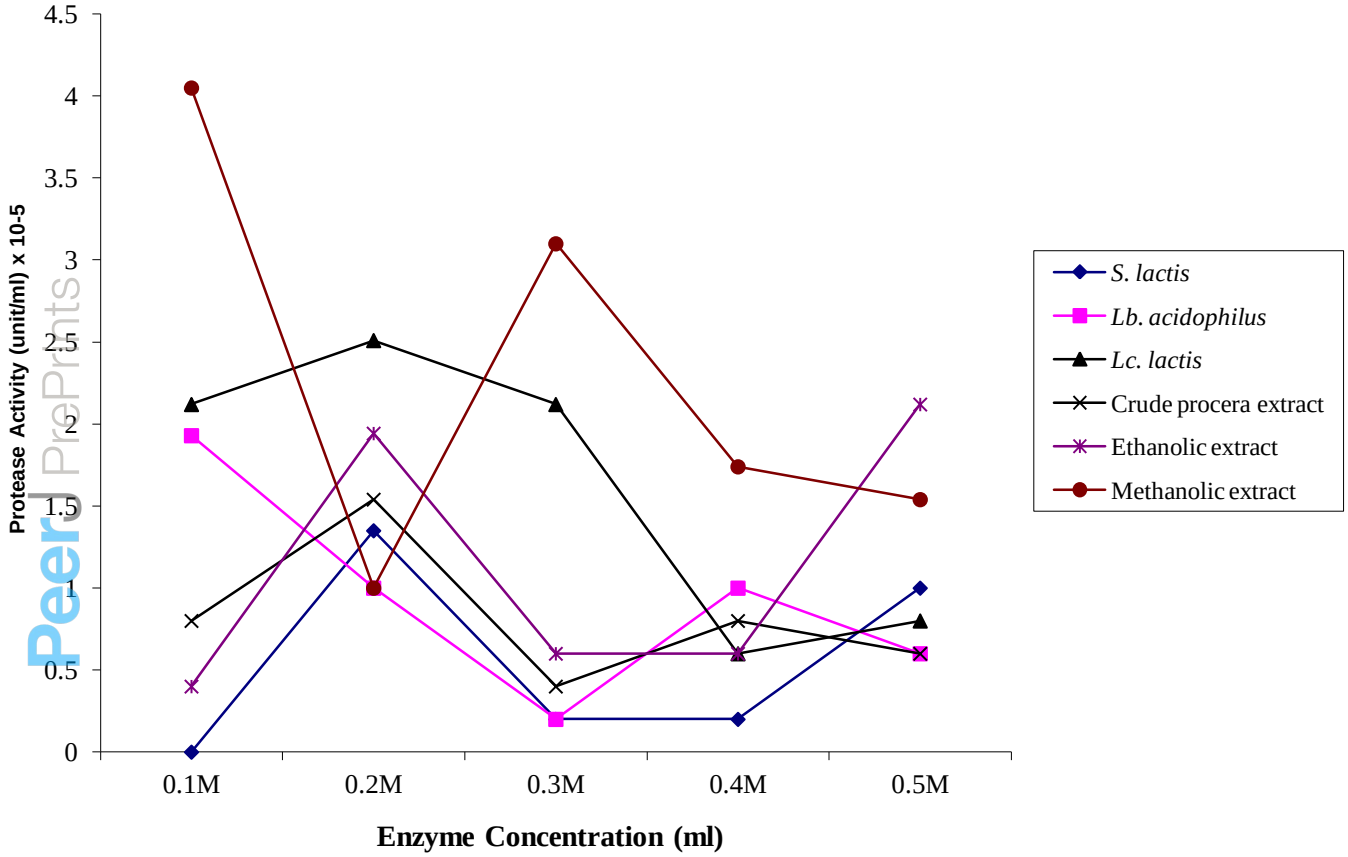

Fig. 34. Effect of $CuSO_4$ Metal Ion on Protease Activity

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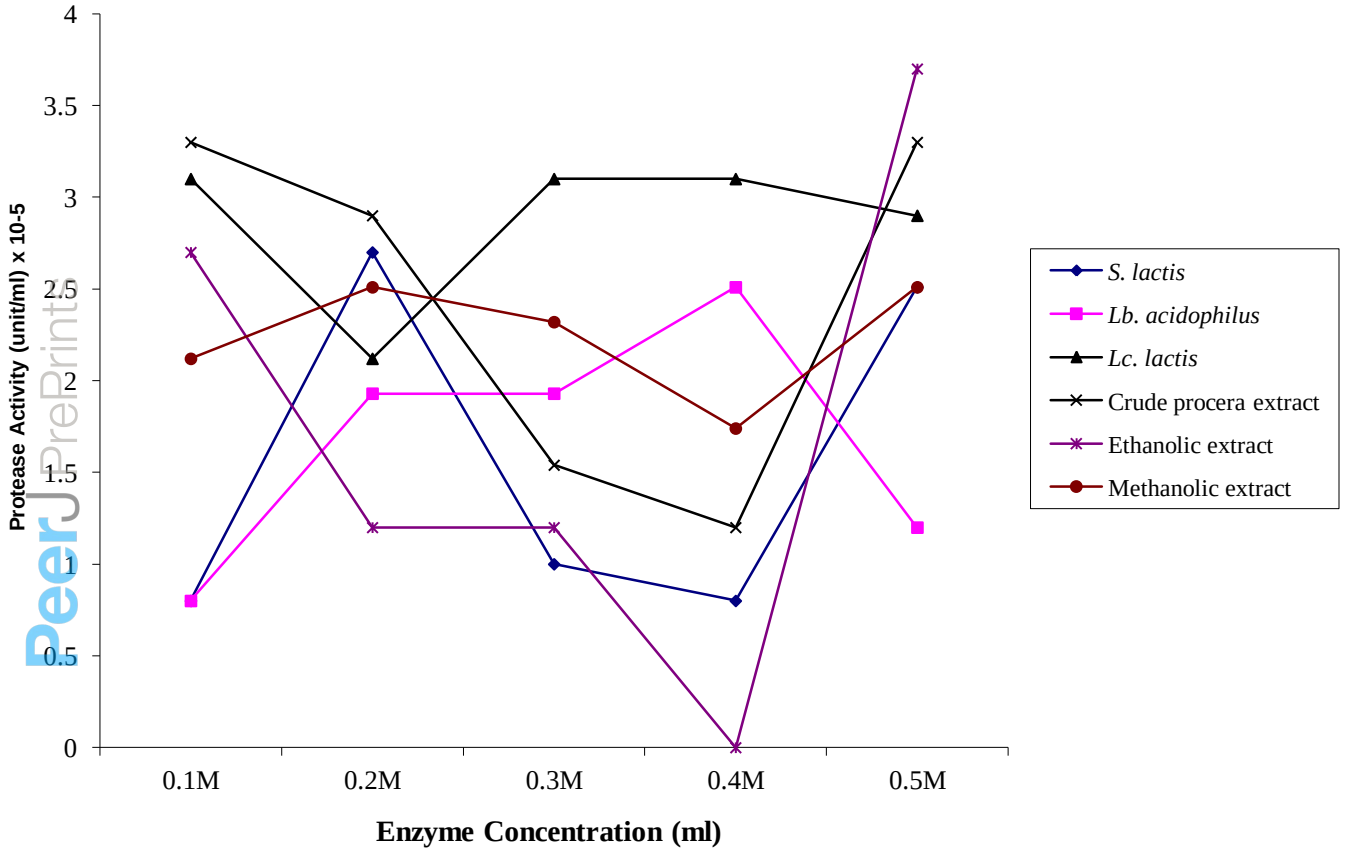

Fig. 35. Effect of NaCl Metal Ion on Protease Activity

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Effect of various Amino Acid on Enzyme activity

Table 4 shows that at 0.1ml concentration Panthetonic acid, Guanine and Leucine gave a high protease activity (3.70) for *Streptococcus lactis* but its maximum (4.44) was attained by tyrosine while the least (0.20) was from tryptophan and Lysine. Lysine and serine gave high activity for *Lactobacillus acidophilus* but Panthetonic acid gave the highest (3.86) with the least activity from methionine and tryptophan. Highest activity (4.44) was recorded for *Lactococcus lactis* with lysine while the least (0.20) was from panthetonic acid. The crude extract of *C. procera* equally had a high activity (3.86) with Lysine but its highest came from glutamic acid (4.44) while its least was from methionine (0.20). The Ethanolic extract also achieved a high activity with Lysine (5.02) while its maximum also came from glutamic acid (5.41) but Leucine gave no activity. Guanine, serine and lysine gave high activity to the methanolic extract but its highest came from panthetonic acid (12.94) while tryptophan had the least (2.90).

At 0.2ml concentration, table 5 shows that *Streptococcus lactis* activity was highly favoured by panthetonic acid and Lysine (3.30) with tyrosine and serine closely behind (2.90) while the least activity was recorded by Glutamic acid. Tyrosine gave the highest activity (4.30) for *Lactobacillus acidophilus* with the least activity (0.80) by Glutamic acid, Lysine and Leucine. *Lactococcus lactis* activity attained its highest (4.30) with serine with Tyrosine closely behind (3.50) while the least was recorded by Leucine Methionine and Tryptophan (0.60). For the crude extract, it attained its maximum activity (5.60) with Leucine while the least activity was attained with Glutamic acid, Panthetonic acid and Guanine. For the ethanolic extract of *C. procera*, glutamic acid gave the highest activity (6.60) and methionine gave the least activity (0.40). The methanolic extract showed its highest activity (1.74) with

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panthetonic acid with tryptophan Leucine closely behind (1.54) while the least activity (0.20) was with lysine.

In table 6 at 0.3ml concentration, high activity with Guanine, Methionine and Glutamic acid was recorded for *Streptococcus lactis* but its maximum activity was with lysine (4.83) while the least was with leucine (1.35). *Lactobacillus acidophilus* attained its highest activity with leucine (3.50) while Guanine, Panthetonic acid, Methionine and lysine had the least activity (1.20). *Lactococcus lactis* had its highest activity (6.20) with lysine and leucine while Panthetonic acid had the least activity (1.20). The crude extract of *C. procera* showed no activity with serine but its highest (5.41) was recorded with methionine and Guanine. For the Ethanolic extract of *C. procera*, no activity was recorded for serine and lysine while its highest activity (5.60) was with Guanine. The methanolic extract also recorded no activity for Glutamic acid and methionine while the highest activity (6.00) was from Leucine and Tyrosine with Panthetonic acid, Guanine and serine following closely behind.

At 0.4ml concentration, table 7 shows no activity for *Streptococcus lactis* with Glutamic acid and Tryptophan while the highest activity (5.60) was observed with Leucine. Guanine gave the highest activity (3.30) for *Lactobacillus acidophilus* with the least activity (0.40) from Lysine and Leucine. No activity was recorded for *Lactococcus lactis* in the presence of Tryptophan but its highest activity (2.51) was with Leucine. No activity was also recorded for the crude extract of *C. procera* in the presence of methionine and Tyrosine but its highest activity (3.10) was with Guanine. The Ethanolic extract of *C. procera* recorded its highest activity (1.74) with panthetonic acid with Tryptophan and Tyrosine closely behind (1.35) but the least activity was recorded with Lysine and Methionine. The Methanolic extract of *C. procera* had its highest activity (1.54) with Glutamic acid while its least activity (0.20) was recorded with both Leucine and Methionine.

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At 0.5ml concentration, table 8 shows *Streptococcus lactis* activity with its highest (2.90) in the presence of Panthetonic acid while the least was recorded with Tryptophan (0.20). *Lactobacillus acidophilus* activity cruised to the highest (3.50) in the presence of Tyrosine and Methionine with Panthetonic acid closely behind but the least was recorded with serine (0.20). *Lactococcus lactis* showed no activity in the presence of Panthetonic acid and Methionine but its highest (3.10) was recorded with serine. The crude extract of *C. procera* showed no activity with Methionine while recording its highest activity (6.20) with Lysine. For the Methanolic extract, its highest activity was recorded in the presence of Leucine (6.20) while its least was with Tyrosine (0.20). The Ethanolic extract also showed its highest activity (6.20) with panthetonic acid while its least was recorded with serine (0.20).

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Table 4: Effect of 0.1ml Amino Acid Concentration on Protease Activity

Extracts	Amino Acids (ml)/Enzyme Activity (units/ml) x 10^{-5}								
	Serine	**Glutamic Acid**	**Lysine**	**Leucine**	**Methionine**	**Tyrosine**	**Tryptophan**	**Panthotenic Acid**	**Guanine**
S. lactis	1.93*	2.32	0.20	3.70	2.90	4.44	0.20	3.70	3.70
Lb. acidophilus	3.50	1.35	3.30	1.20	0.60	2.90	0.60	3.86	1.35
Lc. lactis	3.86	3.50	4.44	1.54	1.20	2.70	2.51	0.20	1.54
Crude Procera Extract	2.12	4.44	3.86	3.10	0.20	0.80	1.00	1.74	2.70
Ethanolic Extract	2.51	5.41	5.02	0.00	3.30	0.20	3.30	1.93	1.93
Methanolic Extract	11.20	3.50	10.04	5.21	4.83	7.72	2.90	12.94	11.40

* Each value is a mean of duplicate determinations.

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Table 5: Effect of 0.2ml Amino Acid Concentration on Protease Activity

Extracts	Amino Acids (ml)/Enzyme Activity (units/ml) x 10^{-5}								
	Serine	**Glutamic Acid**	**Lysine**	**Leucine**	**Methionine**	**Tyrosine**	**Tryptophan**	**Panthotenic Acid**	**Guanine**
S. lactis	2.90*	0.40	3.30	1.00	0.60	2.90	0.80	3.30	0.60
Lb. acidophilus	1.74	0.80	0.80	0.60	3.30	4.30	1.35	1.93	1.20
Lc. lactis	4.30	1.93	1.74	0.60	0.60	3.50	0.60	1.35	2.90
Crude Procera Extract	1.35	0.80	3.50	5.60	1.20	2.32	3.50	0.80	0.80
Ethanolic Extract	4.30	6.60	1.93	3.86	0.40	3.86	3.86	4.44	3.86
Methanolic Extract	0.40	0.60	0.20	1.54	1.20	0.80	1.54	1.74	0.80

* Each value is a mean of duplicate determinations.

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Table 6: Effect of 0.3ml Amino Acid Concentration on Protease Activity

Extracts	Amino Acids (ml)/Enzyme Activity (units/ml) x 10^{-5}								
	Serine	**Glutamic Acid**	**Lysine**	**Leucine**	**Methionine**	**Tyrosine**	**Tryptophan**	**Panthotenic Acid**	**Guanine**
S. lactis	1.74*	3.30	4.83	1.35	3.70	1.74	2.12	1.54	3.86
Lb. acidophilus	2.70	1.74	1.20	3.50	1.20	1.54	2.32	1.20	1.20
Lc. lactis	1.35	1.35	6.20	6.20	3.30	1.54	4.83	1.20	2.70
Crude Procera Extract	0.00	3.10	1.20	2.70	5.41	3.86	1.74	2.32	5.41
Ethanolic Extract	0.00	0.80	0.00	4.10	2.12	1.54	2.70	0.20	5.60
Methanolic Extract	5.60	0.00	2.70	6.00	0.00	6.00	2.51	5.21	5.21

* Each value is a mean of duplicate determinations.

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Table 7: Effect of 0.4ml Amino Acid Concentration on Protease Activity

Extracts	Amino Acids (ml)/Enzyme Activity (units/ml) x 10^{-5}								
	Serine	**Glutamic Acid**	**Lysine**	**Leucine**	**Methionine**	**Tyrosine**	**Tryptophan**	**Panthotenic Acid**	**Guanine**
S. lactis	0.40*	0.00	1.93	5.60	0.40	0.40	0.00	2.32	0.60
Lb. acidophilus	1.74	0.60	0.40	0.40	3.10	0.60	0.60	0.60	3.30
Lc. lactis	2.32	0.20	2.12	2.51	0.40	2.12	0.00	0.20	0.20
Crude Procera Extract	0.80	1.93	2.12	2.12	0.00	0.00	0.20	1.93	3.10
Ethanolic Extract	0.80	0.40	0.20	1.00	0.20	1.35	1.35	1.74	1.20
Methanolic Extract	0.60	1.54	0.60	0.20	0.20	0.80	0.40	0.60	0.40

* Each value is a mean of duplicate determinations.

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Table 8: Effect of 0.5ml Amino Acid Concentration on Protease Activity

Extracts	Amino Acids (ml)/Enzyme Activity (units/ml) x 10^{-5}								
	Serine	**Glutamic Acid**	**Lysine**	**Leucine**	**Methionine**	**Tyrosine**	**Tryptophan**	**Panthotenic Acid**	**Guanine**
S. lactis	0.40*	0.40	0.80	1.20	0.40	0.80	0.20	2.90	1.20
Lb. acidophilus	0.20	0.80	1.20	1.74	2.90	3.50	1.93	2.70	0.60
Lc. Lactis	3.10	1.74	0.60	2.12	0.00	1.35	2.12	0.00	2.70
Crude Procera Extract	0.40	0.60	6.20	2.32	0.00	0.60	2.12	2.32	0.20
Ethanolic Extract	0.20	1.00	1.35	2.70	1.20	2.51	2.32	6.20	1.74
Methanolic Extract	1.74	0.80	0.60	6.20	0.60	0.20	1.00	0.60	1.00

* Each value is a mean of duplicate determinations.

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Separation by Ion – Exchange Chromatography (G – 100)

Figure 36 shows the separation by ion – exchange chromatography (G – 100) of high molecular weight proteins and enzymic activity of the fractions of *Streptococcus lactis* towards casein. Peak A with fraction number nineteen (19) had the protein content of 0.02mg/ml and protease activity of 10.62 units/ml $x10^{-5}$ while peak B with fraction number fifty-one (51) had the protein content of 0.07mg/ml and protease activity of 12.16 units/ml $x10^{-5}$. Figure 37 shows the separation by ion – exchange chromatography (G – 100) of high molecular weight proteins and enzymic activity of the fractions of *Lactococcus lactis* towards casein. Peak A with fraction number thirty-five (35) had the protein content of 0.08 mg/ml and protease activity of 8.50 units/ml $x10^{-5}$ while peak B with fraction number fifty-four (54) had the protein content of 0.10 mg/ml and protease activity of 7.72 units/ml $x10^{-5}$.

Figure 38 shows the separation by ion – exchange chromatography (G – 100) of high molecular weight proteins and enzymic activity of the fractions of *Lactobacillus acidophilus* towards casein. Peak A with fraction number twenty-three (23) had the protein content of 0.23 mg/ml and protease activity of 11.20 units/ml $x10^{-5}$ while peak B with fraction number twenty-nine (29) had the protein content of 0.09 mg/ml and protease activity of 9.85 units/ml $x10^{-5}$. Figure 39 shows the separation by ion – exchange chromatography (G – 100) of high molecular weight proteins and enzymic activity of the fractions of the crude extract of *Calotropis procera* towards casein. Peak A with fraction number eighteen (18) had the protein content of 0.07 mg/ml and protease activity of 6.18 units/ml $x10^{-5}$ while peak B with fraction number twenty-four (24) had the protein content of 0.23 mg/ml and protease activity of 5.41 units/ml $x10^{-5}$. Figure 40 shows the separation by ion – exchange chromatography (G – 100) of high molecular weight proteins and enzymic activity of the fractions of ethanolic extract of *Calotropis procera* towards casein. Peak A with fraction number twelve (12) had the protein

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content of 0.07 mg/ml and protease activity of 3.67 units/ml $x10^{-5}$ while peak B with fraction number fifty-four (54) had the protein content of 0.21 mg/ml and protease activity of 6.76 units/ml $x10^{-5}$. Figure 41 shows the separation by ion – exchange chromatography (G – 100) of high molecular weight proteins and enzymic activity of the fractions of methanolic extract of *Calotropis procera* towards casein. Peak A with fraction number twenty-four (24) had the protein content of 0.05 mg/ml and protease activity of 5.80 units/ml $x10^{-5}$ while peak B with fraction number thirty-one (31) had the protein content of 0.10 mg/ml and protease activity of 6.76 units/ml $x10^{-5}$.

Separation by Ion – Exchange Chromatography (C - 50)

Figure 42 shows the separation by ion – exchange chromatography (C – 50) of low molecular weight proteins and enzymic activity of the fractions of *Streptococcus lactis* towards casein, peak Ba with fraction number seven (7) had the protein content of 0.25 mg/ml and protease activity of 9.46 units/ml $x10^{-5}$. Figure 43 shows the separation by ion – exchange chromatography (C – 50) of low molecular weight proteins and enzymic activity of the fractions of *Lactobacillus acidophilus* towards casein, peak Ba with fraction number ten (10) had the protein content of 0.01 mg/ml and protease activity of 6.95 units/ml $x10^{-5}$. Figure 44 shows the separation by ion – exchange chromatography (C – 50) of low molecular weight proteins and enzymic activity of the fractions of *Lactococcus lactis* towards casein, peak Ac with fraction number fifty-six (56) had the protein content of 0.05 mg/ml and protease activity of 7.72 units/ml $x10^{-5}$

Figure 45 shows the separation by ion – exchange chromatography (C – 50) of low molecular weight proteins and enzymic activity of the fractions of Crude extract of *Calotropis procera* towards casein, peak Bc with fraction number fifty-two (52) had the protein content of 0.27

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mg/ml and protease activity of 6.00 units/ml $x10^{-5}$. Figure 46 shows the separation by ion – exchange chromatography (C – 50) of low molecular weight proteins and enzymic activity of the fractions of the ethanolic extract of *Calotropis procera* towards casein. Peak Bc with fraction number fifty-four (54) had the protein content of 0.18 mg/ml and protease activity of 7.53 units/ml $x10^{-5}$. Figure 47 shows the separation by ion – exchange chromatography (C – 50) of low molecular weight proteins and enzymic activity of the fractions of the methanolic extract of *Calotropis procera* towards casein, peak Ba with fraction number thirty-one (31) had the protein content of 0.07 mg/ml and protease activity of 8.30 units/ml $x10^{-5}$.

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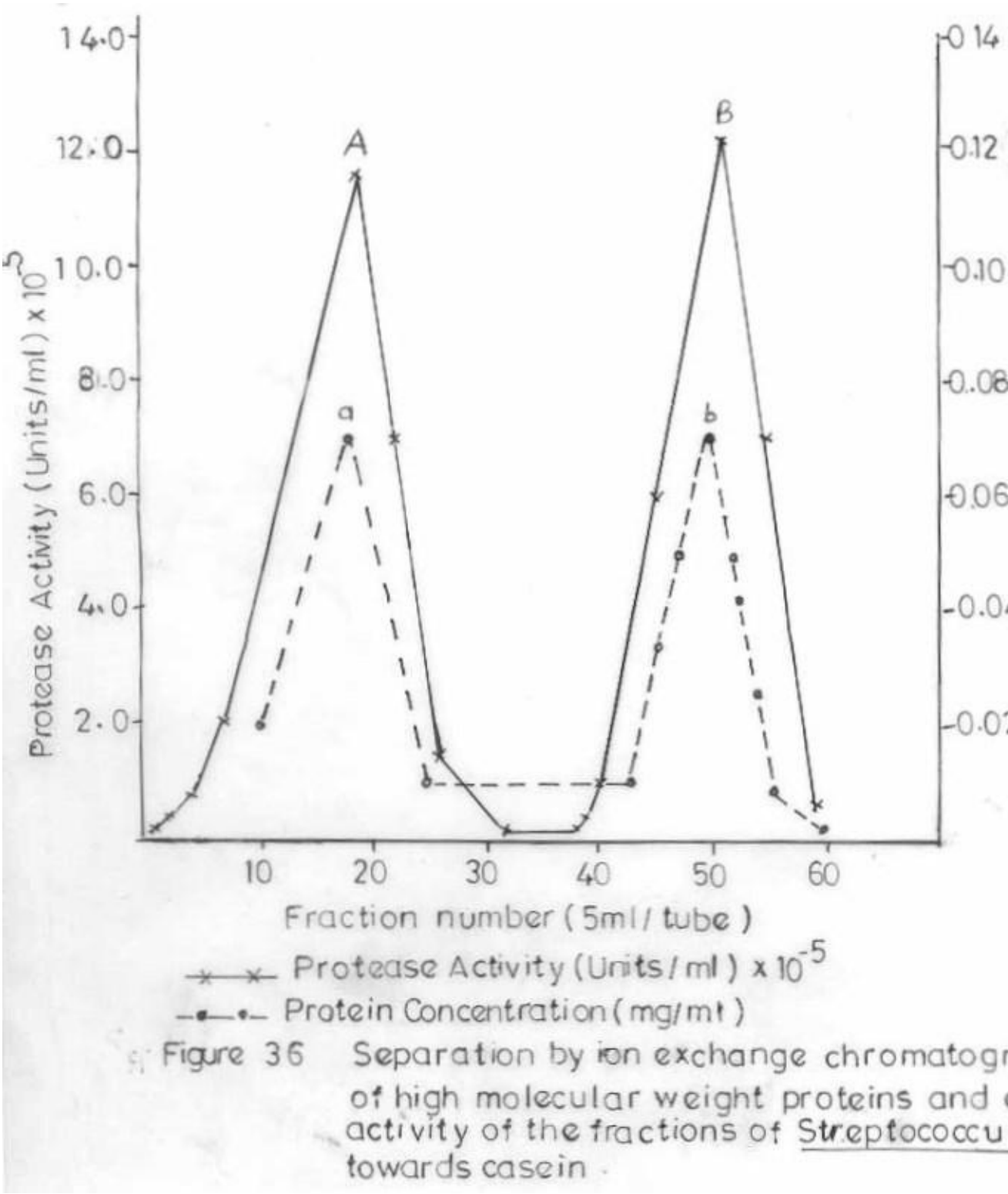

Figure 36 Separation by ion exchange chromatogr
of high molecular weight proteins and
activity of the fractions of Streptococcu
towards casein

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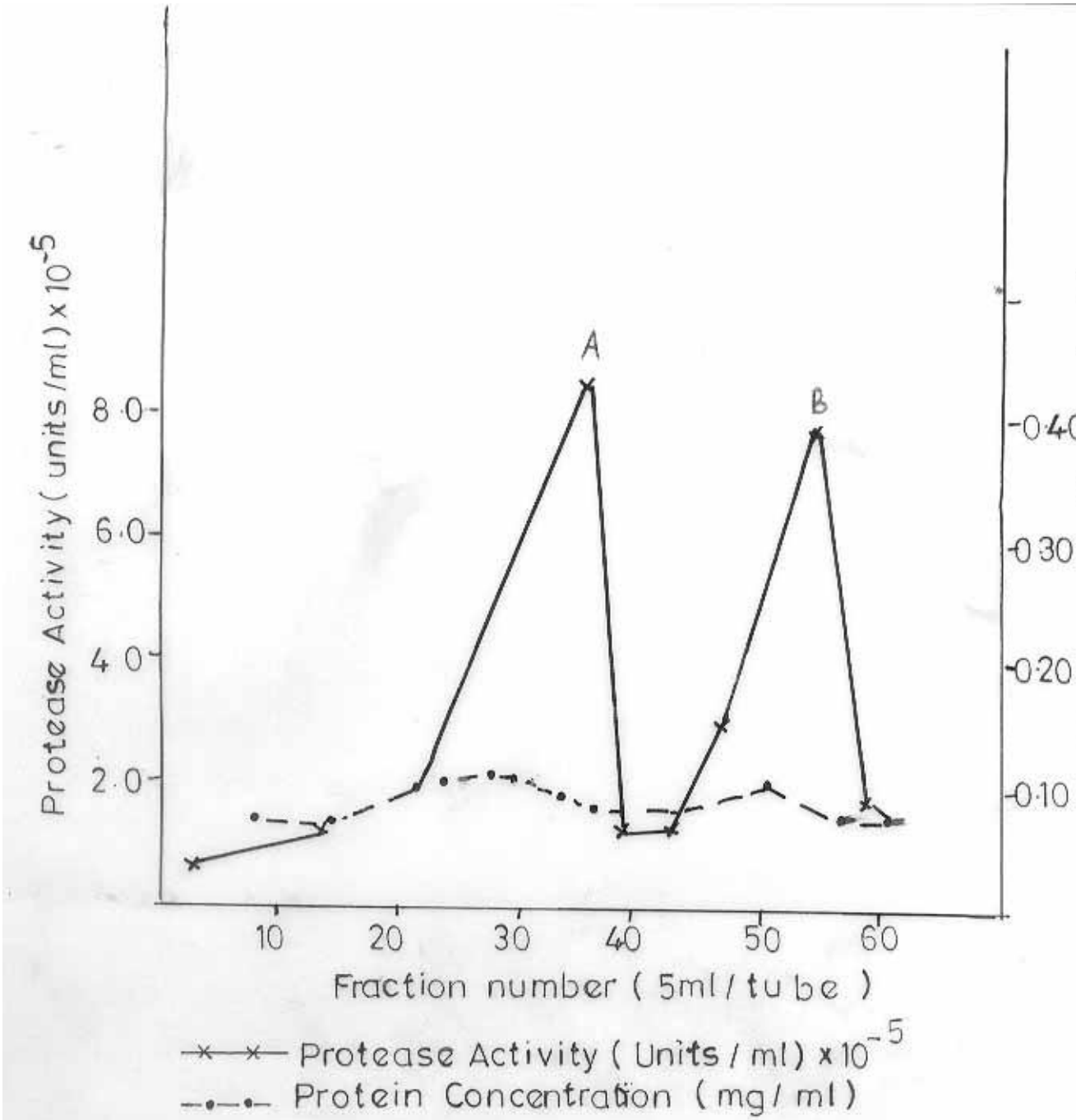

Figure 37. Separation by ion exchange chromatogra
of high molecular weight proteins and enz
activity of the fractions of Lactococcus lac
casein

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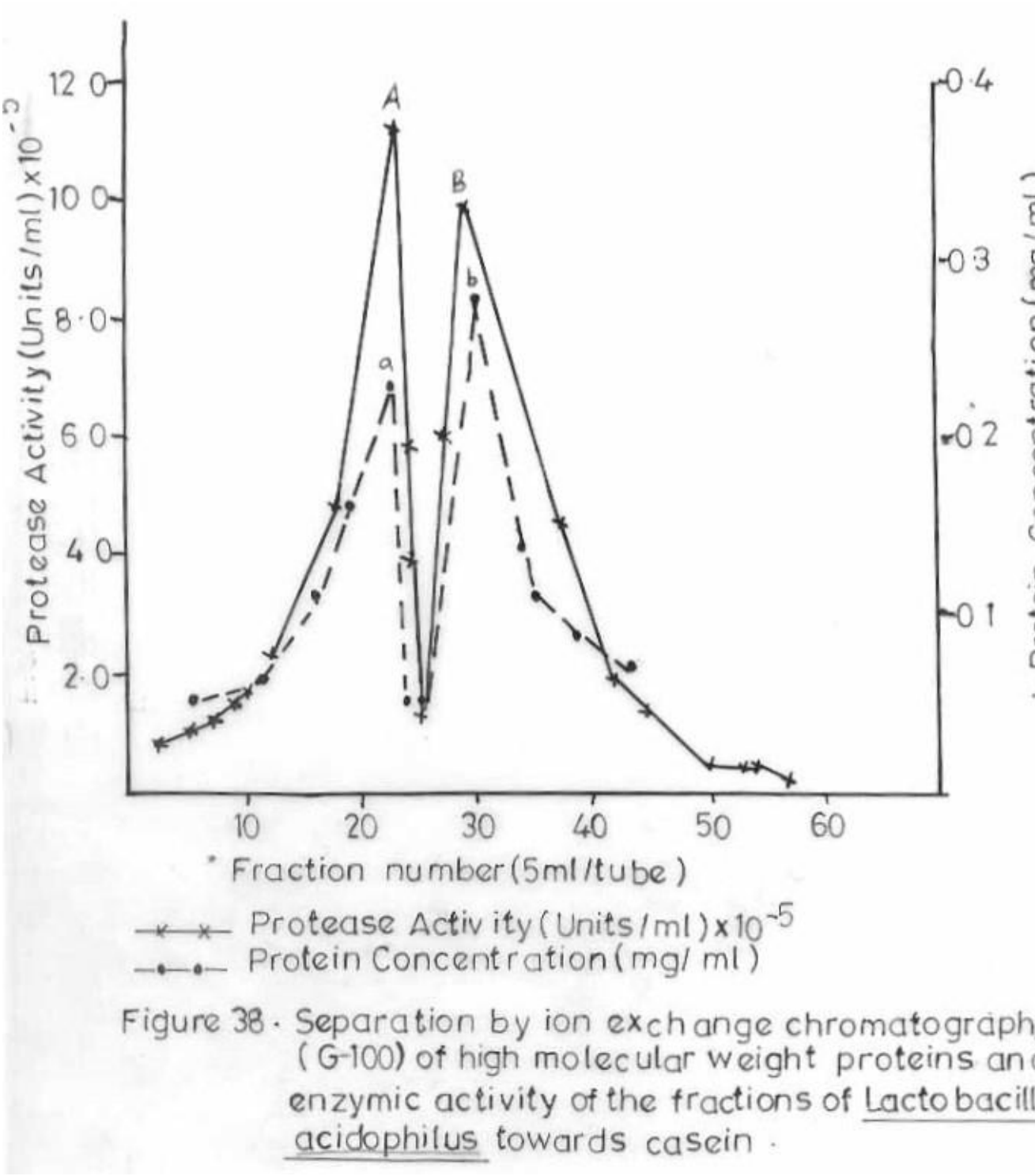

Figure 38. Separation by ion exchange chromatograph (G-100) of high molecular weight proteins an enzymic activity of the fractions of Lactobacill acidophilus towards casein.

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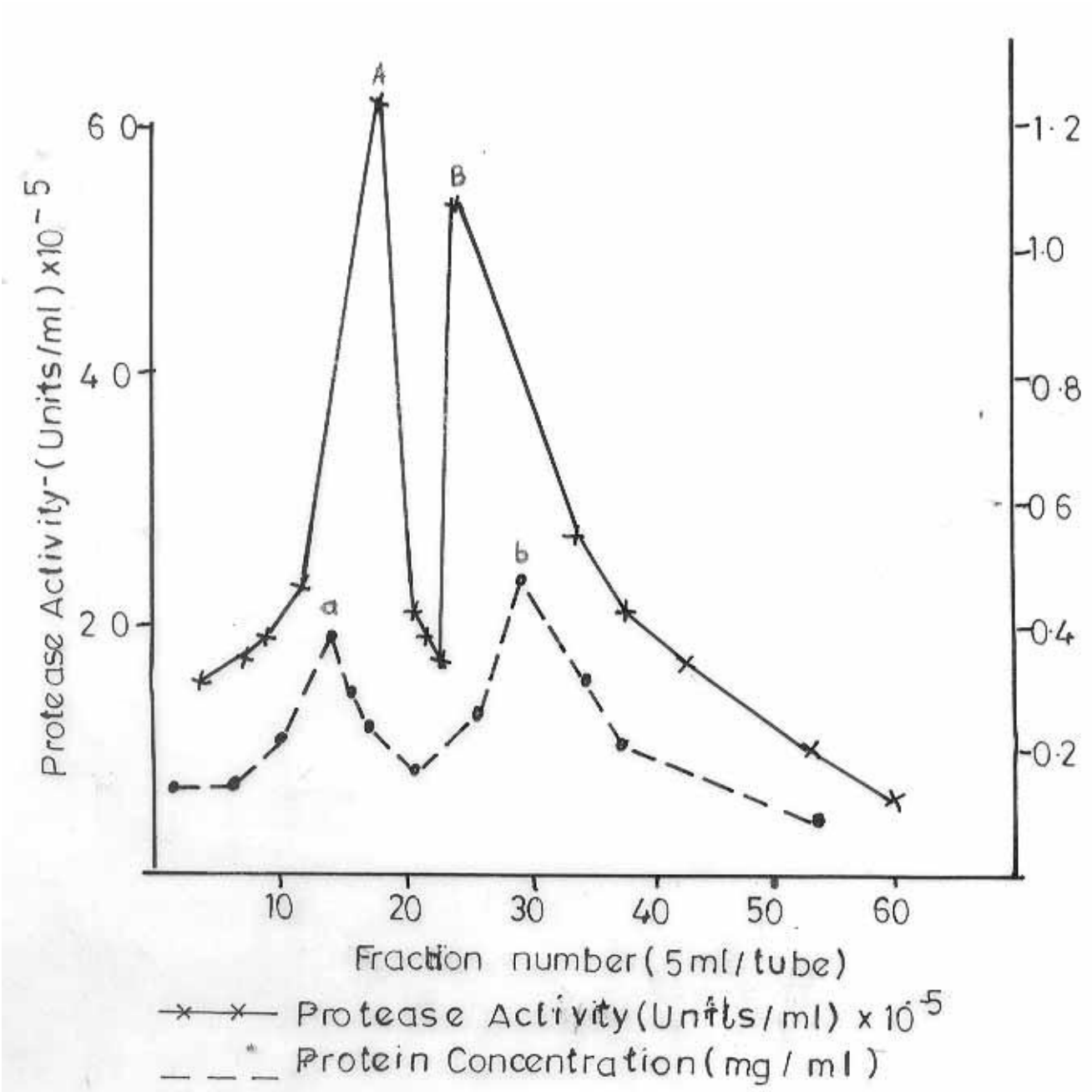

Figure 39. Separation by ion exchange chromatograp
of high molecular weight proteins and e
of the fractions of crude extract from Cal
procera towards casein.

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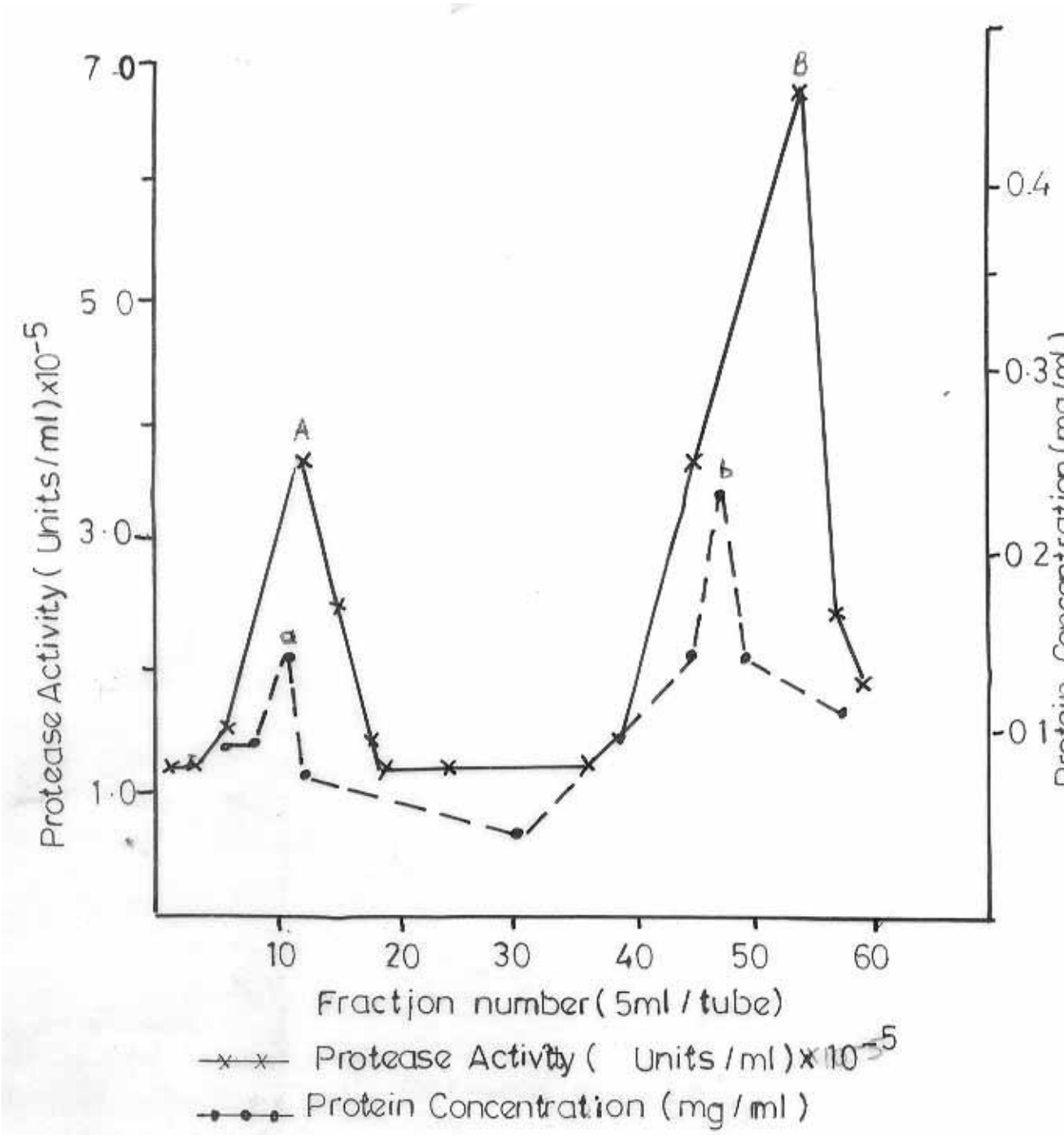

Figure 40. Separation by ion exchange chromatography (G-1
of high molecular weight proteins and enzymic act
of the fractions of Ethanolic extract of Calotropis
procera towards casein

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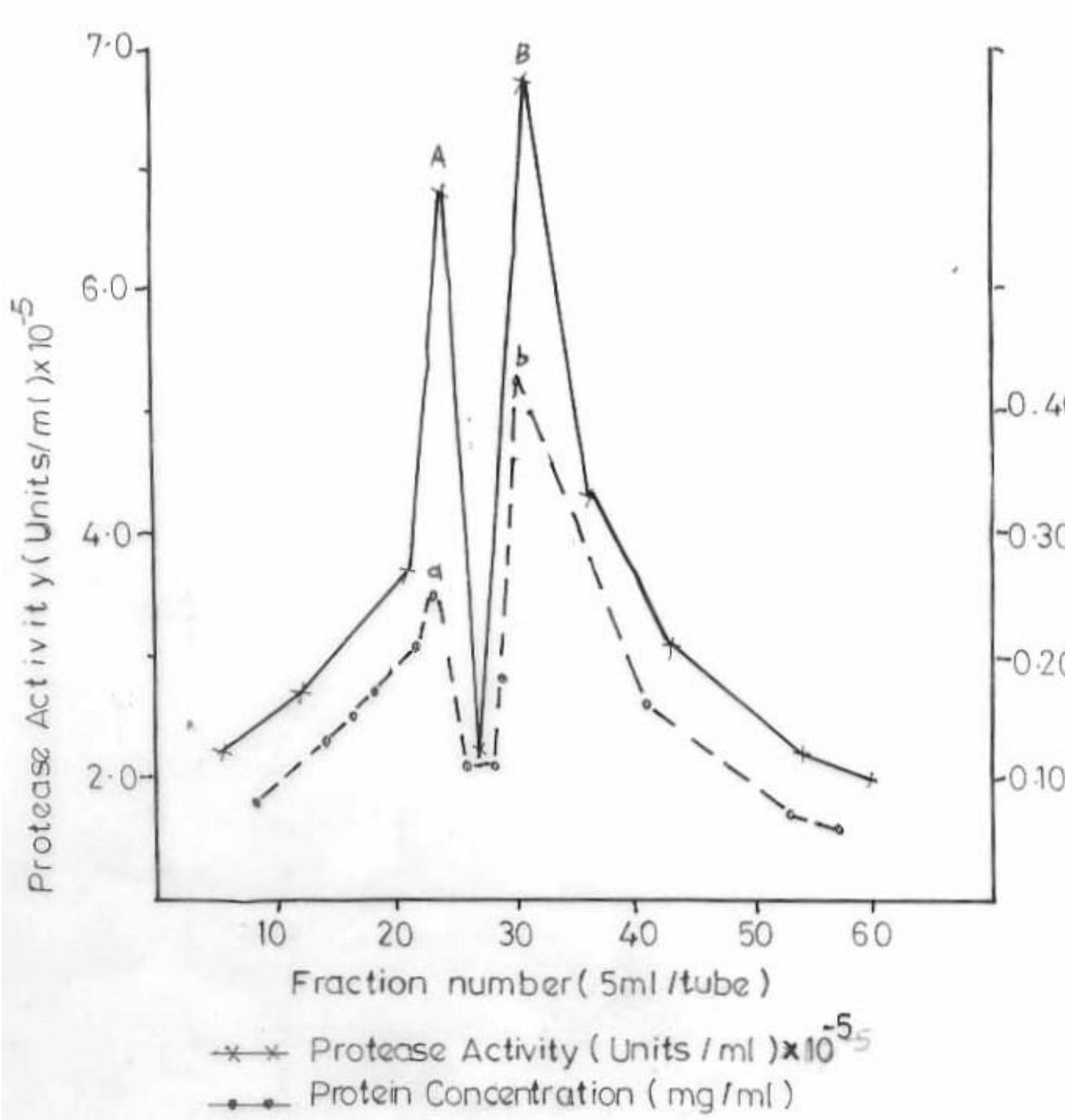

Figure 41 · Separation by ion exchange chromatography (G-10
of high molecular weight proteins and enzymic act
of the fractions of methanolic extract of Calotropis
towards casein ·

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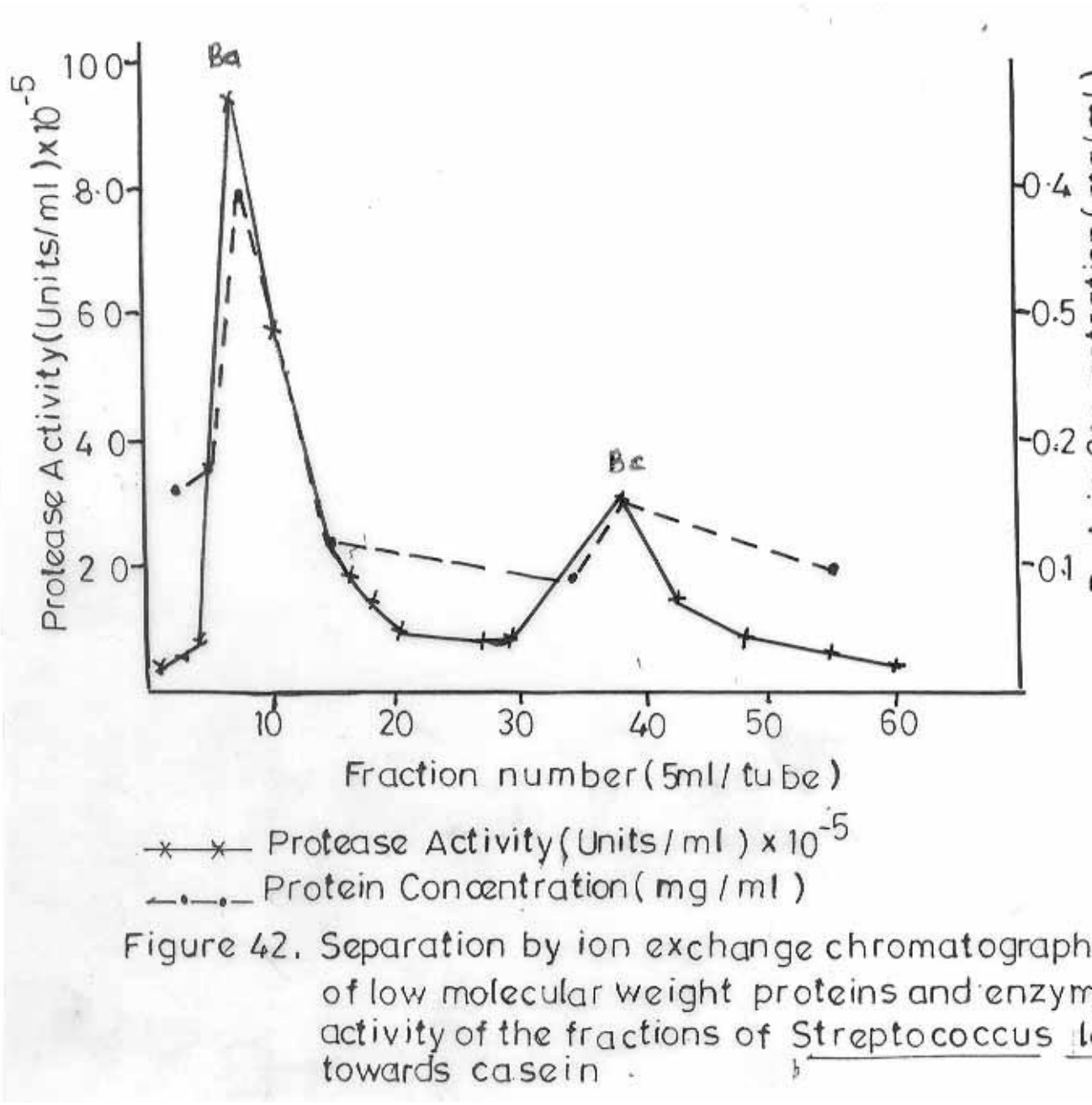

Figure 42. Separation by ion exchange chromatograph of low molecular weight proteins and enzym activity of the fractions of Streptococcus towards casein.

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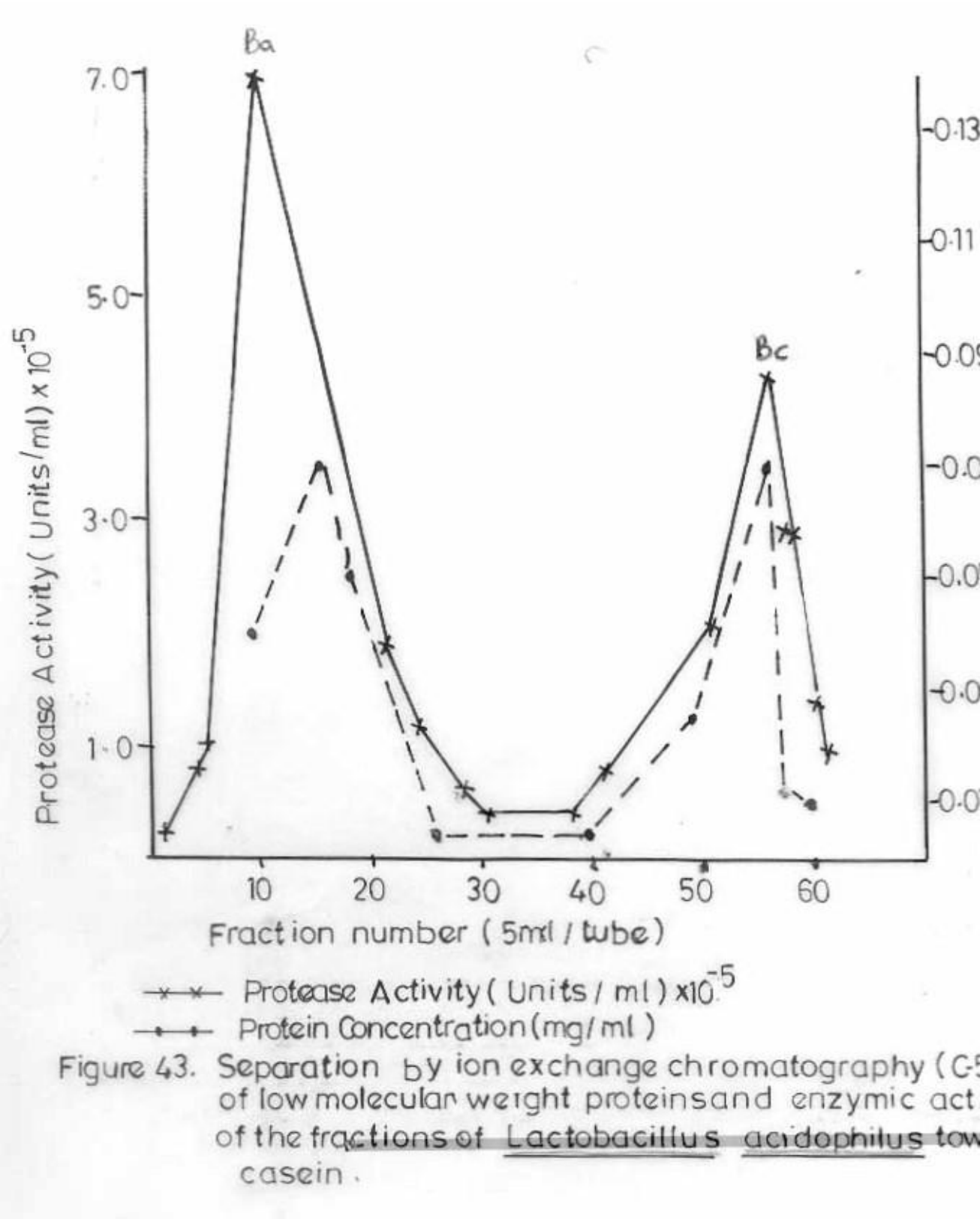

Figure 43. Separation by ion exchange chromatography (G-5
of low molecular weight proteinsand enzymic act
of the fractions of Lactobacillus acidophilus tow
casein.

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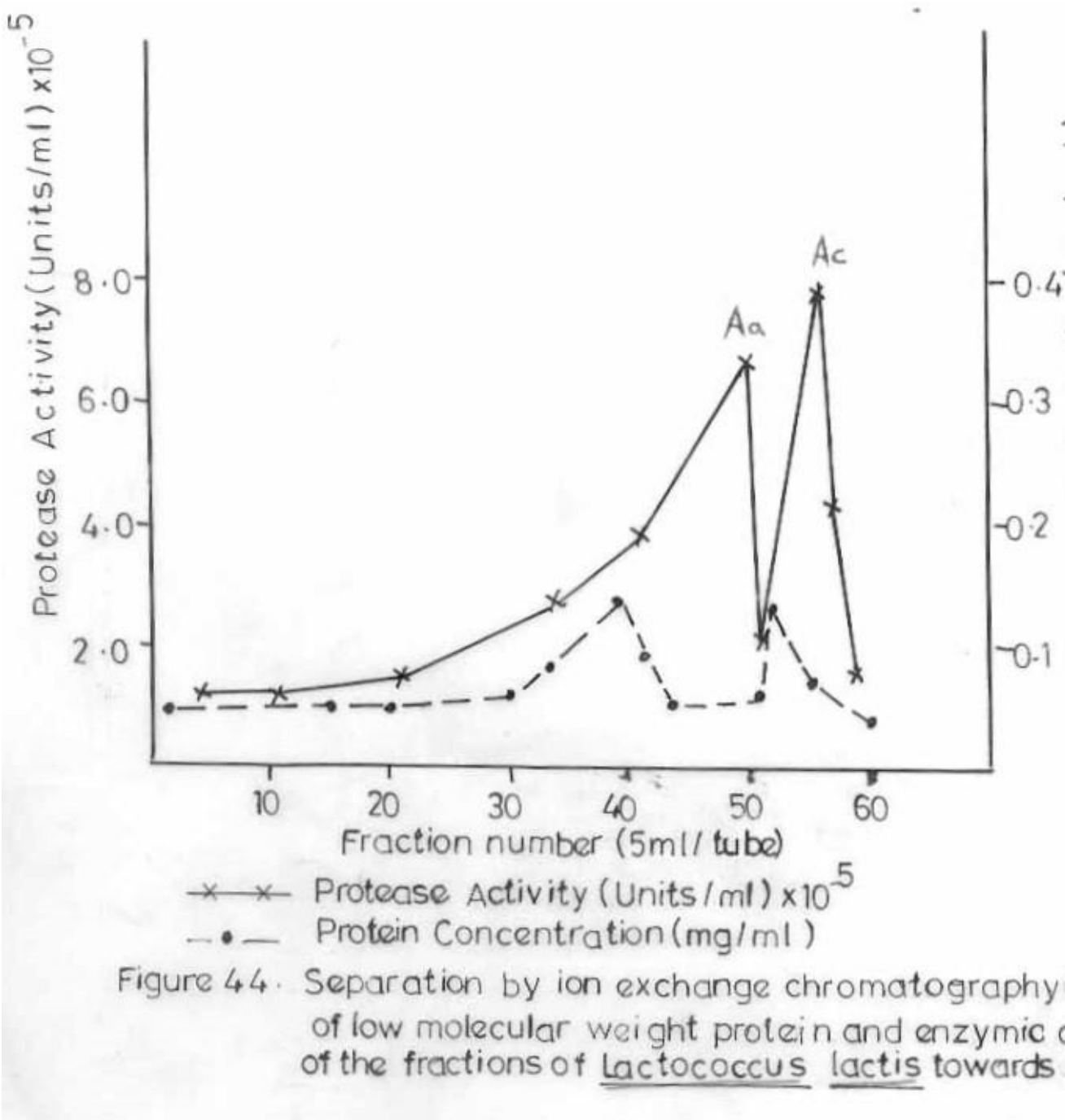

Figure 44. Separation by ion exchange chromatography
of low molecular weight protein and enzymic
of the fractions of Lactococcus lactis towards

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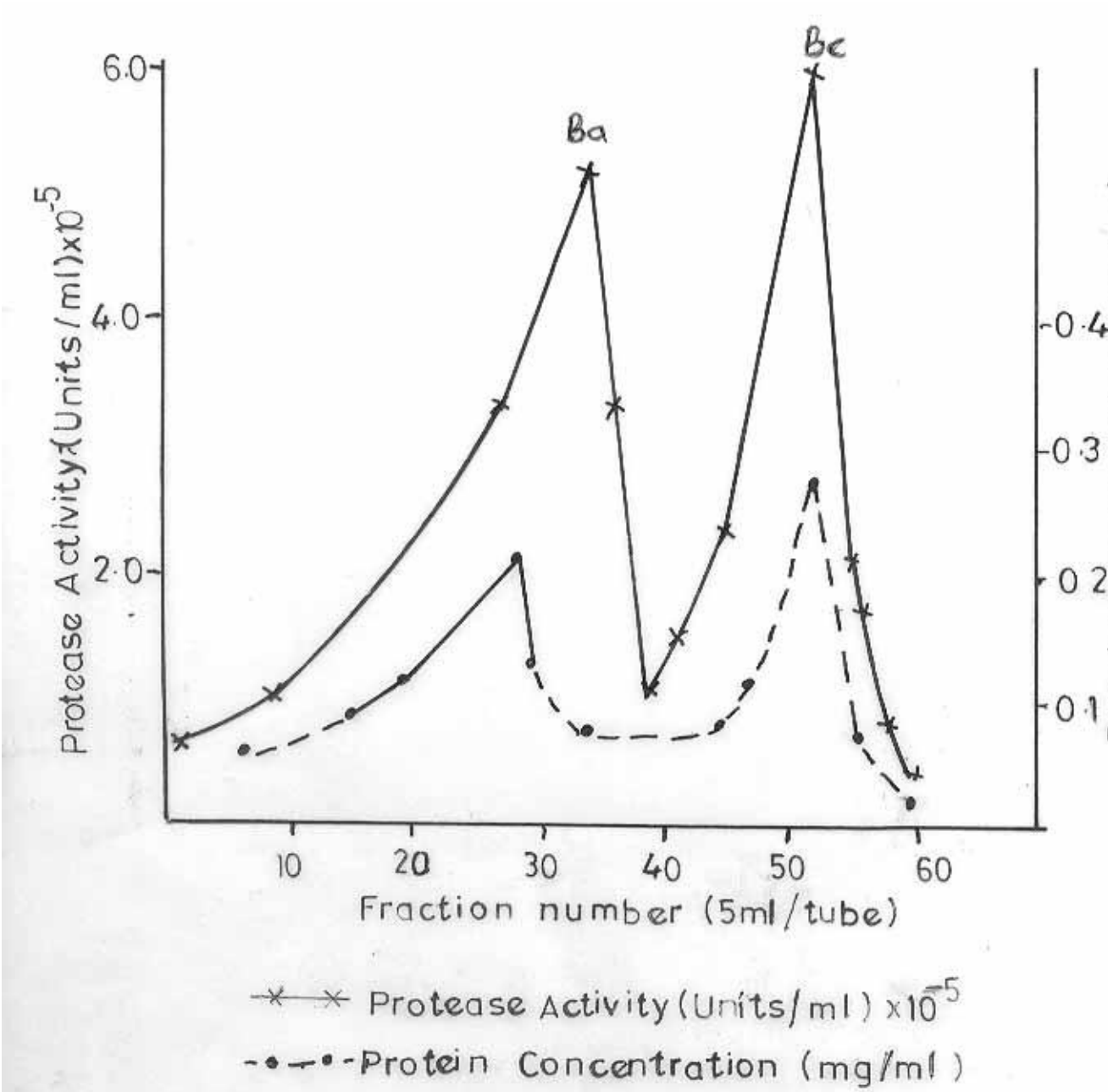

Figure 45. Separation by ion exchange chromatography of low molecular weight proteins and enzymi of the fractions of crude extract of Calotropis towards casein.

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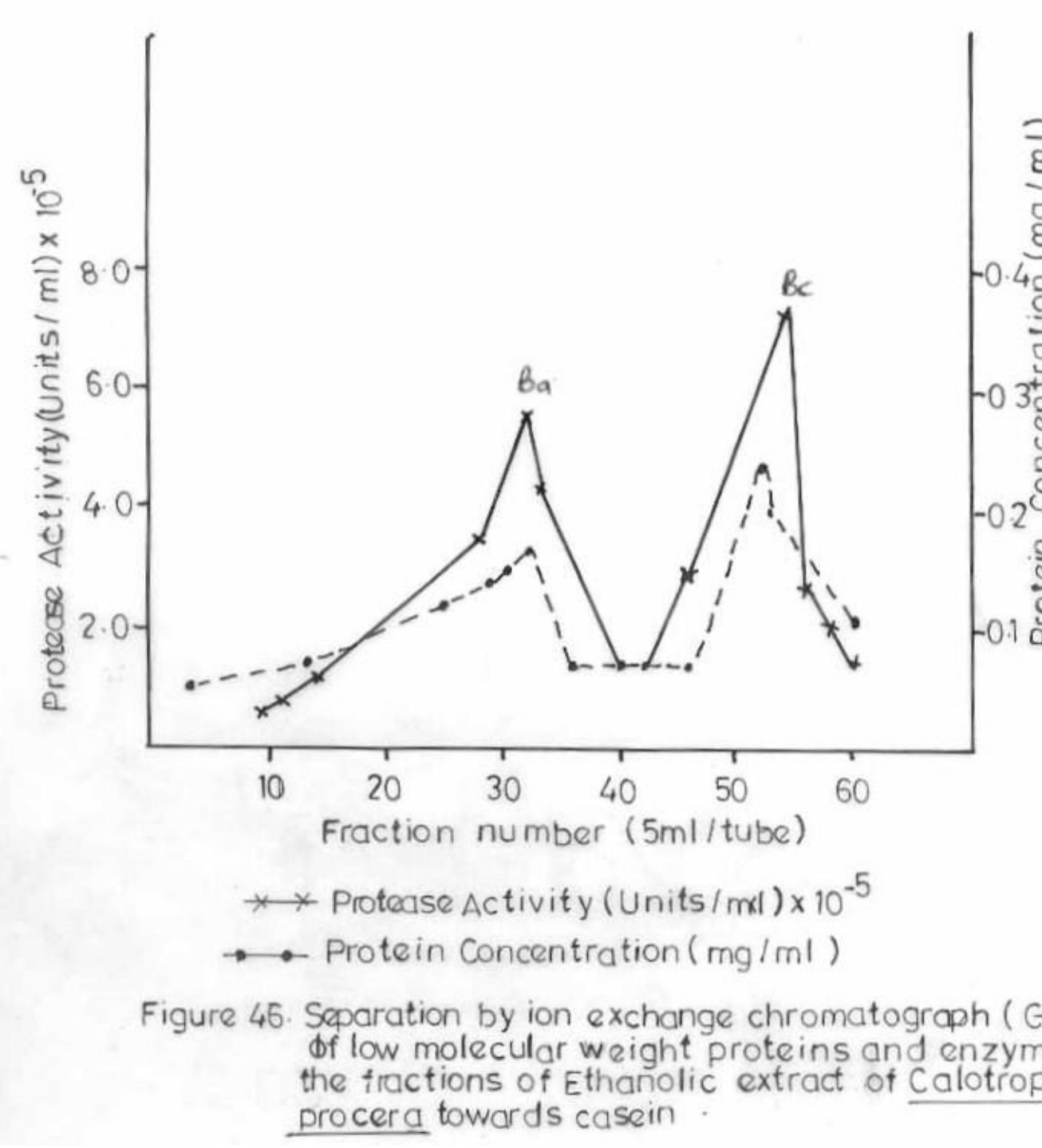

Figure 46. Separation by ion exchange chromatograph (G
of low molecular weight proteins and enzym
the fractions of Ethanolic extract of Calotrop
procera towards casein

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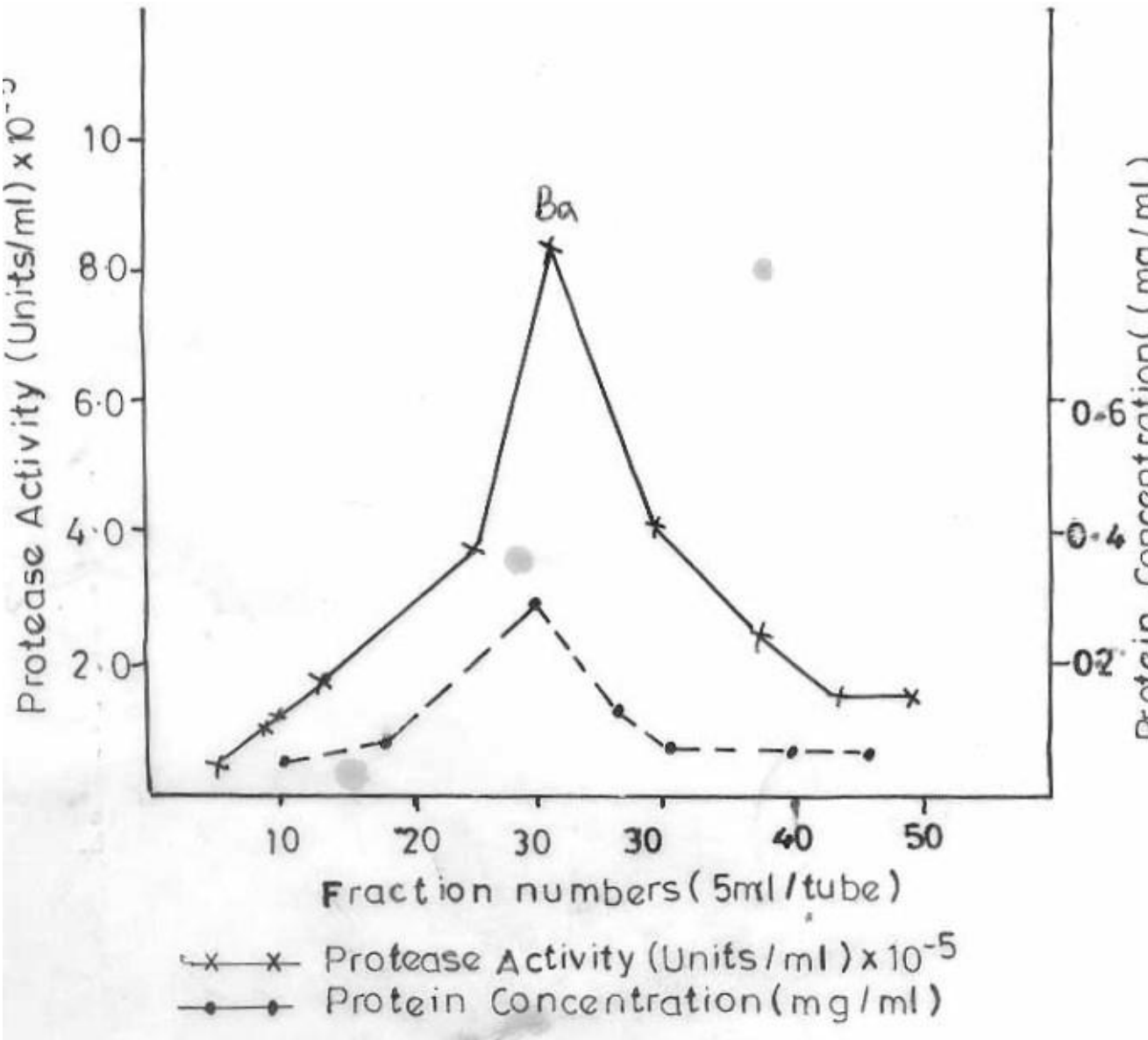

Figure 47. Separation by ion exchange chromatography C-50 of low molecular weight proteins and enzymic activity of the fractions of methanol extract of Calotropis procera towards case

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Table 9: Partial Purification of Protease from *Streptococcus lactis* isolated from "Wara"

Fraction	Volume (ml)	Unit Activity (unit)	Total Activity (unit)	Unit Protein (mg/ml)	Total Protein (mg)	Specific Activity (unit/mg protein)	Yield (%)	Purification fold
Crude extract	100	1.83	183	0.20	20.00	9.15	100	1
$(NH_4)_2 SO_4$ Precipitation (100%)	100	1.20	120	0.37	37.00	3.24	65.57	0.35
Sephadex G-100								
Peak A	2	10.62	21.24	0.02	0.40	53.10	11.61	5.80
Peak B	2	12.16	24.32	0.07	1.40	17.37	13.29	1.90
Sephadex C-50								
Peak Ba	2	9.46	18.92	0.25	5.00	3.78	10.34	0.41

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Table 10: Partial Purification of Protease from *Lactobacillus acidophilus* isolated from "Wara"

Fraction	Volume (ml)	Unit Activity (unit)	Total Activity (unit)	Unit Protein (mg/ml)	Total Protein (mg)	Specific Activity (unit/mg protein)	Yield (%)	Purification fold
Crude extract	100	1.00	100	0.18	18.00	5.56	100	1
$(NH_4)_2 SO_4$ Precipitation (100%)	100	1.40	14.00	0.07	7.70	1.82	14	0.33
Sephadex G-100								
Peak A	2	11.20	22.40	0.23	4.60	4.87	22.40	0.88
Peak B	2	9.85	19.70	0.09	1.80	10.94	19.70	1.97
Sephadex C-50								
Peak Ba	2	6.95	13.90	0.01	0.20	69.50	13.90	12.5

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Table 11: Partial Purification of Protease from *Lactococcus lactis* isolated from "Wara"

Fraction	Volume (ml)	Unit Activity (unit)	Total Activity (unit)	Unit Protein (mg/ml)	Total Protein (mg)	Specific Activity (unit/mg protein)	Yield (%)	Purification fold
Crude extract	100	0.91	91.00	0.25	25.00	3.64	100	1
$(NH_4)_2 SO_4$ Precipitation (100%)	100	0.66	66.00	1.03	103	0.64	72.53	0.18
Sephadex G-100								
Peak A	2	8.50	17.00	0.08	1.60	10.63	18.68	2.92
Peak B	2	7.72	15.44	0.10	2.00	7.72	16.97	2.12
Sephadex C-50								
Peak Ac	2	7.72	15.44	0.05	1.00	15.44	16.97	4.24

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Table 12: Partial Purification of Protease from the Crude Extract of *Calotropis procera*

Fraction	Volume (ml)	Unit Activity (unit)	Total Activity (unit)	Unit Protein (mg/ml)	Total Protein (mg)	Specific Activity (unit/mg protein)	Yield (%)	Purification fold
Crude extract	100	1.35	135	0.30	30.00	4.50	100	1
$(NH_4)_2 SO_4$ Precipitation (100%)	100	1.20	120	0.19	19.00	6.32	88.89	1.40
Sephadex G-100								
Peak A	2	6.18	12.36	0.07	1.40	8.83	9.16	1.96
Peak B	2	5.41	10.82	0.23	4.60	2.35	8.01	0.52
Sephadex C-50								
Peak Bc	2	6.00	12.00	0.27	5.40	2.22	8.89	0.49

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Table 13: Partial Purification of Protease from the Ethanolic Extract of *Calotropis procera*

Fraction	Volume (ml)	Unit Activity (unit)	Total Activity (unit)	Unit Protein (mg/ml)	Total Protein (mg)	Specific Activity (unit/mg protein)	Yield (%)	Purification fold
Crude extract	100	11.80	1180	7.40	740	1.60	100	1
$(NH_4)_2 SO_4$ * Precipitation (100%)	-	-	-	-	-	-	-	-
Sephadex G-100								
Peak A	2	3.67	7.34	0.07	1.40	5.25	0.62	3.28
Peak B	2	6.76	13.52	0.21	4.20	3.22	1.15	2.01
Sephadex C-50								
Peak Bc	2	7.53	15.06	0.18	3.60	4.18	12.76	2.61

* The ethanolic extract did not dissolve the ammonium sulphate crystals thus the extract was used directly on the chromatography column.

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Table 14: Partial Purification of Protease from the Methanolic Extract of *Calotropis procera*

Fraction	Volume (ml)	Unit Activity (unit)	Total Activity (unit)	Unit Protein (mg/ml)	Total Protein (mg)	Specific Activity (unit/mg protein)	Yield (%)	Purification fold
Crude extract	100	10.62	1062	2.12	212	5.01	100	1
$(NH_4)_2 SO_4$ * Precipitation (100%)	-	-	-	-	-	-	-	-
Sephadex G-100								
Peak A	2	5.80	11.60	0.05	1.00	11.60	1.09	2.32
Peak B	2	6.76	13.52	0.10	2.00	6.76	1.27	1.35
Sephadex C-50								
Peak Ba	2	8.30	16.60	0.07	1.40	11.86	1.56	2.37

* The methanolic extract did not dissolve the ammonium sulphate crystals thus the extract was used directly on the chromatography

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In this study, Seven Lactic Acid Bacteria species were isolated from West African soft cheese "wara". The species were identified as *Streptococcus lactis*, *Streptococcus pyogenes*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Streptococcus faecalis.* Lactic Acid Bacteria are commonly found in foods and feeds regularly consumed because of their beneficial status. Their role is to promote sugar fermentation and other modifications of raw materials present in such foods and feeds (Hans, 1993).

According to Herrero *et al.*, (1996), Lactic Acid Bacteria species are widely used empirically or deliberately in the manufacture of dairy products. Traditionally, they are part of the microbiota present on raw materials introduced by contact with contaminated tools and manufacturing equipment. The lactic acid produced acts as a selective trait that allows them to predominate (Buckenhuskes, 1993). The presence of a pyogenic streptococcus – *Streptococcus pyogenes* – may be due to the fact that the cow is a significant source of bacteria in cheese production in which case, contamination may have been from inside or outside the udder; equipment and environment involved in the cheese preparation; individuals involved in its packaging (Akam *et al.*, 1989).

Proteolysis by lactic acid bacteria is initiated by cell – wall associated proteinases, thus several exo- and endopeptidses have been purified from starter bacteria (Kok, 1990; Pritchard and Coolbear, 1993). Proteolysis is the removal of a signal peptide from a newly synthesized protein chain in the rough endoplasmic reticulum. The protein chain is broken down inside the cell to release a supply of amino acids needed during growth (Burgoyne and Duncan, 1998). For the development of an acceptable cheese flavor, a well-balanced breakdown of the curd protein (i.e., casein) into small peptides and amino acids is necessary, these products are then

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flavor compounds by themselves or act as precursors of flavor components during the actual cheese flavor formation (Exterkate, 1987).

Different nitrogen and carbon sources were used to optimize protease production. Total yield for *Streptococcus lactis* (4.25 units/ml) was best with the combination of peptone and casein as nitrogen sources in the presence of glucose as carbon source at 28°C and pH 5.0 after 72 hours of incubation with 0.5ml inoculum size, total protease yield for *Lactococcus lactis* (1.93 units/ml) was best with meat extract as nitrogen source in the presence of glucose as carbon source at 28°C and pH range of 5.5-6.0 after 48 hours with 2.0ml inoculum size while for *Lactobacillus acidophilus* total yield (3.50 units/ml) was best achieved with ammonium citrate as nitrogen source in the presence of starch as carbon source at 40°C and pH of 5.5 after 48 hours with 0.5ml inoculum size. In an experiment by Wellingta and Meire, (2004), maximum protease production was observed with starch (1.14 u/mg protein) and trisodium citrate (1.11u/mg protein) as carbon sources and that 1% glucose (w/v) repressed the synthesis of protease. It was also reported that maximum enzyme activity of 1.1u/mg protein was obtained when ammonium nitrate was used as nitrogen source while ammonium chloride and ammonium citrate brought about a good level (0.76,0.73 u/mg protein) of enzyme activity.

Further studies on the activity of the protease produced was done and it was observed that the optimum conditions were as follow; *Streptococcus lactis* protease had its optimum activity at 28°C with pH 5.0 in the presence of 4% substrate concentration and an enzyme concentration of 2.5ml. Low activity was rather noticed in the presence of low substrate concentration, protease activity was inhibited by nitrophenol while $NaNO_3$ and leucine stimulated high protease activity. According to Neviani *et al*. (1995), certain streptococcal species have complex requirements for nutritional factors and their growth in milk is influenced by the availability of simple and assimilable nitrogen sources in the form of

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individual amino acids. They also found out that peptidase activities of such strains especially those active against leucine and lysine was related to the individual amino acid content of casein and non-protein nitrogen fraction present in milk. *Lactobacillus acidophilus* protease had its optimum activity at 40°C with ph 5.5 in the presence of 2% substrate concentration and an enzyme concentration of 0.5ml.

However, low protease activity was noticed in the presence of high substrate concentration, as earlier reported by Eggiman and Bachmann (1980). $NaNO_3$, NaCl, tyrosine and all the inhibitors stimulated an increase in protease activity. Eggiman and Bachmann characterized a thermostable enzyme and reported that it had pH of 5.5 and optimum temperature was at 47.5^{o}C. Thermostable protease are advantageous in some applications because higher processing temperatures can be employed resulting in faster reaction rates, increase in the solubility of nongaseous reactants and products, and reduced incidence of microbial contamination by mesophilic organisms, proteases secreted from thermophilic bacteria are of particular interest and have become increasingly useful in a range of commercial application (Adams and Kelly, 1998).

Ohta *et al.* (1966) reported the presence of tyrosine in the breakdown of casein into its amino acid constituent. In an experiment they carried out on a thermostable protease from thermophilic bacteria, a two-thirds of tyrosine residue was noticed in the enzyme and according to chromatographic analysis, the tyrosine contents were determined to be 12.54 moles/100g of protein (i.e. 29.10 moles per mole of the enzyme spectrophotometrically). This implied that the thermostable protease exists as a compact structure with many tyrosine residues buried inside the molecule, hence high content of tyrosine may play a role in its stability. *Lactococcus lactis* optimum protease activity was observed in the presence of 5% substrate concentration and 2.0-2.5 ml enzyme concentration at 28°C with pH range 5.5-6.0.

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Little or no activity was observed at low substrate concentration, while activity was strongly inhibited by EDTA- a known inhibitor of metallic proteases (Bernhard and Marc, 1980), thus suggesting the presence of a metallic-protease.

However in the current work, NaCl, $NaNO_3$ and three amino acids (lysine, leucine and serine) stimulated high protease activity. In a study by Tan *et al.*, (1991) it was reported that protease activity of a purified lactococcal enzyme was inhibited by EDTA and that its amino acid composition showed a high concentration of serine residues with an optimum pH of 6.0-6.5 at a temperature of 30-38°C. In a similar report by Robyn and Pritchard (1994), a Lactococcal protease that was highly sensitive to metalloenzyme inhibitors was purified. With respect to amino acid requirements, Neviani *et al.*, (1995) reported the importance of leucine as being essential during initial stages of Lactococcal growth.

The crude extract of *C. procera* had its optimum protease activity in the presence of 1% substrate concentration at 35°C and pH 3.0 with 1.0ml enzyme concentration. Protease activity was inhibited by nitrophenol and EDTA, while $NaNO_3$ and lysine stimulated high activity, though high substrate concentration brought about a decrease in activity. The ethanolic extract had its optimum activity in the presence of 5% substrate concentration and 0.5ml enzyme concentration between 28-35°C with pH 5.0. Activity was inhibited by nitrophenol and EDTA while $NaNO_3$, NaCl and three amino acids (glutamic acid, guanine and panthetonic acid) stimulated high protease activity. The methanolic extract had its optimum activity in the presence of low (1-3%) substrate concentration and an enzyme concentration of 2.5ml at 28°C and pH 3.5. No significant inhibition was noticed with respect to the inhibitors, $NaNO_3$, NH_4Cl, $CuSO_4$ and panthetonic acid stimulated high protease activity while high substrate concentration brought about a decrease in activity.

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Streptococcus lactis substrate concentration had the affinity (km) of 0.42 with a maximum velocity (Vmax) of 2.0. *Lactobacillus acidophilus* with an affinity (km) of 0.77 and a maximum velocity (Vmax) of 2.50. *Lactococcus lactis* had an affinity (km) of 3.3 with a maximum velocity (Vmax) of 0.87. The crude extract of *Calotropis procera* having an affinity (km) of 5.0 with a maximum velocity (Vmax) of 1.54. The Ethanolic extract of *C. procera* having an affinity (km) of 2.44 with a maximum velocity (Vmax) of 8.77 while the methanolic extract of *C. procera* having an affinity (km) of 5.0 with a maximum velocity (Vmax) of 4.17. It can be seen that the protease enzymes were operating at a low velocity when compared with the required Vmax of 11.49 (Prescott *et al.*, 2005). According to Dutta (2000), nitrogen is an essential constituent of all proteins and nitrate of the soil is the main source of nitrogen supply to higher plants. Nitrate is reduced to ammonia in the root and leaf, cellularly, the ammonia is then incorporated into α- ketoglutamic acid and under the action of glutamic dehydrogenase which is widespread in plants, the first amino acid which is glutamic acid is formed. Other important amino acids formed by the transfer of an amino group of the glutamic acid to oxalacetic acid include leucine and tyrosine.

Vadde and Ramakrishna (2005) reported that proteolytic enzymes play a central role in the biochemical mechanism of plant germination to form 10-40% major source of dietary protein. In their study, activity of the protease purified was not affected by EDTA, as observed in the methanolic extract of *C. procera* in this study. Purification of their crude extract showed a specific activity of 0.42 units/mg.

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Conclusion

Oberg and Broadbent (1993) reported that the protease activity of lactic acid bacteria reaches its maximum during the log phase and that low concentrations of molecular nitrogen components such as amino acids and peptides in milk are insufficient for growth during early stages of fermentation. Thus the ability of lactic acid bacteria to grow in milk depends on the amount of free amino acids and peptide present in the medium and since the concentrations of these compounds are low within such medium, then the presence of a functional proteolytic system is of great importance. By the action of proteinases on casein, a major component of milk proteins, peptides of different sizes are released and then hydrolysed. The importance of these has been discussed in a number of reports (Law, 1979; Exterkate and Veer, 1985).

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