

*Isolation and Characterisation of Cassava Linamarase
using Centrifuge and Cross Flow Membrane*

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**A Dissertation Submitted to the Faculty of Engineering and the Built Environment,
University of the Witwatersrand, in fulfilment of the requirements for the Degree of
Master of Science in Engineering**

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

(Signature of candidate)

_____ Day of _____

ABSTRACT

Linamarase application exists in biotechnology such as potentiometric sensors for linamarin by coupling linamarase from cassava leaf with a cyanide ion-selective electrode and to measure glucose in biomedical applications. It is used in a batchwise process to detoxify fermenting cassava during 'garri' production. Linamarase along with its naturally occurring substrates, linamarin and lotaustralin, is found in a variety of edible plant tissues such as those of cassava from which garri is produced. However, the separation and purification of linamarase at reasonable large quantity for these applications from plants has been a challenge. In the study a miniflex Ultrafiltration (UF) Cross Flow obtained from Schleicher and Schuell (Germany) was used for linamarase isolation and purification from cassava tissues. Membranes with different pore sizes of 0.45, 0.2, 0.1 and 0.02 μm , made from polyethersulfon screens and silicone adhesives, with surface area of 2.4 mm^2 , were experimented. Fluxes were observed to decrease very sharply from 0.45 to 0.02 μm membrane pore sizes. No permeate was collected from 0.1 and 0.02 μm membranes due to concentration polarisation and clogging of these membranes. Permeate and retentate from 0.45 and 0.2 μm membrane contained linamarase, while the retentate of the 0.1 and 0.02 μm membranes contained linamarase and that no permeate was collected from 0.1 and 0.02 μm membranes due to the fouling and clogging of the small membrane pores. It was therefore concluded that linamarase was finally purified by the 0.2 μm membrane. A simple mathematical model derived from the Hagen-Poiseuille equation could not predict the linamarase flux data, perhaps due to the effect of concentration polarisation, which led to the proposition of the Langmuir adsorption isotherm. It was interesting to observe that the plot of $1/v$ versus $1/\Delta p$ from the use of the Langmuir equation gave a linear relationship from which the linamarase flux

was predicted. The standard error between the experiment and the model was 0.011, which is a good measure of the agreement between data. The Langmuir adsorption isotherm therefore predicts the fouling and concentration polarisation of the membrane during linamarase purification from cassava tissues. This proposition was supported by the solute deposits on the pores and surface of the membrane where van der Waal forces were created between the molecules, thus resulting in the fouling and chemical polarisation.

DEDICATION

This dissertation is exclusively dedicated to my late mother, Mrs Janet Obazu who instilled in me the desire to love life, even in turbulent times.

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LIST OF ABBREVIATIONS AND SYMBOLS

BCT	Bulk cassava tissue
BCE	Bulk cassava extract
Conc.	Concentration
CCE	Crude cassava extract
CN	Cyanide ions
CNG	Cyanogenic glucosides
HCV	High cyanide cultivar
HCN	Hydrogen cyanide
HNL	Hydrogen nitrilase
LCV	Lower cyanide cultivar
RPM	Revolution per minutes
SDS-PAGE	Sodium dodecyl sulfate-Polyacralymide gel

CHAPTER ONE

INTRODUCTION

1.1 Background and Motivation

Linamarase has been the subject of recent research due to its role in biological processes. Its application exists in biotechnology, such as potentiometric sensors for linamarin, by coupling linamarase from the cassava leaf with a cyanide ion-selective electrode, and to measure glucose in biomedical applications. Linamarase along with its naturally occurring substrates, linamarin and lotaustralin, is found in a variety of edible plant tissues, such as those of cassava (*Manihot esculenta* Crantz), flax (*linum ussitissimum*), white clover (*Trifolium repens*, L.), butter beans (*Phaseolus Lunatus* L.), peaches, apricots, and bitter almonds. The toxicity of these plants is associated with the linamarase-catalyzed hydrolysis of these two structurally-related cyanogenic glucosides, which yields, among other products, HCN, a potent inhibitor of cytochrome oxidase, and other respiratory enzymes (Itoh-Nashida *et al.*, 1987).

According to McMahon *et al.* (1995), all cassava tissues, with the exception of seeds, contain the cyanogenic glycosides linamarin (>90% total cyanogens) and lotaustralin (<10% total cyanogens). Leaves have the highest cyanogenic glycoside levels (5.0 linamarin/kg fresh weight), whereas roots have approximately 20-fold lower linamarin levels. In addition to tissue-specific differences, there are cultivar-dependent differences in the root cyanogens levels. Total root linamarin levels range between 100 and 500 mg linamarin (kg fresh weight) for low and high cyanogenic cultivars, respectively. No cassava cultivars lack cyanogenic glycosides.

However, from the work of Santana *et al.* (2002) it was observed that there were two different patterns of linamarase activity. In the low cyanide type, young leaves displayed very high enzyme activity during the early plant growing stage (3months), whereas in root peel the activity increased progressively to reach a peak in 11 month-old plants. Conversely, in the high-cyanide cultivar (HCV), root peel linamarase activity decreased during the growth cycle, whereas in expanded leaves linamarase activity increased in 11 month-old plants.

Linamarase has been studied previously in leaves at the subcellular levels. White *et al.* (1994) and Gruhnert *et al.* (1994) showed that in more than 50% of the cassava leaves, linamarase was present in the apoplast. The apoplastic location was confirmed by Mkpong *et al.* (1990) who showed that most of the immunogold labelling was present in the cell wall of cassava leaves. Pancoro and Hughes (1992) demonstrated the presence of mRNA in laticifer cells of petiole and leaves, and Elias *et al.* (1997) purified and characterized the laticifer isozyme. This is the first report in which the immunolocalization of linamarase in cassava roots is shown and the location of the enzyme is compared in low and high-cyanide cassava cultivars. The results of immunofluorescence presented (figure 1.1) clearly showed the immunolocalization of linamarase in laticifers of petioles and the roots of both cultivars. The results obtained support the hypothesis proposed first by Pancoro and Hughes (1992), that linamarase may be transported from shoots to roots throughout the branched laticifer network in the cassava plant.

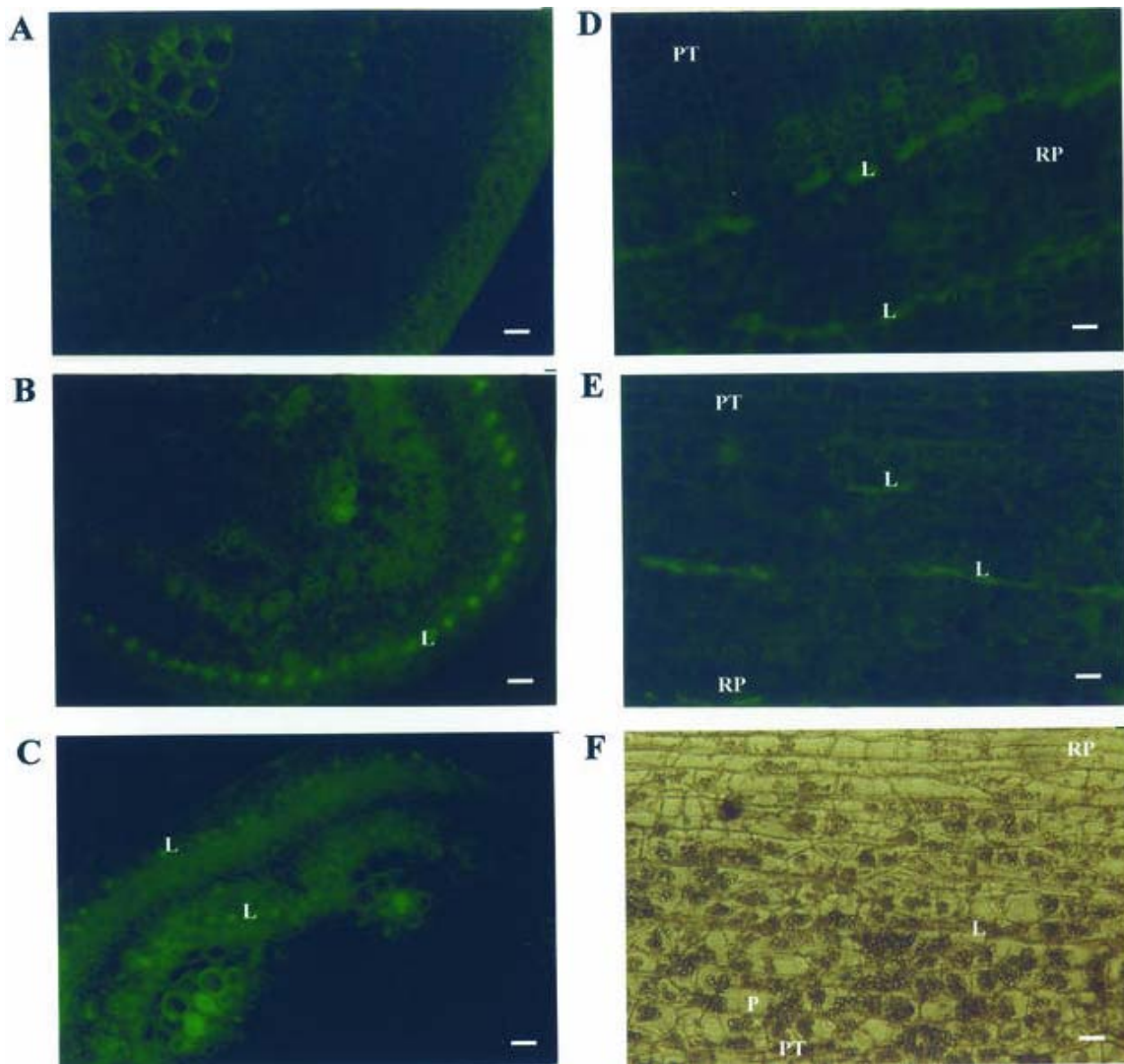


Figure 1.1 The results of immunofluorescence of linamarase in cassava tissues (Santana *et al.*, 2002)

Characteristically, figure 1.1 is explained thus: In situ immunofluorescence of linamarase in cassava tissues. A, Petiole section of mature leaf of low-cyanide content cassava (LCV), incubated with normal serum. As expected, a background tissue fluorescence is observed. B, Petiole section of young leaf of LCV after incubation with anti-linamarase serum. A specific fluorescence corresponding to linamarase was located mainly in laticifers. C, Petiole section of mature leaf of HCV after incubation with anti-linamarase serum. A specific fluorescence corresponding to linamarase was located mainly in laticifers, cambium, and cells surrounding them. D, Root section of LCV. Root peel (RP) and parenchyma tissue (PT) is observed.

Parenchyma (P) cells and laticifer (L) cells are indicated. E, Root section of HCV after incubation with anti-linamarase serum. A specific fluorescence corresponding to linamarase was located mainly in laticifers. F, Root section of LCV after incubation with anti-linamarase serum. A specific fluorescence corresponding to linamarase was located mainly in laticifers. Bars = 100 μm (Santana *et al.*, 2002).

In recent years, linamarase has attracted considerable industrial and academic interest in view of its potential use in the processing of edible cyanogenic plant tissues, particularly cassava, apricots and bitter almonds, and the quantification of bound cyanide in the form of cyanogenic glucosides (CNG) in plant tissues and body fluids. For example, linamarase has been used successfully in a batchwise process to detoxify fermenting cassava during ‘gari’ production. (Ikediobi, *et al.*, 1982, 1985). In order to minimize waste associated with batchwise use of the soluble enzyme, a few attempts have been made to immobilize linamarase with a view to develop a system that enables repeated use of the enzyme (Ikediobi, 1986; Narinsigh *et al.*, 1988; Yeoh *et al.*, 1994)

However, the functions of cyanogenic glycosides remain to be determined in many plants; but in some plants they have been indicated as herbivore deterrents and as transportable forms of reduced nitrogen (Belloti and Arias, 1993; Selmer, 1993; McMahon *et al.*, 1995). It is estimated that between 3,000 and 12,000 plant species produce sequester cyanogenic glycosides, including many important crop species (Kakes, 1990; Poulton, 1990), such as sorghum, almonds, lima beans (nondomesticated), and white clover. The most agronomically important of the cyanogenic crops is the tropical root crop cassava (*Manihot esculenta*, Crantz). Nonetheless, plant β -glucosidases are known to function in the chemical defence of young plant parts against pests by catalyzing the hydrolysis of toxic glucosides. It is also

known that all β -glucosidases isolated from grasses (e.g. maize, sorghum, oat, rice, ginger and indigo) are localized in the plastid (Thayer and Conn, 1981; Esen and Stetler, 1993; Inoue *et al.*, 1996). In contrast, all β -glucosidases isolated from dicots, including *Trifolium repens* (Kakes, 1985), black cherry (Poulton and Li, 1994) and *Brassica napus* (Thangstad *et al.*, 1991; Hoglund *et al.*, 1992), are localized in the cell wall or vacuole (protein bodies). In addition, monocot β -glucosidases are not glycosylated, while dicot β -glucosidases are glycoproteins. Based on the evidence, which shows significant differences between monocot and dicot β -glucosidases, the crucial question is how monocot and dicot β -glucosidases have evolved and acquired differences in terms of physiological substrates, post translational modification, targeting, and function.

More than 153 million tons of cassava are produced annually, and it is the major source of calories for many people living in the tropics, particularly sub-Saharan African (Cork, 1985). Following the trend of past and present research in which linamarase is used as an enzyme that initiates the activities of cyanogenic compounds such as linamarin, lotaustralin etc., linamarase obtained from cyanogenic plants such as cassava is often used in the raw or crude form (impure linamarase). Since its purification process has not been firmly established, this dissertation presents the use of membrane technology to purify linamarase from cassava tissues that have been blended in water and centrifuged. Membrane technology play an increasingly important role as unit operations for resource recovery, pollution prevention, and energy production, as well as environmental monitoring and quality control. They are also key component technologies of fuel cells and bioseparation applications (Wiesner *et al.*, 1989).

This separation technology involves separating components from a solution with mixed molecules sizes by flowing the solution under pressure over the surface of a membrane (Dziezak, 1990; Koseoglu *et al.*, 1991). When the feed stream enters the membrane system, a driving force is applied across the membrane such that solutes, whose sizes are greater than the pore size of the membrane, are retained and concentrated, forming a liquid that can either be called the concentrate, retentate or residue. Water and solutes smaller than the pores pass through the membrane and are called the permeate (Srikanth, 1999; Woerner, 2004; Mohr *et al.*, 1989; Koseoglu *et al.*, 1991).

1.2 Research Problem

The major drawback of linamarase, despite its potentials, is the difficulty in its isolation, purification and characterization. Hence, the various results from several cytotoxicity tests and research using crude linamarase are questionable. Chronic cyanide exposure associated with the consumption of cassava has been associated with a number of cyanide induced disorders, including goiter, dwarfism, and tropical ataxic neuropathy. This is particularly a problem in regions of the world where cassava is a major source of calories (Balagopalan *et al.*, 1988; Oke, 1980; Tewe, 1984; Umoh *et al.*, 1985).

Hence, the interest here is to develop strategies for the effective detoxification of cassava food products which would be compatible with the beneficial aspect of cyanogenesis. The demonstration of infiltration of cassava root tissues with excess linamarase lowers the linamarin content to levels considered safe for human consumption (Mkpong *et al.*, 1989). Despite several attempts to modify the cyanogenic potential of cassava, a comprehensive understanding of the physicochemical characterization of linamarase in cassava root and leaves is necessary.

1.3 Hypothesis

Membrane purified cassava linamarase compares well with commercial available ones in terms of physical and chemical characteristics.

1.4 Justification of the study

Several research works have been carried out on the determination of cyanogenic glycosides using linamarase in its raw or crude form (impure linamarase). The lack of purification process brings about the question of validity of results obtained from various cytotoxicity test. Moreso, several research studies have been reported on the determination, and not on the isolation and purification, of this group of compounds known as beta-cyanogenic glucosides in different naturally occurring plants using various quantitative and semi quantitative means because of the enormous difficulties associated with these processes (Seigler, 1975). It is imperative, therefore, to conduct this study on the isolation, purification and characterization of cassava linamarase using membrane technology. This work is thus expected to demonstrates the purification of the enzyme linamarase from cassava plants using a simple cross flow membrane.

1.5 Scope of the project

In an attempt to establish a possible process for the isolation, purification and characterization of linamarase from cassava, this project would be in three phases:

- (i) physicochemical and bio-chemical study of cassava linamarase,
- (ii) development of an optimum process for its isolation and purification

- (iii) characterization by several techniques of polyacrylamide gel Electrophoresis (SDS-PAGE).

The above mentioned phases would be preceded by the sourcing of cassava stems and the planting of stems in the university garden.

1.6 Research questions

In an effort to devise and establish a possible process for the isolation and purification of cassava linamarase, the following research questions have been considered:

- Would a cross flow membrane be able to isolate and purify linamarase from cassava?
- Is the linamarase isolated from the locally available cassava plant comparable to the commercial one?
- What are the limiting factors on the current method of isolation and how could they be overcome?

1.7 Purpose and Aims

The aim of this research is to explore and establish a simple and fast technique for the isolation and purification of linamarase from cassava tissue using membrane technology.

The research aims to achieve the following objectives:

- Isolation and purification of linamarase from locally available cassava species
- Characterization of linamarase on physical and chemical properties

- Designing a model on the performance and operating conditions of the ultrafiltration membrane.

1.8 Expected contribution to knowledge

It is expected that this research would contribute to knowledge by providing

- Information on the physico-chemical characteristics of linamarase from cassava tissue
- Information on the use of ultrafiltration for the isolation of linamarase
- Information on the bioactivities of linamarase from cassava tissue
- Information for designing reactors and equipment for linamarase purification/production from cassava tissue.

1.9 Dissertation outline

Chapter One

This chapter discusses the background and motivation of this study, research problem, hypothesis, justification of the study, scope of the project, research questions, purpose and aims and the expected contribution to knowledge.

Chapter Two

This chapter focuses on literature review and it will be discussed in two parts. The first part discusses linamarase as a cyanogenic compound from cassava and the function of linamarase. The second part discusses the overview of ultrafiltration in membrane technology.

Chapter Three

This chapter explains the experimental procedure of isolation and characterization of linamarase and the recording of results obtained from various experiments.

Chapter Four

This chapter discusses all experimental results.

Chapter Five

This chapter comprises the conclusion and recommendations of the study.

CHAPTER TWO

LITERATURE REVIEW

2.1 CASSAVA

Cassava (*Manihot esculenta* Crantz) is widely grown for its edible storage roots (Cooke, 1978). The plant tissues contain many macromolecules and minerals, including cyanide and tannin, as can be seen in the summary in Table 2.1 (Iyuke and Idibie, 2007; McMahon *et al.*, 1995). Thus cassava is an important source of calories in tropical countries and it ranks tenth among all crops in worldwide production (McMahon *et al.*, 1995). However, the presence of cyanogenic glycosides, mainly linamarin and its breakdown products in cassava and its processed products, has been a cause for concern from the view point of food safety. Almost all the tissue of cassava contains large amounts of cyanogenic glycosides, such as linamarin and lotoaustralin. Linamarin accounts for 95% of the total cyanoglycosides. Therefore, most of the research on cassava cyanoglycoside has been focused on the biochemistry and metabolism of such compounds (Padmaja, 1995).

Table 2.1 Proximate, mineral and anti-nutrients contents in unfermented cassava products (Iyuke and Idibie, 2007)

Proximate			Mineral			Anti-nutrients		
Sample	% dry matter		Sample	mg/100 g dry matter		Sample	dry matter	
	Flour	Gari		Flour	Gari		Flour	Gari
Protein	4.4	3.6	Zn	13.1	5.8	Cyanide (mg/kg)	21.3	14.6
Crude fat	2.6	3.6	Mg	43.4	27.7	Tannin (%)	0.2	0.2
Ash	2.1	1.9	Fe	26.0	2.3			
Crude fibre	3.8	3.7	Ca	61.6	16.7			
			Na	43.8	51.4			
			K	49.8	55.6			

Cyanogenesis is initiated in cassava when the plant tissue is damaged. Rupture of the vacuole releases linamarin, which is hydrolysed by the linamarase, a cell wall associated with β -glycoside (McMahon *et al.*, 1995). Linamarase (β -D-glucosylhydrolase. EC 3.2.1.21) along with its naturally occurring substrates, linamarin (2-hydroxyisobutyro-nitrile- β -D-glucopyranoside) and lotaustralin (2-hydroxy-2-methylbutyro-nitrile- β -D-glucopyranoside), is found in a variety of edible plant tissues such as those of cassava (*Manihot esculenta* Crantz) flax (*Linum usitatissimum*), white clover (*Trifolium repens*, L.), butter beans (*Phaseolus Lunatus* L.) peaches, apricots, and bitter almonds. The toxicity of these plants is associated with the linamarase-catalyzed hydrolysis of these two structurally-related cyanogenic glucosides, which yields, among other products, HCN, a potent inhibitor of cytochrome oxidase, and other respiratory enzymes (Itoh-Nashida *et al.*, 1987).

Lately, linamarase has attracted significant industrial and academic interest in view of its potential use in the processing of edible cyanogenic plant tissues, particularly cassava, apricots and bitter almonds, and in the quantification of bound cyanide in the form of cyanogenic glucosides (CNG) in plant tissues and body fluids. For example, linamarase has been used successfully in a batchwise process to detoxify fermenting cassava during 'garri' production (Ikediobi *et al.*, 1982, 1985). In order to minimize waste associated with batchwise use of the soluble enzyme, few attempts have been made to immobilize linamarase with a view to developing a system that enabled repeated use of the enzyme (Ikediobi *et al.*, 1986, Narinsigh *et al.*, 1988, Yeoh *et al.*, 1994).

There is great interest among cassava growers and breeders to screen their germplasm collection for varieties with low linamarin (cyanide) content. Varieties with high linamarin content are also sought after as they tend to show greater resistance to pests and diseases, since there is strong evidence that cyanogenesis (the ability of some plants to synthesize

cyanogenic glucosides, when enzymatically hydrolyzed, released cyanohydric acid, known as prussic acid) can provide the plant a protective device against predators such as herbivores (Ilza and Mario, 2000).



Figure 2.1: Cassava tubers (roots)

2.1.1 Cassava and its cyanogenic nature

Naturally, cassava is a cyanogenic plant and constitutes two cyanogenic glucosides, linamarin (2- β -D-glucopyranosyloxy-2-methylpropanenitrile) and lotaustralin [(2R)-2- β -D-glucopyranosyloxy-2-methylbutyronitrile] derived from valine and isoleucine, respectively (Peifan *et al.*, 2004; Koch *et al.*, 1992). The cyanide potential of a specific cassava line varies, depending on soil type and nutrient supply (de Bruijn, 1973; Bokanga *et al.*, 1994b). But in sorghum (*Sorghum bicolor*) and dhurrin (cyanogenic glycoside), synthesis is induced by nitrogen supply in plants above the age of 8 weeks (Busk and Moller, 2002). In a similar series of experiments, the cyanide potential was determined from 6-week-old cassava plants

following the administration of nitrogen. The cyanide potential in leaves and tubers from the same plant may also differ (de Bruijn, 1973; Riis *et al.*, 2003).



Figure 2.2a: Cassava planted at two months old (Wits agricultural garden)



Figure 2.2b: Cassava planted at nine months old (Wits agricultural garden)

Linamarin in itself is not toxic and is an unlikely source of cyanide exposure in humans (Mlingi *et al.*, 1992). It has been observed that part of the ingested linamarin in cassava products passes through the human body unchanged within 24 hours when excreted in human urine (Brimer and Roseling., 1993; Carlson *et al.*, 1995). Linamarin has been known since 1891 (Nestel, 1973), and it had been implicated as an aggravating factor in iodine deficiency

disorder as a result of malnutrition. Its role in neurological disease and some tropical variants of diabetes mellitus has been reported (Grindley *et al.*, 2002). Its role in leaves and roots has been reported (Liangcheng *et al.*, 1995). However, these compounds have been reported to be present in all the tissues of the plant (Conn, 1980), with the content of linamarin varying widely in different tissues of the cassava plant: leaves (Figure 2.2), stem and root peel contains higher level of the glucoside than the edible tuber (De Bruijn, 1973; Nambisan and Sandaresan, 1994). Varieties of different cassava also show wide variation in tuber linamarin content (range, 25-450 µg cyanide equivalent/g), which could be due to different rates of biosynthesis, degradation or transport (Elias *et al.*, 1997). Environmental factors, the cultivar and the growth condition have all been implicated as well (Cooke, 1978; Bradbury *et al.*, 1991).

Translocation of linamarin takes place from leaves to roots (Nambisan and Sandaresan, 1994). Santana *et al.* (2002) reported that cassava linamarin synthesis suggests that the cyanoglucosides accumulate in roots and are synthesized in shoots and then transported to roots where they are stored. In contrast, Elias *et al.* (1997) reported that there is no progressive accumulation of linamarin in tubers which is an indication that linamarin is not stored passively in the tissue, but is mobilized and utilized.

2.1.2 Cassava cyanogenocyt (hydrolysis of linamarin)

Besides cyanogenic glucosides, cassava tissues are also composed of an endogenous hydrolyzing enzyme known as linamarase (β -glucosidase). When cassava root tissue is damaged, mainly by mechanical action (e.g., during processing or preparation for consumption) or microbial action (e.g., during fermentation process or deterioration owing to

poor-harvest storage), the enzyme (linamarase) comes in contact with the linamarin, resulting in its hydrolysis and the subsequent release of hydrogen cyanide (cyanogenesis) (Yeoh *et al.*, 1998). The hydrolysis of linamarin is a two-step reaction involving the formation of an intermediate, acetonecyanohydrin, which breaks down spontaneously or by hydroxynitrilelyase action to form acetone and hydrogen cyanide (Yeoh *et al.*, 1998).

Cyanoglucoside, linamarin and amygdalin (laetrile) are chemically related but have different molecular weights. Linamarin has never been used in cancer metabolic therapy in the United States of America, but amygdalin and linamarin, derived from the seeds of almond, apricot and peach (Nahrstedt, 1987), and cassava extracts (Yeoh *et al.*, 1998), respectively, were used for cancer control, though not adequately proven scientifically linamarin has been in existence in the Chinese herbal history (Iyuke *et al.*, 2004).

A good correlation between leaf and tuber cyanide potential in cassava grown in the field has been reported (Cooke *et al.*, 1978), but the existence of an association between root and leaf cyanide potential has subsequently been refuted (Ayanru and Sharma, 1984/1985; Makame *et al.*, 1987; Mkong *et al.*, 1990; Bokanga *et al.*, 1994). In the tissue culture of cassava, cyanogenesis arises late in the regeneration process (Joseph *et al.*, 2001). No cyanide potential was detectable in the embryos, and the cyanide potential rises slowly during the development of plantlets. In contrast, seedlings derived from cassava seeds are cyanogenic from the onset of germination (Koch *et al.*, 1992). However, cyanogenic glucoside synthesis in shoots is initiated differently, whether the shoot arises from stakes or seeds. Cyanogenic glucoside synthesis is not initiated at a subsequent developmental stage in the selection of cassava lines with greatly reduced cyanide potential. It is pivotal to be certain that cyanogenic glucoside synthesis is not initiated at a subsequent developmental stage.

Hydrolysis of linamarin yields an unstable hydroxynitrile intermediate, acetone cyanohydrin, plus glucose. Acetone cyanohydrin spontaneously decomposes to acetone and HCN at pH > 5.0 or temperatures > 35 °C and can be broken down enzymatically by hydroxynitrile lyase (Cutler and Conn, 1981; Yemm and Poulton, 1986; Wajant and Mundry, 1993; Wajant *et al.*, 1994; White *et al.*, 1994; White and Sayre, 1995; Zheng and Poulton, 1995; Hasslacher *et al.*, 1996; Wajant and Pfizenmaier, 1996). The activity of linamarase on cyanogenic compounds such as linamarin is shown in figure 2.3.

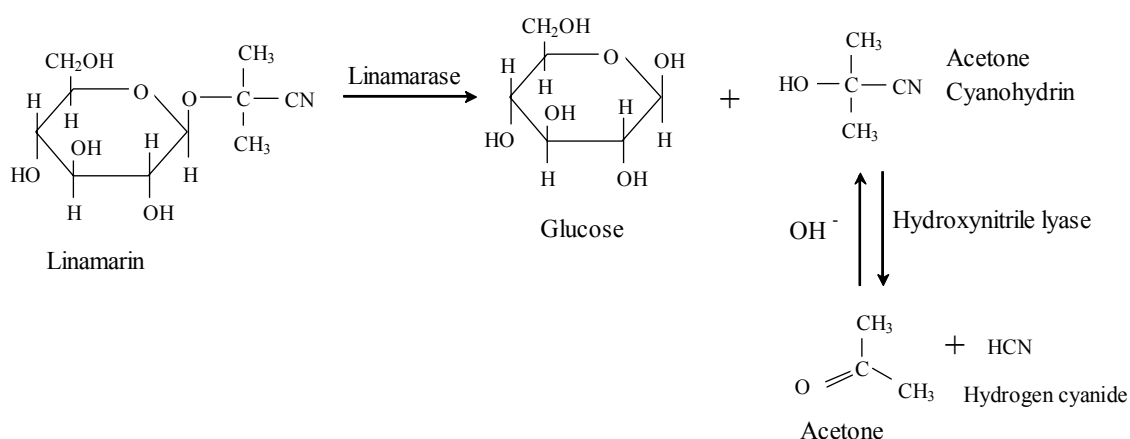


Figure 2.3: Linamarin hydrolysis

In a plant, linamarase (β -glucosidases) plays an important role in its defence against some pathogens and herbivores by releasing hydroxamic acids, coumarins, thiocyanates, terpenes, and cyanide from the corresponding glucosides (Fenwick *et al.*, 1983; Neimeyer, 1988; Hruska, 1988; Jones, 1988; Poulton, 1990; Oxtoby, 1991). Plant β -glucosidases also function in the hydrolysis of conjugated phytohormones (i.e., glucosides of gibberellins, auxins, abscisic acid, and cytokinins) (Nowachi *et al.*, 1980; Schliemann, 1984; Wiese and Grambow, 1986; Brzobohaty *et al.*, 1993).

Even though linamarin and linamarase are present in most of the plant tissue, no hydrogen cyanide (HCN) is detected under physiological conditions, suggesting that the enzymes and

their substrate exist in two different compartments. Previous studies on compartmentalization of cyanogenic glycosides and their degrading enzymes have shown that in leaves, 50% to 70% of the linamarase activity was apoplastic and located in cell walls (Mkpong *et al.*, 1990; Gruhnert *et al.*, 1994), Pancoro and Hughes (1992) demonstrated that leaf laticifer cells were enriched with linamarase using an antisense linamarase riboprobe. This isoform of linamarase was purified and characterized by Elias *et al.*, (1997), which confirm the location of the enzyme in laticifers and in cell walls of leaves. However, very little information is available on the location and expression of the linamarin-degrading enzymes in cassava roots.

Various health disorders are associated with the consumption of cassava, which contains residual cyanogens. These disorders includes include hyperthyroidism, tropical ataxic neuropathy, and konzo (Osuntokun, 1981; Cock, 1985; Tyllskar *et al.*, 1992; Rosling *et al.*, 1993). Cyanide poisoning from high-cyanogenic cassava is typically associated with insufficient consumption of amino acid like cysteine and methionine in the diet. Reduced sulfur-containing compounds are substrates for the detoxification of cyanide catalyzed by the enzymes rhodanese and or β -cyanoalanine synthase (Castric *et al.*, 1972; Kakes, 1990; Nambisan, 1993). Until recently, it had been assumed that all of the residual cyanogens present in cassava foods was in the form of linamarin. This assumption was based on the observation that acetone cyanohydrin is unstable and that the cyanide generated from acetone cyanohydrin is readily volatilized during food processing. However, it was demonstrated that the major cyanogens present in some poorly processed cassava roots was acetone cyanohydrin, not linamarin (Tyllskar *et al.*, 1992). These results suggested that the spontaneous (high pH and/or temperature) and enzymatic breakdown of acetone cyanohydrin was reduced or inhibited in roots. In part, the high, residual acetone cyanohydrin levels could be attributed to the low-pH conditions established during the soaking (fermentation) of roots

for food preparation. This hypothesis, however, does not address the contribution of hydrogen nitrilase (HNL) activity to root acetone cyanohydrin turnover and root cyanogenesis.

2.1.3 Physicochemical characteristics of linamarin

Linamarin [2-(-D-glucopyranosyloxy)-2-methylpropanenitrile] is also called phaseolunatin. It has an empirical formula of $C_{10}H_{17}NO_6$, with a molecular weight of 247.24 g/mol. It has an elemental composition of C 48.58%, H 6.93%, N 5.6% and O 38.83%. It is soluble in water and appears as a white solid. Linamarin can be found in any of these families: Compositae, Leguminosae, Euphorbiaceae, Linaceae and Papaveraceae (Seigler, 1975) with all of them possessing acetonecyanohydrin-glucoside.

2.1.4 Linamarin analysis

Determination of cassava cyanogenic potential (linamarin) has been on the trend, either with the use of an enzyme (linamarase) or sulfuric acid. Over the past 25 years, many appropriate analytical methods of determining the cyanogenic potential of cassava have been well published (Yeoh *et al.*, 1998), some of which are known to be quantitative methods (Bradbury *et al.*, 1991; Yeoh and Truong, 1993) while others are semi-quantitative (Indira *et al.*, 1969; Ikediobi *et al.*, 1980). The principle behind them has, however, been reported to be the same (Yeoh *et al.*, 1998) i.e. linamarin is hydrolyzed by the enzyme (Linamarase). This is followed by detection of the hydrogen cyanide or glucose released. The semi-quantitative procedure involves direct damaging of the cassava root tissue by the addition of some organic solvents causing the endogenous enzyme to hydrolyze the linamarin. This is followed by alkaline picrate paper test and consequently detecting the liberation of hydrogen cyanide in the course of the reaction (Egan *et al.*, 1998; Yeoh *et al.*, 1998) However, Yeoh *et al.*, (1998)

submitted that the reliability of such methods had often been questioned. The quantitative method of analysis involves the extraction of linamarin from cassava roots and cassava processed products, and then the hydrolysis by the addition of exogenous linamarase (Yeoh *et al.*, 1998). This is followed by the determination of hydrogen cyanide (linamarin equivalent) in several routes such as amperometry (Tetsu *et al.*, 1996), potentiometry (Yeoh and Truong, 1993), spectrophotometry (Cooke 1978; Bradbury *et al.*, 1991) and biosensory (Yeoh *et al.*, 1998). However, quantitative procedures can't perform numerous analysis for they are not designed to handle large numbers of samples. This is because the analysis requires the use of cassava root extracts, and their preparation is a rate-limiting step (Yeoh *et al.*, 1998). Research findings and recommendations have been made on the newly modified picrate assay, that the newly modified picrate assay should be used for the rapid screening of cassava cyanogens in all cases, except when a significantly high proportion of low-cyanogen clones ($0-50 \text{ mgkg}^{-1}$) are used (Gerard *et al.*, 1994).

2.1.5 Anti cancer potential of nitriloside linamarin

An exciting, important feature of the nitriloside has been unveiled exclusively with regard to malignant lesion that are known to be almost completely deficient of the detoxification enzyme, 'rhodenase,' although rich in the hydrolyzing enzyme ' β - glucosidase' or ' β - glucuronidase' (Roger, 1996a; Roger, 1996b). "Nitriloside is hydrolysed selectively at malignant lesion by β -glucosidase in the rhodenase deficient lesion. By this way the CN ion is brought to the malignant cell in a highly concentrated and selective manner" (Roger, 1996a). Figure 2.4 represents the detoxification reaction of cyanide by the rhodenase enzyme. There are a number of normal tissues in the body that carry both beta-glucosidase and beta-glucuronidase but also carry equal concentration of rhodanase, which completely protects

such normal somatic tissues from the action of any cyanide ion that the beta-glucosidase or beta-glucuronidase component of the tissue causes to be released from the hydrolysed nitriloside (Roger, 1996b). Figure 2.4 represents the detoxification reaction of cyanide by the rhodenase enzyme.

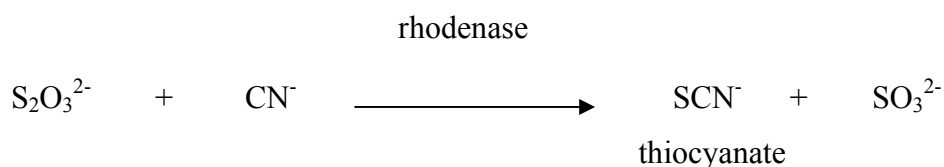


Figure 2.4: Detoxification reaction of hydrogen cyanide by rhodenase

Dr. Krebs Jr compares linamarin to sodium chloride saying if it is literally true to say that linamarin contains cyanide, a deadly poison? Then it is also true to say that table salt, sodium chloride, contains the deadly poison, chlorine. Under normal conditions, the chlorine in salt and the cyanide in linamarin are tightly bound, and therefore in no danger of suddenly leaking out. However, from the reference and report of Dr Krebs Jr, one could ask the question: how can a compound that is totally non-toxic be relevant to a disease as serious as cancer, a disease perhaps as fatal as deadly anemia once was? Would we not expect that very powerful cytotoxic compounds would be required to destroy cancer cells? Would these not be compounds like the nitrogen mustard, the antimetabolites, the cyclophosphoramides, methotrexate, 5-flourouracil, 6-chloropurine, 6-mercaptopurin, azaserine, triethylenphosphamide, the nitrosoguanidines, and countless other compounds so toxic that some kill almost 25 percent of the patients treated directly or indirectly through toxicity alone? For an agent to be effective, it must be non-toxic to normal somatic cells and yet present powerful cytotoxins to neoplastic cells (Krebs Jr, 1970). Linamarin as a nitriloside containing vitamin B₁₇ is expected on hydrolysis to release a specific and powerful cytotoxin, the HCN. The neoplastic (cancer) cell that is almost completely deficient of the detoxification

enzyme (rhodenase) but extremely rich in the hydrolyzing enzyme will be exposed to the toxic effect of the cyanide released, thereby killing the cancer cell. If some cyanide “spill out” from the cancer cells, adjacent normal cells will then be able to detoxify it through their highly rich rhodenase enzymes (Figure 2.4) that are widely distributed in all the body tissues (Krebs Jr, 1974; Oke, 1969). If detoxification is equal to absorption, no death or damage occurs, no matter the quantity of cyanide absorbed (Oke, 1969).

The antineoplastic (cancer) potential of cassava linamarin can therefore never be undermined. This is in accordance with Krebs Jr., (1974). However, according to fountain of life (<http://www.thefountainoflife.ws/cancer/nocancer.htm>), “the Indians of North America are people who are remarkably free from cancer. The American Association (AMA) went as far as conducting a special study in an attempt to ascertain why there was little to no cancer amongst the Hopi and Navajo Indians. The February 5, 1949 issue of the journal of the American Association affirmed that they found 36 cases of malignant cancer from a population of 30,000. In the same population of white persons there would have been about 1800. Dr Krebs research later found that the typical diet for the Navajo and the Hopi Indians consisted of nitriloxide-rich foods such as cassava. He premeditated that some of the tribes would consume the equivalent of 8000 mg of vitamin B₁₇ per day from their diet”. Hence, what can prevent has the potential to cure. The chemical and structural taxonomy of linamarin 2-(-D-glucopyranosyloxy)-2-methylpropanitrile is quite similar to those of amygdalin and methyl- α -glucoside, due to the common location of glucose moiety as presented in Figure 2.4 (Iyuke *et al.*, 2004; Lei *et al.*, 1999; Zubay, 1993). One other main resemblance between linamarin and amygdalin, both being cyanoglucosides, is that they do not contain free cyanide. Their cyanide ions (CN⁻) only turn out to be freed when they undergo hydrolysis. In the case of linamarin, the hydrolysis occurs when it makes contact

with linamarase to generate glucose, acetonecyanohydrin, which soon after decomposes to hydrogen cyanide and acetone as shown earlier in Figure 2.3. On the other hand, methyl- α -glucoside is being used to study the transport of glucose as a common carbon and energy source for many cells (Zubay, 1993). Taking the structural similarities of methyl- α -glucoside with linamarin, it is suggested that linamarin may transport glucose into cells as well (Iyuke *et al*, 2004). Methyl- α -glucoside is a non-metabolisable analogue. But the study by Hagihira *et al.* (1963), concerning glucose transport system of *Escherichia coli* by following intracellular accumulation of ^{14}C -labelled- α -methyl glucoside, observed that α -methyl glucoside was partially phosphorylated. It was also highlighted that the glucoside was not integrated into the cellular constituents or metabolized for energy. These observations could then be implied to have resulted from the usual favoured bond split at α -positions, leading to the glucose phosphorylation. Correspondingly, linamarin molecules would be glucose transporters and the resultant bond cleavage is at the α -position, to generate glucose phosphorylation, and at the appropriate pH conditions, the nitrile component would dissociate to the usual hydrogen cyanide and ketone. The inference of this is that when linamarin is transported into the cell, the cell will try to phosphorylate the glucose moiety by splitting the α -bond, and the cyanide ion will be deposited within the cytoplasm, in turn imposing a toxic effect on the cell, which will lead to cell apoptosis (Iyuke *et al.*, 2004). Therefore, a neoplastic (cancer) cell which needs more than normal cells to support its unusual and rapid growth and proliferation is expected to be extremely vulnerable to linamarin intracellular transport into the cancer cells, simply described as a 'suicidal case'. In essence, linamarin should be credited as a strong weapon that could arrest and destroy cancer.

However, from the view point of food safety, the presence of cyanogenic glucosides, mainly linamarin, and their breakdown products in cassava and its processed products, has been a

cause for concern. Hence, many methods for measuring total cyanide content are available (Cooke 1978; Bradbury *et al.*, 1991; Bradbury and Egan 1992; Yeoh and Truong 1993; Yeoh 1993; Bradbury *et al.*, 1994; Yeoh and Tan 1994a, 1994b; Brimer 1994; Tatsuma *et al.*, 1996; Yeoh *et al.*, 1996). Several of these methods are based on the enzymatic hydrolysis of linamarin, followed by spectrophotometric, potentiometric or amperometric measurement of the cyanide released. Unfortunately, enzyme-based methods cannot be used in many laboratories in developing countries, either due to lack of the equipment needed for enzyme preparation using existing procedures (Wood, 1966; Cooke *et al.*, 1978; Cooke, 1979; Eksittikul and Chulavanatol, 1988; Yeoh, 1989; Mpkong *et al.*, 1990) or because the cost of commercial linamarase is prohibitive. The one solution to this problem was to develop an acid hydrolysis method (Bradbury *et al.*, 1991) which removed the need for enzyme. Another approach would be to develop a simple and cheap procedure for linamarase isolation that could be used by laboratories in developing countries, bearing in mind that they may lack instruments for protein isolation.

Quantitative hydrolysis (or autolysis) of these cyanogenic glycosides is difficult and the existing methods of assay are unreliable and lack sensitivity. More importantly, the crude form usage of linamarase in the past brings one to the question of the validity and reliability of the results obtained. Hence, the use of centrifuge and membrane technologies to isolate and purify linamarase from cassava tissues would be of importance for health services.

2.2 The Principle of centrifugation

A centrifuge is an instrument designed to produce a centrifugal force far greater than the earth's gravity, by spinning the sample about a central axis. Particles of different size, shape

or density will thereby sediment at different rates, depending on the speed of rotation and their distance from the central axis.

If a heterogeneous or multiphase mixture needs to be separated, then separation can be done physically by exploiting the differences in density between the phases. Separation of the different phases of a heterogeneous mixture should be carried out before homogeneous separation, taking advantage of what already exists. Phase separation tends to be easier and should be done first. The phase separations likely to be carried out are:

- Gas – liquid (or vapour – liquid)
- Gas – solid (or vapour – solid)
- Liquid – liquid (immiscible)
- Liquid – solid
- Solid – solid.

The simplest type of centrifugal device is the cyclone separator for the separation of solid particles or liquid droplets from a gas or vapour. This consists of a vertical cylinder with a conical bottom. The mixture enters through a tangential inlet near the top, and the rotating motion created develops a centrifugal force that throws the dense particles radially toward the wall. The entering fluid flows downward in a spiral adjacent to the wall. When the fluid reaches the bottom of the cone, it spirals upward in a smaller spiral at the center of the cylinder. The downward and upward spirals are in the same direction. The particles of dense material are thrown towards the wall and fall downward, leaving at the bottom of the cone (Robin, 2005). The Beckman J2-21 high speed centrifuge is used in this study. Standard features include imbalance detection, auto temperature compensation, selectable breaking modes, auto over speed compensation, and built in diagnostics.

2.3 Membrane technology

A membrane is a thin barrier through which fluids and solutes are selectively transported when a driving force is applied across the barrier. Membrane technology is a separation and purification technology that applies a positive barrier or film in the separation of unwanted particles, micro-organisms and substances from water and effluents.

Membrane separation technology involves separating components from a solution with mixed molecules sizes by passing the solution under pressure over the surface of a membrane (Dziezak, 1990; Koseoglu *et al.*, 1991). Membrane technologies play an increasingly important role as unit operations for resource recovery, pollution prevention, and energy production, as well as environmental monitoring and quality control. They are also key component technologies of fuel cells and bio-separation applications (Wiesner and Shankararaman, 1999). The technology enables industrial users to concurrently focus, fractionate and purify their products (Dziezak, 1990). The technology can be operated at ambient temperature and does not require phase change (Cheryan, 1986). Thus for heat sensitive products, this becomes certainly very useful. Presently, the sensitivity of membrane technology research is the formulation of the membrane themselves. The scope of applications is being enlarged, and costs are being reduced through the development of membranes that have reduced thickness and greater compatibility with oxidants such as chlorine (Wiesner and Shankararaman, 1999).

Membrane processes separate molecules on the basis of size and molecular weight (Dziezak, 1990). When the feed stream enters the membrane system, a driving force is applied across the membrane such that solutes, whose sizes are larger than the pore size of the membrane,

are retained and concentrated, forming a liquid that can either be called the concentrate, retentate or residue. Water and solutes smaller than the pores pass through the membrane and are called the permeate (Srikanth, 1999; Woerner, 2004; Mohr *et al.*, 1989a; Koseoglu *et al.*, 1991b). A simple membrane concept is shown in Figure 2.5.

2.3.1 Membrane filtration in comparison with conventional filtration

According to Woerner (2004), there are two basic differences between membrane filtration and conventional filtration. Firstly, the membranes are asymmetric with the small side of the pore facing the feed. This feature minimizes the pressure drop across the membrane, and eliminates any tendency to plug the membrane. Membrane systems operate with a strong cross flow over the surface of the membrane which limits the build up of a filter cake or concentration polarization layer in membrane to a few microns.

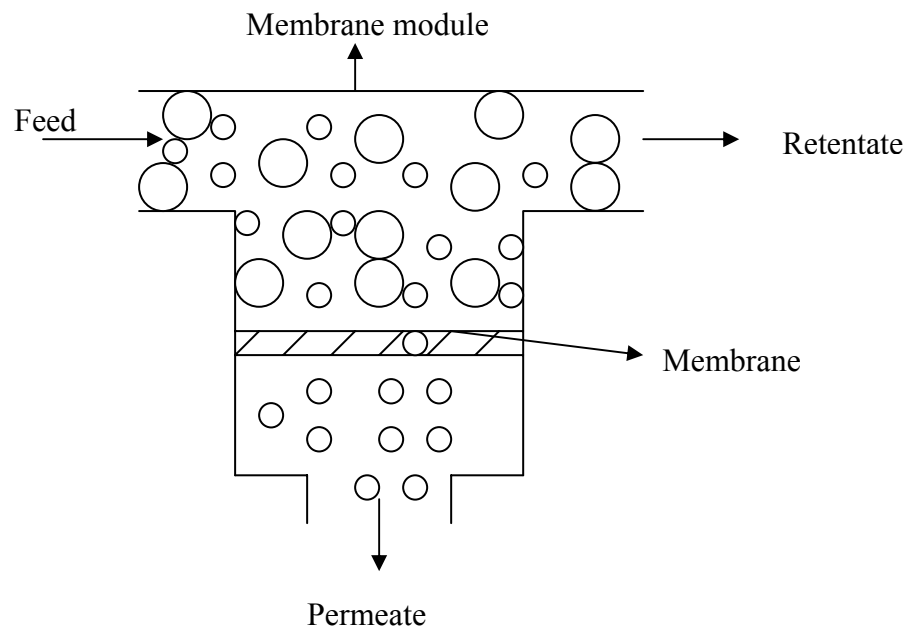


Figure 2.5: Simple Membrane Concept (Mohr *et al.*, 1989a)

The four general pressure membrane processes are microfiltration, ultrafiltration, reverse osmosis and nanofiltration (Woerner, 2004; Srikanth, 1999; Koseoglu *et al.*, 1991b). These processes are all well established. The difference in the pore diameter of the membranes used in each one of these processes produces remarkable differences in the way the membranes are used (Baker, 2004).

2.3.2 Membrane classification

The structure of the membrane can be distinguished generally as either micro porous or asymmetric (Baker, 2004; Paulson *et al.*, 1984; Srikanth, 1999). A microporous membrane, also called the isotropic membrane, behaves almost like a fibre filter and separates by sieving mechanism determined by the pore diameter and particle size distribution (Srikanth, 1999; Baker, 2004). Materials such as ceramics, graphite, metal oxides, and polymers, etc. are used in making such membranes.

Asymmetric membranes also classified as anitropic membranes are the most important type comprising of a thin (0.1-1.0 micron) skin layer on a highly porous (100-200 microns) thick substructure. These membranes merge the high selectivity of a dense membrane with the high permeation rate of a very thin membrane. The resistance to mass transfer is steady to a large extent by the thin top layer (Majid, 2001; Srikanth, 1999). Figure 2.6 below represents the schematic diagram of the principal membrane types.

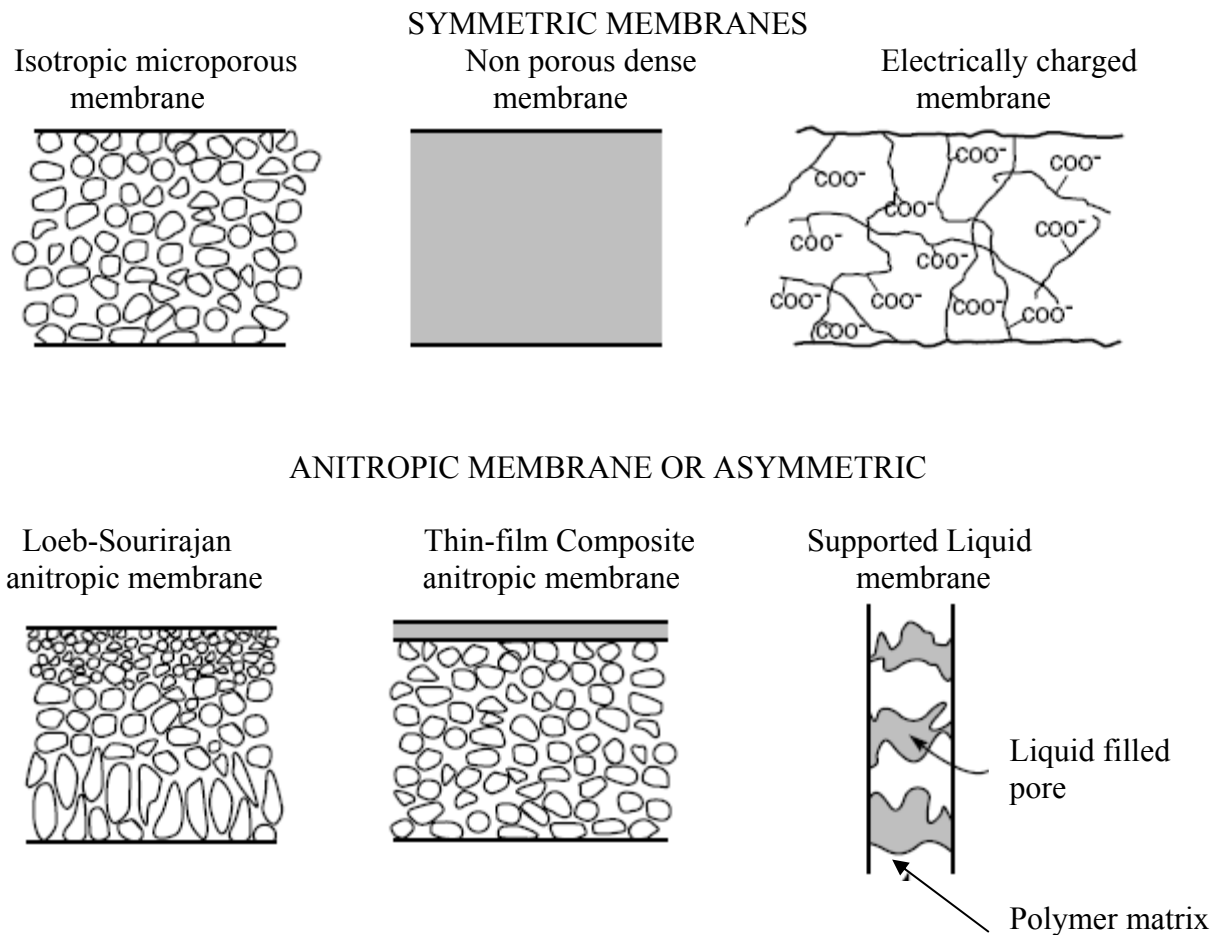


Figure 2.6: Schematic diagrams of the principal types of membrane (Baker, 2004).

The selection of the appropriate membrane requires consideration of operating conditions such as temperature, pressure, pH of the feed stream and chemical compatibility of the membrane with the feed stream (Paulson *et al.*, 1984). Applegate (1984) also stated that the performance of a specific membrane system is affected by the membrane composition, temperature, pressure, velocity of flow and interactions between components of the feedstock and with the membrane material. Thus, good membranes should have high permeability and good mechanical stability.

2.3.3 Overview of membrane processes

Membrane technology is becoming progressively more attractive as a low-cost broad separation technique that enables processors to concentrate, fractionate, and purify their products (Dziezak, 1990; Koseoglu *et al.*, 1991b). This is an energy saving process technology that does not require phase change, heat source or any ancillary equipment, and it is a process that has a minimal effect on the micro environment of the product, such as its pH or ionic strength (Paulson *et al.*, 1984; Cheryan 1986).

Membrane processes can be subdivided based on their driving forces. As driving forces, gradients in pressure (ΔP), concentration (ΔC), temperature (ΔT) and electrical potential (ΔE) are used. Table 2.2 summarizes the different membrane processes with their driving force. (Braeken, 2005).

Table 2.2: Different membrane processes for liquid separations and their driving force (Braeken, 2005).

Driving force	ΔP	ΔC	ΔT	ΔE
Process	Microfiltration Ultrafiltration Nanofiltration Reverse osmosis	Pervaporation Dialysis Liquid membranes	Thermo-osmosis Membrane distillation	Electrodialysis Electro-osmosis

2.3.4 Pressure driven membrane processes

Based on pressure as the driving force, four different membrane processes can be distinguished: microfiltration, ultrafiltration, nanofiltration and reverse osmosis using porous or dense membranes. Figure 2.7 illustrates the separation possibilities of the four processes.

Microfiltration (MF) is used for the separation of particles. The average pore size of microfiltration membranes ranges from 0.1 to 10 μm and separation is obtained by sieving. The hydrodynamic resistance of these membranes is low and therefore low pressure gradients (0.1-2 bar) are sufficient to obtain high fluxes ($\gg 50 \text{ l m}^{-2} \text{ h}^{-1}$). Microfiltration is frequently used as a pretreatment step for nanofiltration or reverse osmosis (Braeken, 2005).

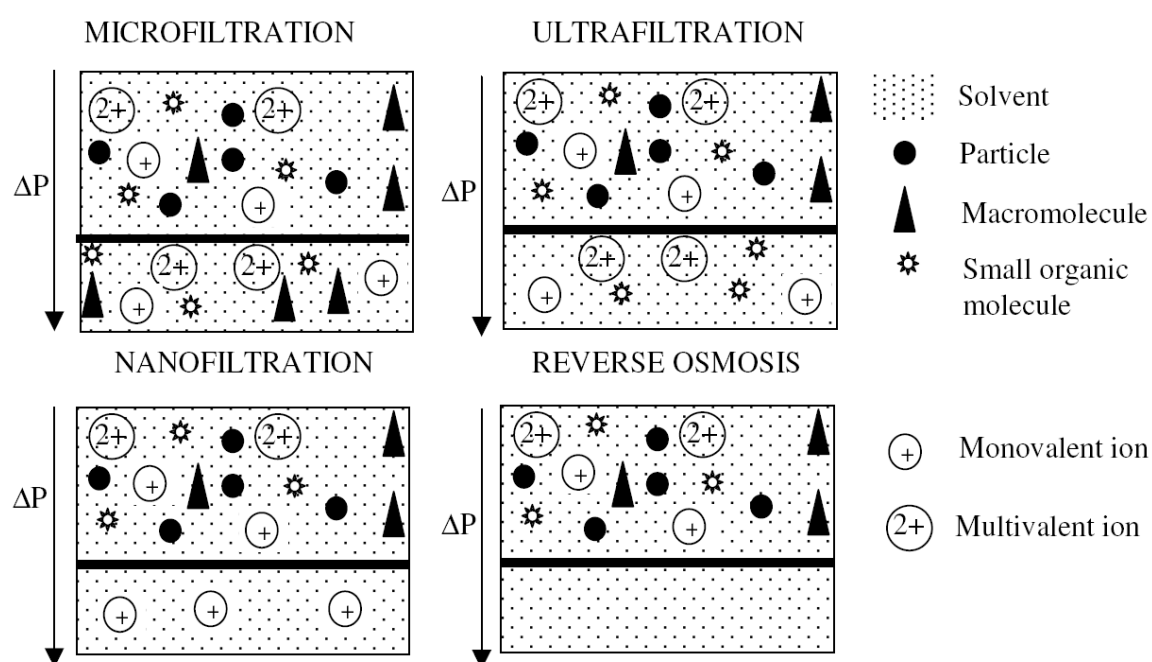


Figure 2.7: Schematic representation of microfiltration, ultrafiltration, nanofiltration and reverse osmosis (Braeken, 2005).

Reverse osmosis (RO) can be used to separate small organic molecules and ions from a solution. In this process, dense membranes are used (no measurable pores), resulting in a high hydrodynamic resistance so that high-pressure gradients (10-100 bar) are required and fluxes are low (0.05-1.4 l m⁻² h⁻¹). Separation is obtained due to sorption and diffusion through the membrane. Typical applications are seawater desalination and the production of ultra pure water for the electronic industry (Braeken, 2005).

Nanofiltration (NF) was developed in the 1970s and 1980s through the modification of reverse osmosis membranes. Nanofiltration membranes require much lower pressures (5-20 bar) than reverse osmosis and combine high permeabilities (1.4-12 l m⁻² h⁻¹) with high retentions of organic molecules with a molecular weight above 200 g mol⁻¹. Therefore, significant energy saving can be obtained by using NF instead of reverse osmosis. The MWCO of nanofiltration membranes lies between 150 and 1000. Multivalent ions are highly rejected, but for monovalent ions only moderate retentions are obtained. The separation results from a combination of diffusion, convection and charge effects, which will be discussed further later on. With these specific properties, nanofiltration can be used in a wide range of applications (Forstmeier *et al.*, 2002; Braeken *et al.*, 2004):

- Removal of surfactants from waste water
- Purification of the percolate of composting installations
- Removal of dyes from textile waste water
- Removal of heavy metals from rinsing water of electroless nickel plating process
- Water softening (removal of Ca²⁺ and Mg²⁺)
- Purification and reuse of brewery waste water
- Removal of endocrine disruptors and pharmaceutical compounds from surface water.

These applications are always in aqueous solutions. During the last five years, polymeric nanofiltration membranes for solvent applications have also been developed, but the stability of these membranes is lower than in aqueous solution applications and low fluxes combined with relatively low retentions are obtained (Schaep, 1999). Ceramic membranes can solve this problem, but they are still in development and not yet commercially available.

2.4 Ultrafiltration

Ultrafiltration (UF) is used to separate macromolecules from an aqueous solution by sieving. Salts are hardly retained. The membranes are characterized by their molecular weight cut-off (MWCO), i.e. the molecular weight of a solute that is retained for 90%. Components with a molecular weight (MW) above the MWCO have a high retention, whereas molecules with a MW below the MWCO are barely retained. Generally, the MWCO of an UF membrane lies between a few 1000 and 100 000 g mol⁻¹, which corresponds with pore sizes between a few nanometer and ca. 0.1 µm. The applied pressure ranges between 1 and 5 bar and fluxes between 10 and 50 l m⁻² h⁻¹ are obtained (Braeken, 2005).

Ultrafiltration is most commonly used to separate a solution that has a mixture of some desirable components and some that are not sought-after. Typical rejected species include sugars, biomolecules, polymers and colloidal particles (Srikanth, 1999). Ultrafiltration is somewhat reliant on the charge of a particle, but is much more dependent on the size of the particles. Literature has revealed that ultrafiltration process operates at 1.03-13.8 bar, though in some cases up to 25-30 bar has been used, and the driving force for transport across the membrane is a pressure degree of difference (Koseoglu *et al.*, 1991b; Srikanth, 1999). Figure

2.8 shows a schematic diagram of ultrafiltration crossflow system and below is an explicit outline of the membrane used in this study.

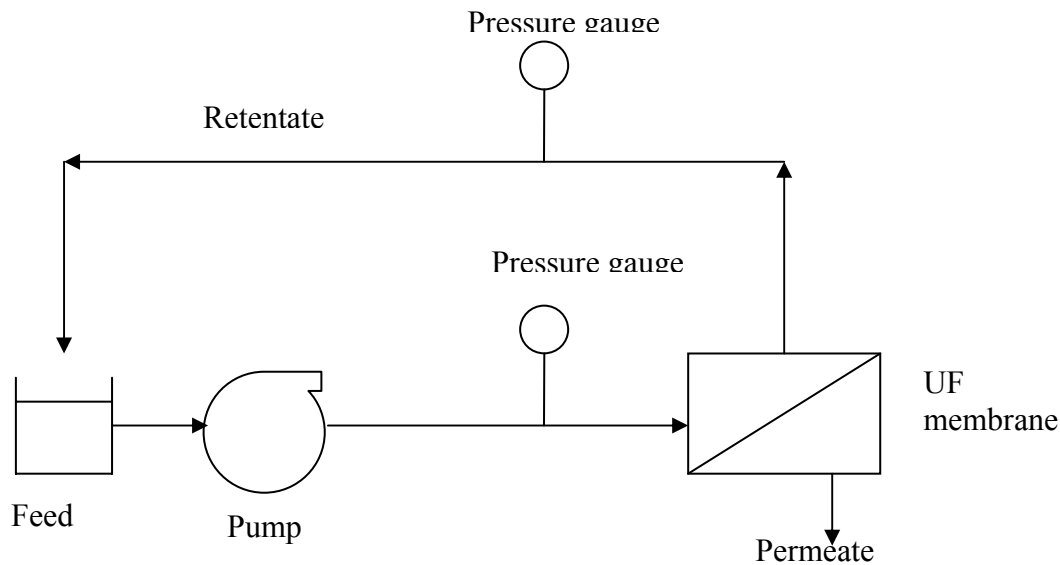


Figure 2.8: Schematic diagram of ultrafiltration crossflow system

It has been found that whenever the solvent of a mixture flows through the membrane, retained species are locally concentrated at the membrane surface, thereby resisting the flow. In the case of processing solution, this localized concentration of solute normally results in precipitation of a solute gel over the membrane. Then, when there is suspended solid in the process, the solids collect as a porous layer over the membrane surface. In view of this, it is clear that the permeate rate can be efficiently controlled by the rate of transport through the polarization layer rather than by membrane properties. For this reason, ultrafiltration through out depends on the physical properties of the membrane, i.e. permeability, thickness, process and system variable like feed consumption, feed concentration, system pressure, velocity and temperature (Srikanth, 1999). Nevertheless, it is important during ultrafiltration to balance speed with retention to attain optimum performance. Operating parameters such as pressure,

concentration, temperature, and pH and fouling have an effect on the flux of a membrane, which is the flow rate divided by the membrane area. The flux is directly proportional to the pressure gradient across the membrane and inversely proportional to the viscosity and the resistance to flow of the membrane and the solid accumulation on the surface of the membrane. The twofold restrictive factors of ultrafiltration cannot be undermined. These are concentration polarization and membrane fouling, both of which have a negative influence on the permeation rate (Nakao and Kimuras, 1981; Haris *et al.*, 1986; Haris and Dobos, 1989). Concentration polarization is a boundary layer phenomenon (Majid, 2001). According to Field (1993), a boundary layer is that region within a fluid, adjacent to a surface, across which there is momentous change in velocity, concentration or temperature. Koseoglu *et al.* (1991b) referred to concentration polarization as the collection of a layer of the solvent and its content on the surface of the membrane by hydrogen bonding or other attractive forces. Solving this problem, Smith and Gregorio (1970) suggested that polarization can be reduced by establishing turbulent mixing in the system to decrease the concentration profile along the flow channel. However, efforts to control the polarization have included adoption of high tangential velocities and membrane configurations to increase surface turbulence and shear. This method includes the use of turbulence promoters, high flow rates, and operation at the maximum temperature permitted by the membrane materials and the membrane modules (Koseoglu *et al.*, 1991b; Mohr *et al.*, 1989a).

2.5 Mechanisms of flux decline.

Different mechanisms of flux decline can be distinguished. Adsorption inside the pores or at the membrane surface narrows the pores. When the molecules have the same size as the pores, permeation can lead to pore blocking, a phenomenon that can be enhanced or caused by adsorption. Concentration polarization results in an increased concentration of solutes at the membrane surface; this high concentration of dissolved molecules provides an extra

barrier for mass transport. Eventually, the concentration at the membrane can become so high that a gel layer is formed, preventing mass transport. This effect is somewhat comparable to the deposition of suspended solids on the membrane surface. Each of these mechanisms corresponds to an increase in the total resistance for mass transport (Mulder, 1998; Vander, 2000).

Fouling, however, refers to the build-up of impermeable particles onto the membrane surface, which could be the possible crystallization and precipitation of smaller solutes that are normally permeable in the membrane pores (Moubois, 1980; Merin and Cheryan, 1980). The accumulation of this deposit interferes with flux (Paulson *et al.*, 1984) and causes a continuous decline in flux and separation. Foulants consist of inorganic salts, macro molecules, colloids and micro organisms (Majid, 2001). Moreso, fouling is an irreversible occurrence, concentration polarization is a reversible occurrence (Kun-pei and Munir, 1983). Recommended methods in controlling and reducing the extent of fouling comprise pretreatment of the sample, adjustment of membrane properties, membrane cleaning, modification of operating conditions, and optimization of membrane module design (Mohr *et al.*, 1989a). The most important approach to the prevention/reduction of fouling at the membrane surface is an increase in cross-flow velocity or the use of turbulence promoters (Da Costa *et al.*, 1993; David *et al.*, 1971).

The total resistance is the sum of all individual resistances, and this is shown in Figure 2.9 (R_p : resistance due to pore blocking; R_a : resistance due to adsorption inside the pores; R_m : membrane resistance (intrinsic); R_g : resistance caused by the formation of a gel layer; R_{cp} : concentration polarization resistance; R_i : specific interactions; R_d : resistance from deposits on the membrane).

The gel layer resistance, the adsorption resistance, the pore blocking resistance, the deposition resistance and the concentration polarization resistance depend strongly on the type of feed solution that is used. Generally, two factors play an important role: the properties of the components that are present in the feed solution and their respective concentrations (Mulder, 1998).

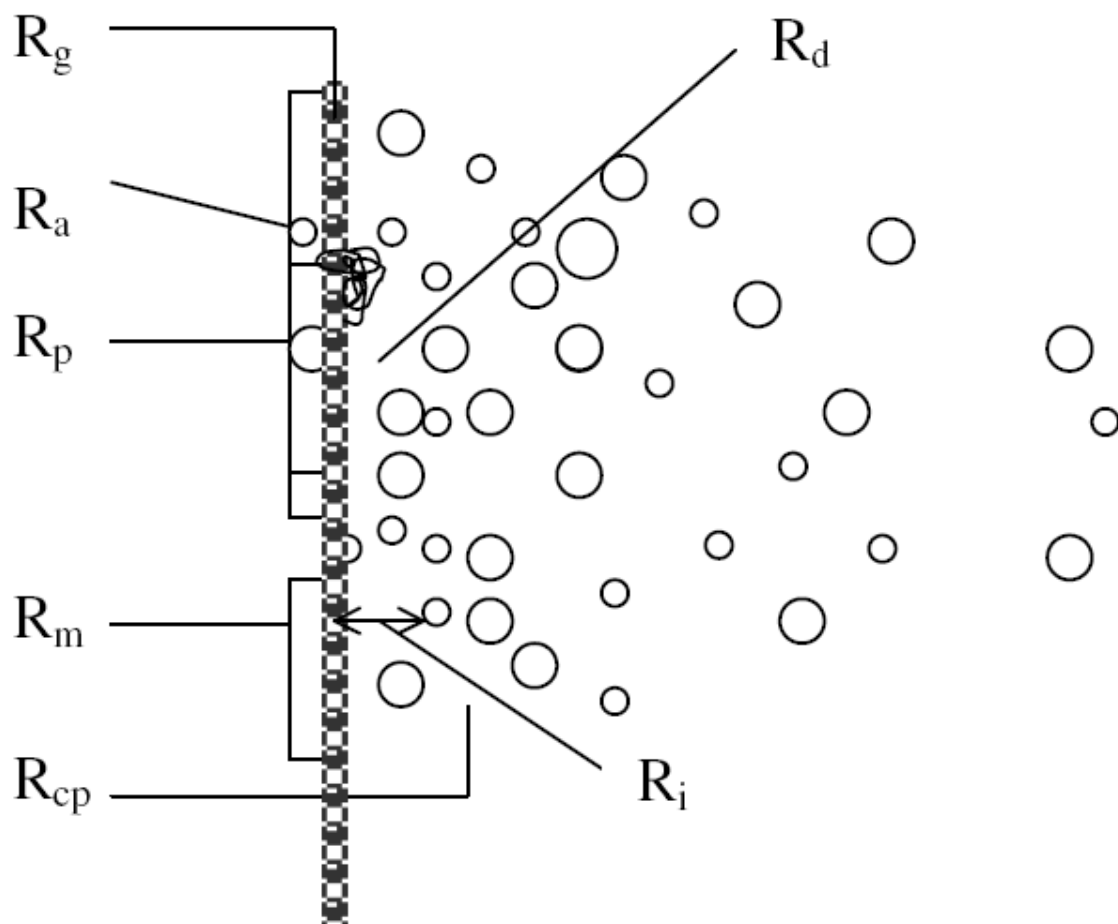


Figure 2.9: Mechanisms contributing to the total resistance towards mass transport

2.6 Characteristics of membrane

The significant characteristics of membrane materials are porosity, morphology, surface properties, mechanical strength and chemical resistance. Polymeric materials such as polysulfone, polypropylene, nylon 6, polytetrafluoroethylene (PTFE), PVC, acrylic copolymer etc., have been used effectively as ultrafiltration membranes. Inorganic materials such as ceramics, carbon based membranes, zirconia etc., have been commercialized by several vendors (Srikanth, 1999). Table 2.3 shows the characteristics of ultrafiltration membranes.

Table 2.3: Characteristics of the ultrafiltration membrane

Process	Membrane and pore radius	Membrane materials	Process driving force	Application
Ultrafiltration	Asymmetric, micro-porous 1-10nm	Polysulfon, polypropylene, nylon, PTFE, PVC, Acrylic copolymer	Hydrostatic pressure, difference at approximately 0.1-1.0Mpa	Separation of macro molecular solutions

Membrane modules are obtainable in four basic designs which include hollow fibre, spiral, plate and frame, and tubular (Srikanth, 1999; Mohr *et al.*, 1989a). The techno-economic factors for the selection, design and operation of membrane modules include cost of supporting materials and enclosure (pressure vessel), power consumption in pumping and ease of replace ability (Srikanth, 1999). Capillary membranes are also being used extensively in industrial applications.

2.7 Ultrafiltration applications

Ultrafiltration has a wide range of applications, namely:

1. Oil emulsion waste treatment
2. Concentration of biological macromolecules
3. Electro-coat paint recovery
4. Concentration of textile sizing
5. Concentration of heat sensitive proteins for food additives
6. Concentration of gelatin, enzyme and pharmaceutical preparations
7. Pulp mill waste treatment
8. Production of ultra pure water for electronic industry
9. Macro molecular separations replacing conventional change of phase method
10. Refining of oil.

Categorically, refining oil (Koseoglu, 1996b; Wiesner and Shankararaman, 1999) to bioprocesses involves the separation and concentration of biologically active components. (Koseoglu, 1991a). In accordance with Haralson and Jondahl (1983), large quantities of spent water from electronic plant can be purified for reuse by both reverse osmosis and ultrafiltration. The food industry has its specific application in the processing of meat, dairy, fruits/vegetable, sugar, grain mill, beverages and fats/oils in terms of protein concentration for gelatin manufacture, preconcentration of milk for cheese manufacture, fractionation and concentration of whey protein, clarification of fruit juices (apple, cranberry), concentration of tomatoes, pineapple and peach juice, natural colour recovery from cranberry waste, recovery of sugar from confectionary equipment wastewater, preconcentration of dilute sugar juice

prior to evaporation/crystallization, enzyme separations, removal of alcohol for low-or non alcoholic beer and wine and vegetable protein preparation (Mohr *et al.*, 1989b).

Cleaning of the membrane involves four methods which are (i) hydraulic (ii) mechanical (iii) chemical and (iv) electrical cleaning. Back flushing is an example of hydraulic cleaning in which the cleaning is carried out by reversing the direction of flow of the membrane. This dislodges the foulant from the membrane and enables the flux to be restored to a near initial value. Chemical cleaning involves exposing the module to a cleaning solution for a period of several hours (Majid, 2001). Cleaning the membrane with suitable acids, detergents or enzymes can loosen or dissolve the fouling matter from the surface of the membrane or dislodge the foulant from within the pores (Mohr *et al.*, 1989a; Porter, 1990). While mechanical cleaning involves the use of oversized sponge balls which is only applicable in tubular systems, electrical cleaning is the application of a pulsed electric field (Majid, 2001).

CHAPTER THREE

3.1 Ultrafiltration and SDS-PAGE

Ultrafiltration separates dissolved solutes from 0.002 to 0.2 μm , which corresponds to a molecular cut-off weight of approximately 500 to 300,000 MW (Dziezak, 1999; Paulson *et al.*, 1984). Cut-off is defined by the molecular size or weight, mass of the components that are retained at 90 – 95% retention coefficient or rather more than 90% rejection i.e. if the components with a molecular weight of 50,000 daltons are retained at 90–95%, the cut-off is 50,000 daltons (Srikanth, 1999; Koseoglu *et al.*, 1991b). Figure 3.1 is a picture of the membrane system used in the entire process of purification in this study.



Figure 3.1: ULTRAN[®]-MiniFlex is a CrossFlow system

ULTRAN[®]-MiniFlex is a CrossFlow system designed for rapid laboratory concentration and diafiltration of biological solution volumes up to 250 ml. In contrast to single pass filtration, cross flow involves recirculation of the feed stream across the membrane surface. Polarization is minimized due to the cross flow velocity generated by a recirculation pump. In

operation, the *retentate*, including all particles and molecules retained by the membrane, continues through the recirculation path, while the solvent and all solutes which pass through the membrane are collected in a reservoir, the so-called *permeate*.

The ULTRAN[®]-MiniFlex system includes a peristaltic pump, feed and retentate pressure indicators, mounted on a convenient base plate, retentate and permeate reservoirs and back pressure valve. All components are connected using flexible silicone tubing and fitting. MiniFlex filter cassettes are available in a wide range of ultrafiltration and microfiltration pore sizes. The filtration unit can easily be assembled in a horizontal or vertical position. It constitutes of cassettes of 24 cm² membrane area per cassette. The hold up volume of retentate per cassette is 0,5 ml and the minimal hold up volume of the system is 2,5 ml. However, the recommended operating pressure and torque are 1,0 – 1,8 bar and 3 – 5 Nm, respectively.

The SDS-PAGE technique involves running the electrophoresis after denaturing the proteins with the detergent sodium dodecyl sulfate. Commonly known as PAGE-SDS (polyacrylamide gel electrophoresis in sodium dodecyl sulfate), it is a high-resolution method. It has two major advantages compared with simple electrophoresis. One is that aggregates and insoluble particles often cause bad results with native gels by blocking the pores; when fully denatured, these aggregates are solubilized and converted to single polypeptides. The other advantage is that the mobility is related to the polypeptide size, thus an immediate indication of molecular weight for each component is provided. Although separation is only on the basis of size (whereas native gel electrophoresis also depends on charge), resolution of closely similar components is excellent.

Dodecyl sulfate binds strongly to proteins, so that only 0.1% dodecyl sulfate is sufficient to saturate the polypeptide chains, with approximately 1 detergent molecule per 2 amino acids residues. Any oligomeric protein with polypeptides that are not covalently linked are dispersed as individual subunits. In order to disrupt any disulfides, β -mercaptoethanol (ca. 1% v/v) is added, and to ensure complete denaturation, the mixture is boiled for a few minutes. Each dodecyl sulfate carries a negative charge, so a typical polypeptide of molecular weight 40,000 acquires about 180 negative charges, far in excess of any net charge that might exist (at neutral pH) on the polypeptide chain originally. Consequently, the charge/size ratio is virtually identical for all proteins, and separation can occur only as a result of the molecular sieving through the pores of the gel. Despite the fact that the potential of separation of proteins of identical size is not possible in this system, it nevertheless appears to give the sharpest overall resolution and the cleanest zones of any method. By making a comparison with a mixture of standard polypeptides of known molecular weight, the whole gel can be calibrated in terms of mobility against size. The equipment used for the above procedure in this study is the Experion™ Pro260 Bio-Rad (Figure 3.2)



Figure 3.2 Experion™ Pro260 Bio-Rad

The Experion system includes the following components: (1) Automated electrophoresis station, (2) Priming station, (3) Vortex station for RNA analysis, (4) System operation and data analysis tools, and (5) Analysis kits, which include the (a) chips and (b) reagents for protein (Pro260 kit), standard-sensitivity RNA (StdSens kit), and high-sensitivity RNA analysis (HighSens kit).

3.2 Experimental

This section describes the experimental approach used in the preparation and characterization of the cassava based linamarase. The approaches adopted include the following: isolation of the enzyme linamarase from the cassava leaves and stem; membrane separation test, characterization and quantification of the amount produced from different concentration of crude cassava extract, and each source-type.

3.3 Materials and Method

Reagents and chemicals (all chemicals are of analytical grade between 98 and 99.5% purity). Sodium acetate buffer (pH 5.5), methanol, ammonium sulphate, sodium phosphate buffer (pH 6.0) and sodium carbonate were obtained from Merck Chemicals Pty (RSA). Brilliant blue (CBB – G250), sodium dodecyl sulphate-polyacrylamide gel and carbonic anhydrase were obtained from the USB Corporation (USA).

A miniflex Ultrafiltration (UF) Cross flow system (Figure 2.9) was obtained from Schleicher and Schuell (Germany). It is built around a tubular module and contained a polyethersulfon membrane of different pore sizes (0.2-0.45 μm including 50 and 500KD). These membranes are fabricated using polypropylene screens and silicone adhesives, with a nominal molecular cut-off weight of 0.2 μm , and a membrane surface area of 2.4 mm^2 . The membrane system includes other parts such as 3R VL 100 constant pressure variable speed, peristaltic pump, pressure gauge, connecting tubes for feed flow, 140 ml $\times$ 2 graduated container, and a VOLTcraft switching power supply.

The Experion System for proteomic and genomic expression analysis (Germany) consist of an automated electrophoresis station, priming station, vortex station for RNA analysis, kits like chips and reagents for protein (Pro 260 kit), standard-sensitivity RNA (StdSens kit), and a high-sensitivity RNA analysis (HighSens kit).

3.4 Preparation of crude cassava linamarase extract

20g of freshly harvested and deveined leaves and stem were homogenized using a blender for 3 minutes with 200 ml of 0.1 M sodium acetate buffer (pH 5.5). The green slurry-like mixture was preliminary-filtered using a flour sack-cloth and centrifuged using a Beckman J2-21

model at 1000 rpm for 30 minutes. The clear supernatant was decanted. This was followed by the addition of 12 ml 2 M (NH₄)₂SO₄ and it was allowed to stand for 16 hrs at 4 °C in order to precipitate any dissolved solutes. The solution was again centrifuged at 1000 rpm for 1 hr and dissolved in a 25 ml 0.1 M sodium phosphate buffer (pH 6.0). This was subjected to further purification. Due to high content of linamarase this experiment was repeated for 40 and 60 g of freshly harvested and deveined leaves and stems.

3.5 Purification of crude cassava linamarase with ultra-filtration

The crude cassava linamarase extract was purified by passing the solution through membrane of different pore sizes: 0.45µm, 0.2µm, 0.02 µm (corresponding to about 50 KD cut off weight) and 0.1 µm (corresponding to about 500 KD cut off weight) using the miniflex ultrafiltration (UF) system at set pressure.

The experiment started with the recycling of the grinded cassava petioles in aqueous solution across the membrane surface. This operation was carried out at a set pressure (1.5 bar) with varying membrane pore sizes, under ambient temperature. During this process, however, the enzyme (linamarase) was excluded based on its molecular weight difference over membrane pore size as retentate, while the solvent and solutes, such as linamarin, lotaustralin, riboflavin and other lower molecular weight components that initially constitute the impurities of the enzyme, passed through the membrane and collected as permeate. Thus, the membrane performance on linamarase purification was evaluated based on its flux, where flux was calculated as:

$$Flux = \frac{\text{volume of permeate}(ml)}{\text{Membrane area}(mm^2) \times \text{Time}(min)}$$

The varied parameters on the permeate flux were evaluated in three different types of experiments:

- a. Effect of transmembrane pressure on permeation flux
- b. Effect of time on the permeation flux by continuous recirculation of the permeate and retentate to the feed tank for 15 hours at fixed operating conditions of transmembrane pressure (1.5 bar) and room temperature (25⁰C)
- c. The effect of water and methanol as washing solvents on the membrane after continuous use.

As the purification process progresses with decreasing membrane pore sizes, the enzyme solution turned yellow, from a slightly tinted yellow to a clear solution. The purification rate tends to vary with membrane pore sizes used in which samples were collected across the different pore size membranes and enzyme purity analysed using the SDS-PAGE Electrophoresis. More so, the percentage purity was calculated using the following equation.

$$\frac{C_{pL}}{C_{pL} + C_{pTot}} \times 100$$

Where C_{pL} = Linamarase Concentration and C_{pTot} = Total Protein Concentration or Initial Concentration of Protein.

3.6 SDS-PAGE analysis of purified samples

This is a technique used for the separation of deoxyribonucleic acid, ribonucleic acid, or protein molecules using an electric current applied to a gel matrix. It is usually performed for analytical purposes, but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for

further characterization. Its applications is found in forensics, molecular biology, genetics, microbiology and biochemistry. The following below is the usage procedure:

Before the run of the samples, the gel cassette was removed from the storage container and rinsed with deionized water. The bottom seal was later peeled off. This was followed by pulling out carefully the cassette comb. Two gels were placed into the criterion cell tank slot, ensuring that the upper buffer chamber of the gel is facing the center of the cell. The upper bath chamber was filled with about 60 ml 1X running buffer. This was then followed by loading the purified enzyme solution samples into the wells using 20 ml pipette tips. Caution was taken in loading unused wells with the SDS-containing buffer used to prepare the sample. This was in order to avoid air bubbles in an attempt to prevent the appearance of uneven width of protein bands. Furthermore, a lid was placed on the tank and the colour-coded banana plugs and jacks were put in aligned with colour. Electrode cables were connected and power switched on while samples were run. After 90 minutes samples were removed and examined.

3.7 Preparation of picrate paper

Picrate paper is used in determining the presence or absence of a compound like linamarin from cassava, protein etc in a solution or reaction mixture. Sodium alkaline picrate papers were prepared using alkaline sodium picrate solution prepared by taking 25 g of Na_2CO_3 and 5 g picrate acid and dissolved in 1 litre of distilled water. Whatman number 1 filter papers were dipped into the solution, and immediately turned the papers from white to yellow. After 30 minutes the papers were removed and air dried for use.

3.8 Analysis of linamarase present in the purified extract

Linamarase activity was determined by estimating the hydrogen cyanide (HCN) liberated during the linamarin/linamarase hydrolysis reaction (Figure 2.3). Reactions were performed at room temperature with 13 mM of linamarin in the presence of 100 mM sodium phosphate buffer, pH 6.0, in a closed vessel. A time course of 30 minutes with different dilutions of the extract was used for the measurements of activity. Excess cassava linamarase was used for quantification of tissue cyanoglycosides. In accordance with the work of Hughes *et al.* (1992), the cyanide was measured spectrophotometrically at 585 nm using a cyanide determination kit. Potassium cyanide was used as a standard. All assays were carried out with three different species of the same age, grown under the same conditions. Protein was determined in triplicate with a protein determination kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions, using bovine serum albumin (BSA) as the standard (Bradford, 1976).

Immediately before use, a solution containing 0.42 units/ml of the enzyme was prepared to determine its specific activity (unit/mass of protein) using 0.08 g of protein. This gave a specific activity of $52 \times 10^{-6} \text{ mol lg}^{-1} \text{ min}^{-1}$ of linamarase [1 enzyme unit (EU) = 1 lmol min^{-1} ($1 = 1\mu\text{m} \times 10^{-6}$)]. Thus, solutions containing initial concentration of $15.2 \times 10^{-4} \text{ mol L}^{-1}$ of linamarin substrate were reacted with a fixed amount ($0.11 \times 10^{-2} \text{ L}$) of purified enzyme in solution representing 20, 40 and 60 gram of crude cassava extract and K and Z representing source types from leaves and stems, respectively treated for linamarase. According to the method proposed by Egan *et al* (1998), this process was followed by the addition of 10 ml of 0.2 M phosphate buffer (pH 7.2). A yellow picric paper cut to size was suspended above the samples and the vials were immediately stopped. The vials were then incubated at 30°C for

30 mins. The change in colour from yellow to orange and to brown was observed as described by Egan *et al.* (1998) indicating the formation of product HCN. The papers were removed and immersed in 50 ml distilled water. After 30 minutes, the elutes were collected and the absorbance read at 510 nm against a blank picric paper using a UV Spectrophotometer (4802 UV/VIS). By means of linamarin calibration curve (Figure 3.3) that was developed by Iyuke and Idibie (2007), the concentration of HCN produced per unit time was determined.

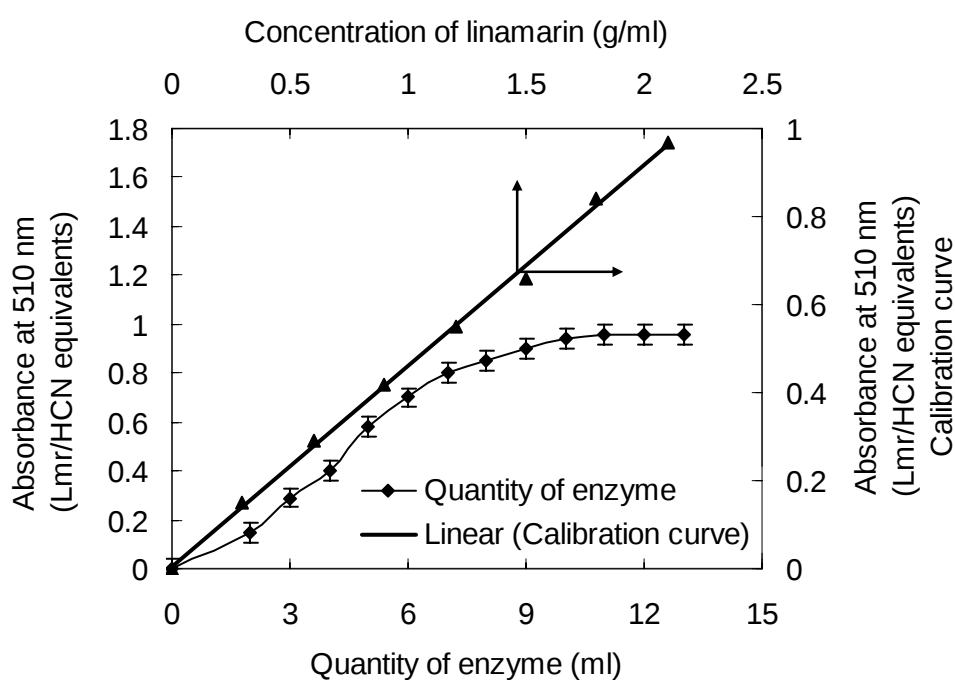


Figure 3.3: Determined standard parameters: optimum quantity of linamarase activity (error bars represent standard deviation of $n = 3$ experiments) linamarin calibration curve with standard deviation = 0.2 (Iyuke and Idibie, 2007)

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Membrane testing and linamarin used in the experiments

The samples of linamarin that were used in this study were earlier prepared from a previous study by Idibie (2007). The separation procedure started with a preliminary experiment with 0.45 μm , 0.2 μm and 50 kD membranes which were carried out using 1000 g bulk cassava tissue (same stock). At 1.5 bar, the result gave diverse concentration of linamarin with different rate of isolation (0.04 g/ml and 1.52 ml/min, 0.07 g/ml and 1.44 ml/min and 0.08 g/ml and 0.62 ml/min for 0.45 μm , 0.2 μm and 50 kD, respectively). Since the concentration of linamarin for 0.2 μm is ± 0.01 of 0.08 (the maximum concentration) but with higher rate of isolation, 0.2 μm membrane thus became the suitable membrane for this experiment. The bulk cassava extracts were earlier obtained from different weights (1000, 1500 and 2500 g) of bulk cassava tissue (BCT). Ultrafiltration with 0.2 μm membrane was found very functional in the isolation of impure linamarin in the form of crude cassava extract (CCE). The presence of linamarase in the solution isolated was established when portions of the samples isolated were treated with picrate paper test analysis as described in chapter three. The change in color from yellow to brown confirmed its presence. This is in line with the work and findings of Cooke (1978), Bradbury *et al.* (1991) and Egan *et al.* (1998). Figure 4.1 shows the kinetics of linamarin isolation using the 0.2 μm cross flow membrane. The rate of linamarin isolation from the bulk of cassava extract was calculated from Figure 4.1 data (appendix 2) and was found to be higher (1.44 ± 0.01 ml/min) when 1000 g of bulk cassava tissue was used than when 1500 g (1.03 ± 0.01 ml/min) and 2500 g (0.8 ± 0.41 ml/min) were used. This could be as a result of high concentration of cassava extracts that reduced the membrane flux

(Srikanth, 1999; Mohr *et al.*, 1989a). The gradual decrease of membrane performance as observed during the process can as well be attributed to membrane fouling and concentration polarization ((Nakao and Kimuras, 1981; Haris *et al.*, 1986; Haris and Dobos, 1989). These dual factors unavoidably reduced linamarin isolation during the process with time.

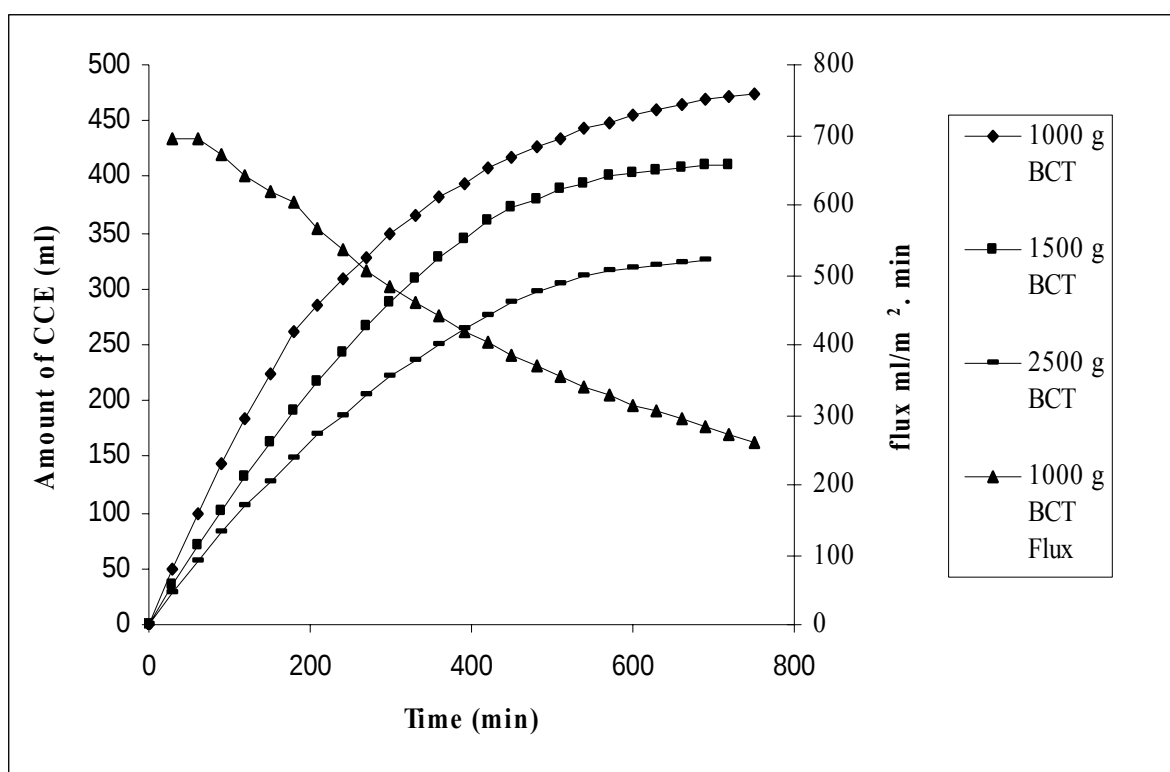


Figure 4.1: Kinetics of linamarin isolation (error bars represent standard deviation for 3 experiments (Idibie 2007))

4.2 Optimum amount of linamarase determination during linamarin hydrolysis

Categorically, to study the linamarase activity by increasing the amount of enzyme upon the rate of hydrolysis of linamarin using linamarase, the substrate was made to be present in an excess amount such that the reaction should be independent of the substrate concentration, linamarin. Hence, the quantity of linamarase present in the reaction was measured by the activity of linamarin it hydrolyses to release HCN, which was determined by the spectrophotometer method. Figure 4.2 shows the optimum activity of linamarase with respect to concentration used. It was confirmed (Idibie 2007) that the activity of linamarase as an enzyme that breaks down linamarin by hydrolysis to release HCN (Yeoh *et al.*, 1998; Bradbury *et al.*, 1991) as shown in Figure 2.3 previously in literature review. The activity of linamarase on linamarin hydrolysis is proportional to the quantity of the enzyme used. Figure 4.2 also shows the highest activity of linamarase(0.96 absorbance of HCN released)which was noted using 11 ml of enzyme solution with 2 g of linamarin, against 0.85 and 0.8 absorbance of HCN released for 2.5 and 1.5 g. This is regarded as the HCN equivalent concentration released from linamarin, even though the amount of enzyme was increased (Egan *et al.*, 1998; Yeoh *et al.*, 1998; Bradbury *et al.*, 1991). Since linamarin was used in excess (2.5 g), 11 ml of the enzyme solution was determined to be the optimum hydrolyzing amount on linamarin in all processes runs.

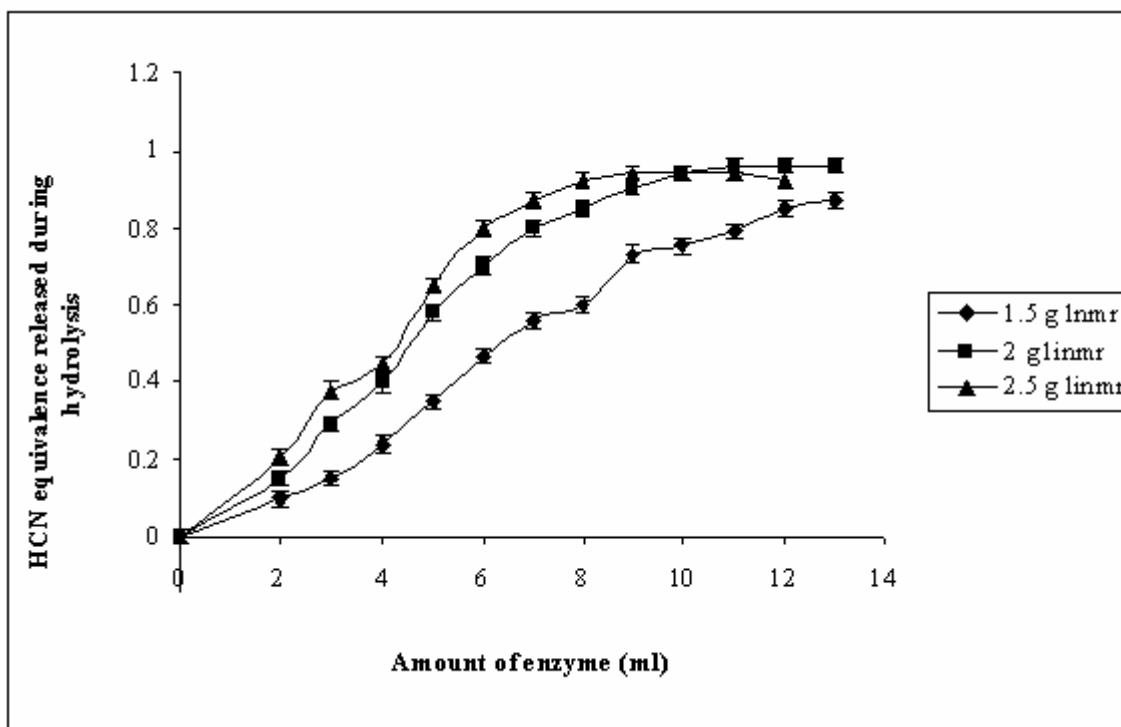


Figure 4.2: Optimum amount of enzyme determination (error bars represent standard deviation for 3 experiments)

4.3 Effect of membrane process on the quality of linamarase

In most of the available literature (Wood, 1966; Cooke *et al.*, 1978; Cooke, 1979; Eksittikul and Chulavanatol, 1988; Yeoh, 1989; Mpkong *et al.*, 1990), chromatography and other complex techniques were used in the isolation of cassava linamarase, whereas in this study, isolation of linamarase from the cassava tissues and the associated proteins was achieved by simple membrane filtration. In this study, therefore, linamarase (β -glucosidase) was obtained in high purity as shown by the gel electrophoretic data and its high specific activity. The molecular weight of the purified linamarase was found to be 65 KD. Figures 4.3 a and 4.3b present the SDS-PAGE spectrum obtained from 0.45 μ m and 0.01 μ m membrane sizes, respectively. Figure 4.3 a shows the bands for linamarase, linamarin, riboflavin and several

impurities, while Figure 4.3 b presents the bands for linamarase with 65 KD only. Figure 4.4 presents the colours of samples collected at different stages of purification.

In comparison with the method adopted by Idibie *et al.*, (2007), it is noted that while they isolated and purified the compound linamarin using activated carbon and had percentage purity in the range of 0.04–0.06 %, this study (Table 4.2), in which linamarase from the same source material is isolated and purified using ultrafiltration, had percentage purity of 1.7–3.5 %. This is as a result of the membrane ability to separate and purify materials at both macro and micron pore structures.

In the same light, linamarase obtained experimentally was compared to commercial linamarase and was discovered to compare well. The experimentally obtained linamarase had the same pH (5.5) and temperature range of 40 - 55°C. But it was slightly different in its absorbance using spectrophotometer. The commercial linamarase has an absorbance of 602 nm while the experimentally obtained linamarase had 585 nm. However, the half-life of the obtained linamarase, pre-incubated at 40 °C and pH 5.5, was about 180 minutes, which was the same with the commercial linamarase. This is in line with the work of Petruccioli *et al.*, (1999).

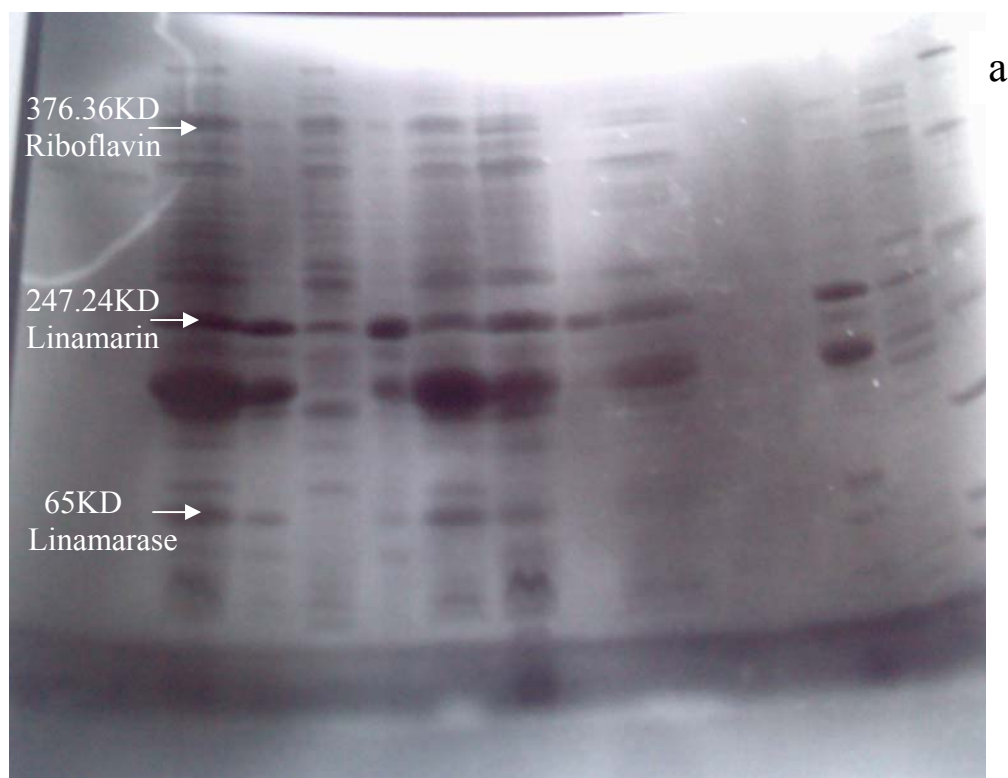
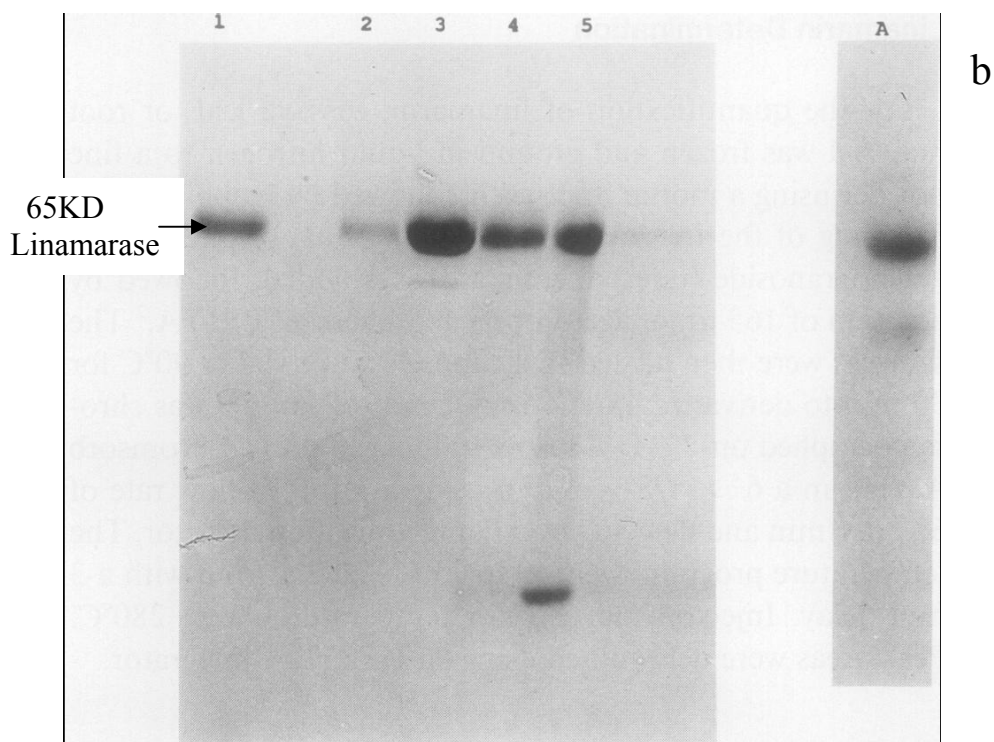


Figure 4.3: SDS-PAGE spectra of linamarase samples collected from (a) retentate and permeate of 0.45 membrane pore sizes (b) permeate of 0.2 μm membrane pore sizes.

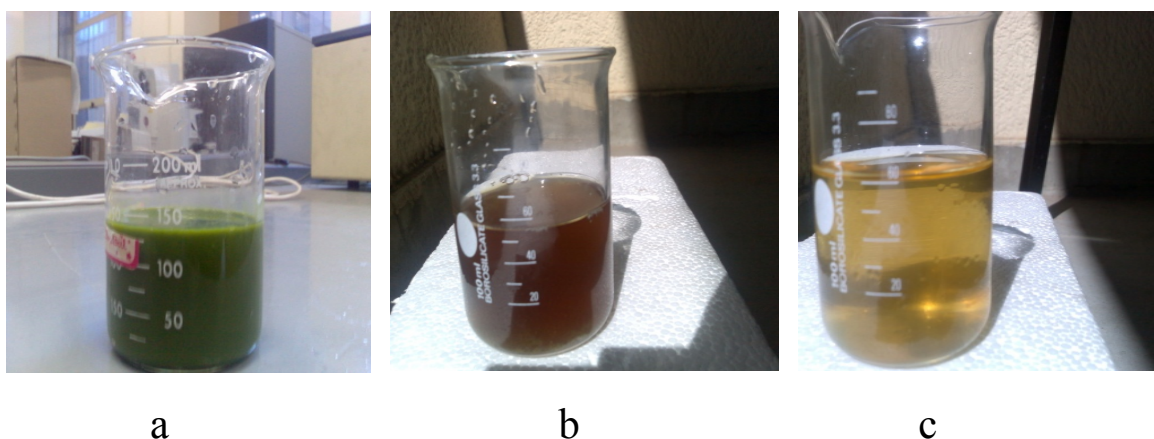


Figure 4.4: Colours of extracts, from left: (a) crude sample, (b) precipitated sample with ammonium sulphate and (c) sample collected at the permeate of 0.2µm membrane pore size

Figure 4.4 presents the different colours for the crude and treated samples. Figure 4.4a is the representation of the crude sample after the cassava leaves and stems were homogenized using a blender with 200 ml of 0.1 M sodium acetate buffer (pH 5.5). Then a green slurry-like mixture was collected and filtered using flour sack cloth and centrifuged at 1000 rpm for 30 minutes and the clear supernatant was decanted. Figure 4.4b is the decanted sample with addition of 12 ml 2 M $(\text{NH}_4)_2\text{SO}_4$ and allowed to stand for 16 hours at 4 °C in order to precipitate any dissolved solutes and the solution was centrifuged at 1000 rpm for 1 hour and dissolved in a 25 ml 0.1 M sodium phosphate buffer (pH 6.0). While Figure 4.4c presents the sample collected as retentate of 0.1 and 0.02 µm pore size membrane.

4.4 The effect of membrane pore sizes on linamarase purification

Membranes with different pore sizes of 0.45, 0.2, 0.1 and 0.02 µm were coupled into the miniflex UF system at set pressure and used in the purification process. As shown in Figure 4.5, the flux decreased sharply for 0.45 to 0.02µm pore membranes from 50 to 200 minutes of filtration at atmospheric pressure. The fluxes for membrane pores of 0.1 and 0.02µm were

not shown in Figure 4.5 because no permeate was collected due to immediate clogging of these membranes. This phenomenon is represented by the transmembrane pressures also presented in Figure 4.5, which reflect the pressure build up between 750 and 780 minutes of filtration, where the transmembrane pressure was observed to be constant at 1 bar for 720 minutes (0.45 μm) and 690 minutes (0.2 μm), where it increased to 1.1 bar due to concentration polarization. However, for 0.1 and 0.02 μm , there was a fluctuation on the build up pressure, which might be due to its inability to permeate, for there was no permeate collected but retentate. As shown in Table 4.1, it was observed that the permeate and retentate for the 0.45 and 0.2 μm membrane contained linamarase, while the retentate of 0.1 and 0.02 μm membranes contained linamarase and that no permeate was collected in 0.1 and 0.02 μm membranes due to the fouling and clogging of the small membrane pores. It was therefore concluded that the cut-off pore size for linamarase purification was 0.2 μm .

Table 4.1: Presence of Linamarase in the retentate and permeate streams of different membrane pore sizes

Stream	Membrane pore size (μm)			
	0.45	0.2	0.1	0.02
Permeate	Linamarase present	Linamarase present	No permeate, no linamarase	No permeate, no linamarase
Retentate	Linamarase present	Linamarase present	Linamarase present	Linamarase present

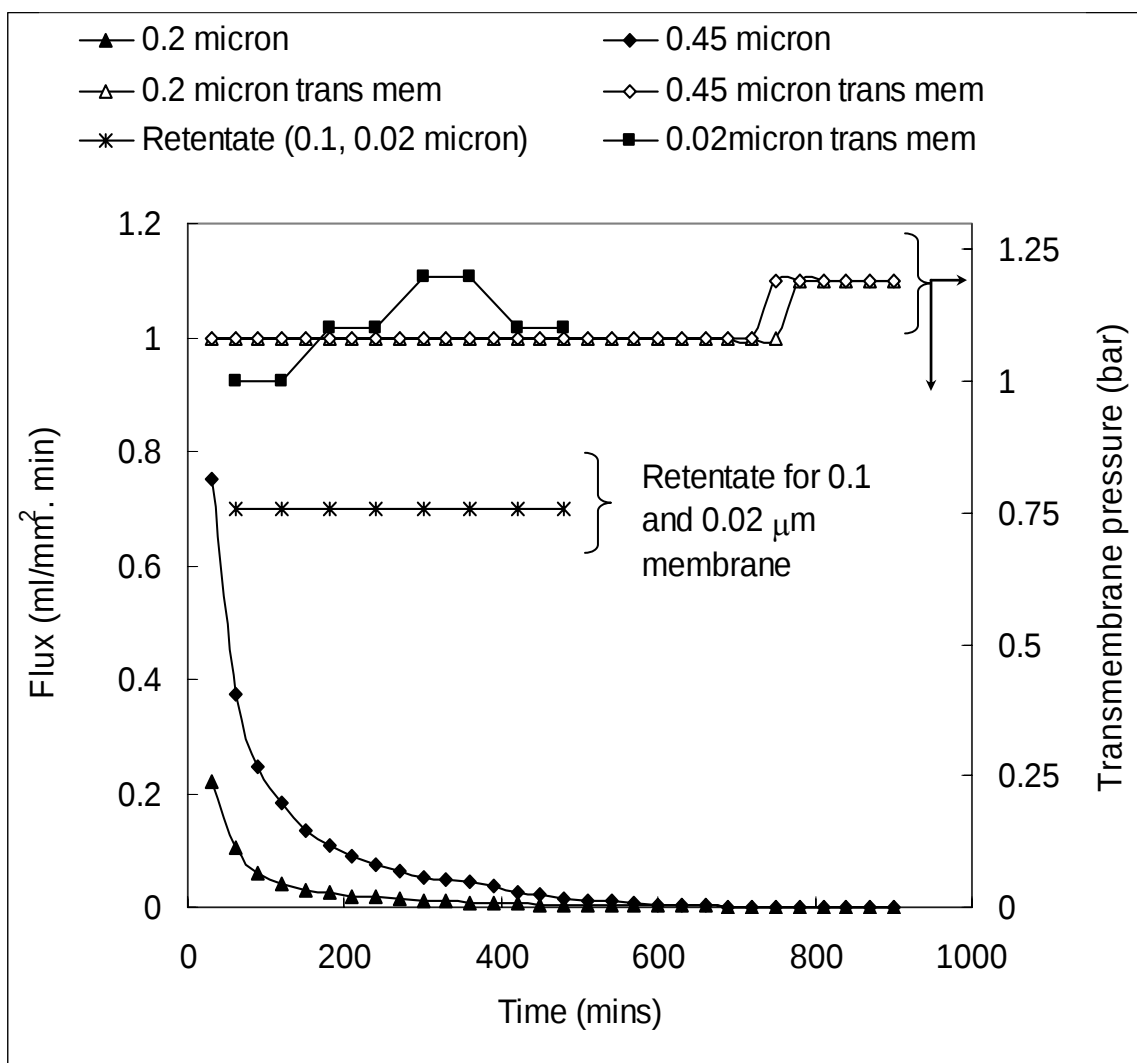


Figure 4.5: Effect of membrane pore sizes on purification of Linamarase

As shown in Figure 4.5, 0.45 μm membrane pore size showed decrease of permeation flux and transmembrane pressure with respect to time from initial 0.75 $\text{ml/mm}^2\cdot\text{min}$ at a transmembrane pressure of 1.2 bar, 0.1208 $\text{ml/mm}^2\cdot\text{min}$ in the 210 minutes and then 0.004 $\text{ml/mm}^2\cdot\text{min}$ after 10 hours of continuous recirculation of the permeate and retentate to the feed tank. Interestingly, at 15 hours of the filtration process, filtration has stopped due to clogging and membrane fouling.

In order to restore the membrane activity after its saturation that led to fouling, the membrane was washed to continue further with the isolation and purification process as discussed in the next section.

4.5 Membrane cleaning

Experiments on cleaning of the membranes involved washing with water and methanol as solvents. In the washing process the solvents were pumped through the membrane with a peristaltic pump in the reverse direction of the process presented in Figure 3.1. The 0.45 and 0.2 μm membranes were cleaned with these solvents since they were observed to be the ones suitable for purification of the linamarase. The system was flushed with deionised water to remove any residual cassava tissues. Then methanol of analytical grade or water was circulated for 30 minutes. This time was arrived at because the membrane was fully cleaned and regenerated within this time, at room temperature. Since the cassava tissues are known to be soluble in water, the temperature of cleaning was left at room temperature.

As shown in Figure 4.6a, cleaning of the membrane is an important procedure in restoring the membranes function. After washing, the initial permeation flux was almost restored.

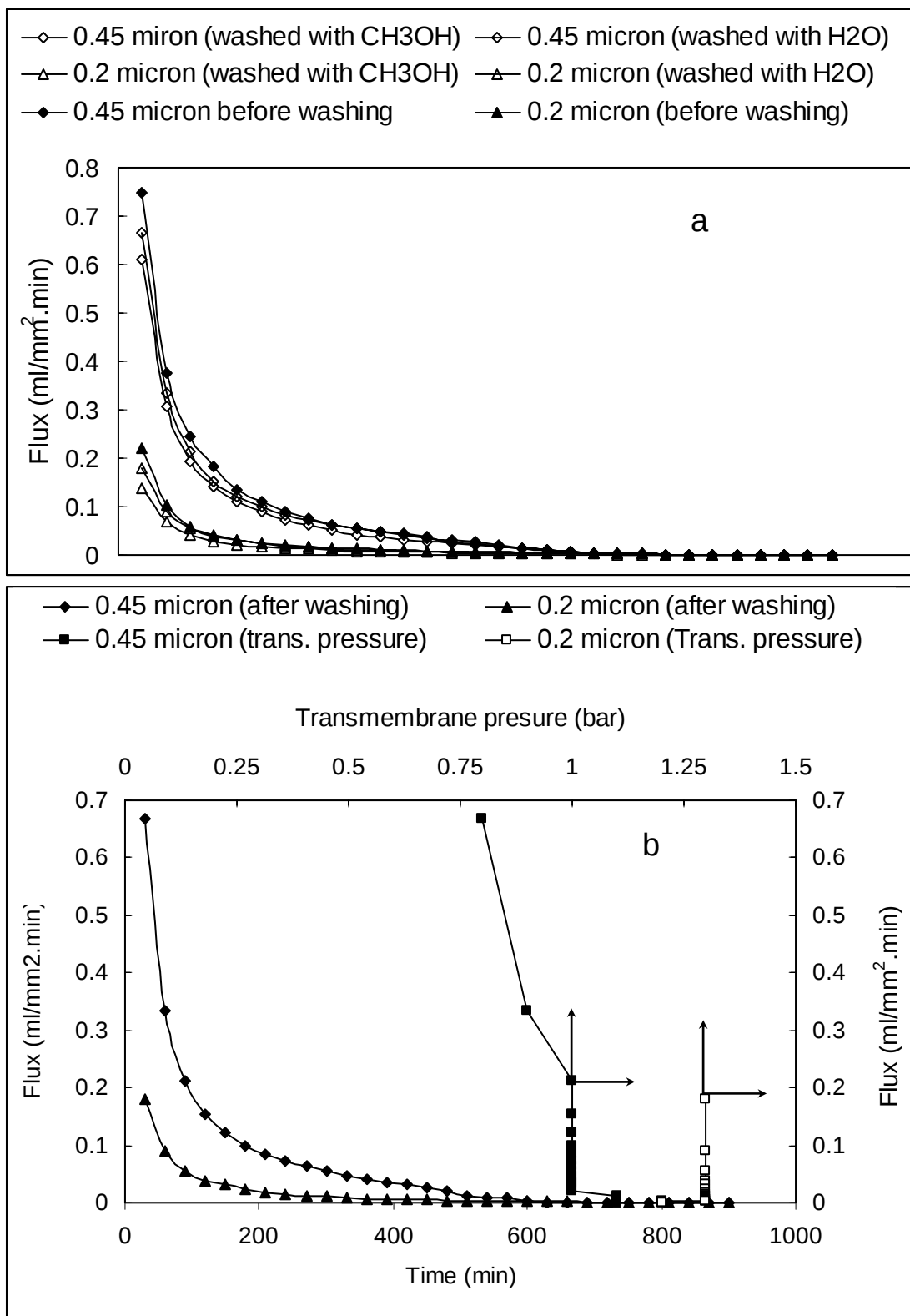


Figure 4.6: Membrane cleaning (a) effect of methanol and water (b) effect of transmembrane pressure

As expected the 0.45 μm membrane has a higher flux than that of 0.2 μm membrane. Before washing with water, the fluxes of the 0.45 and 0.2 μm membranes decreased due to fouling from 0.75 and 0.22 $\text{ml/mm}^2\cdot\text{min}$ to 0.01 and 0.004 $\text{ml/mm}^2\cdot\text{min}$, respectively, at 510 minutes. While after washing the fluxes of the 0.45 and 0.2 μm membranes decreased due to fouling from 0.67 and 0.18 $\text{ml/mm}^2\cdot\text{min}$ to 0.01 and 0.004 $\text{ml/mm}^2\cdot\text{min}$, respectively, at 510 minutes of recalculating filtration.

Figure 4.6 shows that water is a better washing solvent than methanol during linamarase purification from cassava tissues. This is because it restores flux rates quickly and enhances membrane efficiency and also because it has the ability to maintain the membrane lifespan since water has no detrimental effects on the membrane and can be used and reused for the serial cleaning of multiple membrane vessels. Furthermore, when water was used 94% of the membrane was regenerated, while methanol gave 76%. Thus, water was used in the rest of the cleaning experiments. Figure 4.6b presents the effects of transmembrane pressure on the flux (according to arrows it shows ΔP vs flux) behaviour for 0.45 and 0.2 μm membranes with time. For 0.45 μm membrane increase of transmembrane pressure from 0.8 to 1.0 bar resulted in flux decrease from 0.67 to 0.21 $\text{ml/mm}^2\cdot\text{min}$. The flux then dropped sharply to zero at 870 minutes, due to membrane fouling resulting in complete pore blockage. A similar phenomenon can be seen with the 0.2 μm membrane, of which the flux dropped sharply from 0.18 $\text{ml/mm}^2\cdot\text{min}$ at 1.3 bar to zero at 840 minutes.

4.6 Quantitative measurement of enzyme present in different extracts purified

The need to determine the amount of enzyme present in each of the crude cassava samples purified is necessary for scaling up consideration. In this, quantitative analysis of the purified enzyme was carried out via enzyme activity on linamarase or assay. Enzyme activity is the moles of substrate converted per unit time which is equal to the rate multiplied by the reaction volume. Hence enzyme activity is a measure of the quantity of enzyme present in a medium or solution. Table 4.2. shows the result of the quantity of purified enzyme present in each of the samples considered. The result shows that the quantity of enzyme increases with increase in crude cassava extract, i.e. the more the extract, the more the quantity of enzyme present, considering the concentration of crude cassava extract treated. The amount varies from 2.6 to $6.3 \times 10^{-6} \text{ mol min}^{-1}$. The result also showed that the enzyme linamarase is more in the leaves than in the stem. This is in agreement with the recent findings of Bokanga (2006).

Table 4.2: Quantification of linamarase in different extracts and source type

Samples (g)	Initial Conc. (mol/L)	Quantity of Enzyme (mol/min)
20 (root source)	15.2×10^{-4}	2.6×10^{-6}
40 (root source)	15.2×10^{-4}	3.2×10^{-6}
60 (root source)	15.2×10^{-4}	5.5×10^{-6}
Leaves Source	15.2×10^{-4}	6.3×10^{-6}
Stem Source	15.2×10^{-4}	5.5×10^{-6}

4.7 Comparison of experiment with ultrafiltration theories

McCabe *et al.* (2005) emphasized that the performance of an ultrafiltration membrane can be characterized by the permeate flux, the percentage rejection, and the concentration of the solute in the retentate stream. Normally the permeate flux decreases with time because of

membrane fouling, but fouling may increase the rejection. It has also been applauded in the open literature (McCabe *et al.*, 2005) that the modified Hagen-Poiseuille equation gives the volumetric flux v (ml/mm².min), which is the superficial permeate velocity normal to the surface of the membrane as:

$$v = \frac{(\Delta p - \Delta \pi) D^2 \varepsilon}{32 L \tau \mu} \quad (1)$$

where ε is the void fraction of the membrane; D , average pore; Δp , pressure difference; $\Delta \pi$, difference in osmotic pressure; L , nominal thickness of the active layer of the membrane; τ , tortuosity factor; and μ , permeate viscosity. However, since ε , D , τ , and L are difficult to measure, these parameters can be incorporated in the membrane permeability, Q_m , which is the flux of pure water at room temperature per unit pressure drop, or the membrane resistance R_m , which is also the reciprocal of Q_m (McCabe *et al.*, 2005), expressed as:

$$v = Q_m \Delta p = \frac{\Delta p}{R_m} \quad (2)$$

In prolonged membrane filtration as is the case in this study, where gel layer resistance, R_{gel} , is developed due to the additional hydraulic resistance of the gel layer that may form on the surface at high flux, R_m is preferably used. The general equation for ultrafiltration is therefore normally expressed as:

$$v = \frac{\Delta p - \Delta \pi}{R_m + R_{gel}} \quad (3)$$

Rearranging Equation 3 gives;

$$R_{gel} = \frac{\Delta p - \Delta \pi}{v} - R_m \quad (4)$$

Using the data presented above in Figure 4.6b and the pure water filtration using the 0.2 μm membrane into Equation 4, Figure 4.7 is obtained where parameters such as $\Delta \pi = 0.79$ bar, $R_m = 0.357(\text{mm}/\text{min}.\text{bar})^{-1}$ and $R_{gel} = 1.21(\text{mm}/\text{min}.\text{bar})^{-1}$ are calculated. Table 4.3 therefore compares the operational data in this study and those of the literature.

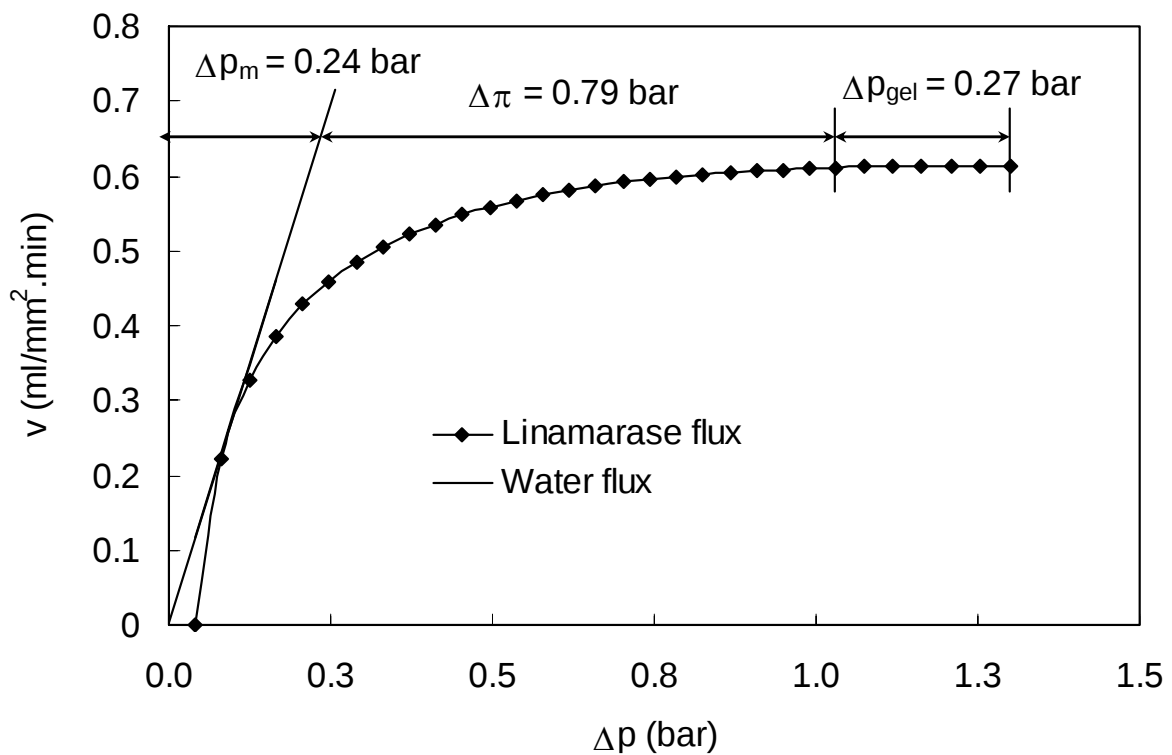


Figure 4.7: Permeate and pure water fluxes in determining independent measurements of ε , D , τ and L of Equation 1 for 0.2 μm membrane.

It is obvious from Figure 4.7 that the resistance imposed on flux after 600 minutes was much greater than that imposed on the pure membrane. However, a closer look at the data of the linamarase flux presented in Figure 4.7 does not agree with Equation 2. While water flux agrees well with Equation 2 in terms of the linear relationship between v , and Δp , the data of

linamarase flux is nonlinear. Interestingly, one could observe that the data of linamarase flux in Figure 4.7 exhibits the common Langmuir adsorption isotherm behaviour.

Table 4.3 Comparison of operational data in the study with literature

$\Delta\pi$	R_m	R_{gel}	Reference:
0.79	0.357	1.21	Present study
0.961	0.327	158.7	Jianhua <i>et al.</i> , 2005
0.854	0.401	170.0	Jianhua <i>et al.</i> , 2005
0.0348	0.93	0.0111	Joshua and Micheal, 2007

where: $\Delta\pi$ = change in osmotic pressure; R_m = membrane resistance
 R_{gel} = gel layer resistance

4.8 Prediction of membrane fouling and chemical polarisation with adsorption isotherm

Normally, as one would expect with solutions of polymers, proteins (as in this study), and other large molecules, the osmotic pressure difference $\Delta\pi$ depends on the solute concentration at the membrane surface, which is often much greater than the bulk concentration, especially when the permeate flux is high while the solute diffusivity is low (McCabe *et al.*, 2005). As shown in Figure 4.7, because of concentration polarisation, the permeate flux is a nonlinear function of Δp , and according to McCabe *et al.*, (2005), a trial-and-error solution is needed to calculate v for s given Δp . When the maximum flux is reached, the flux is limited by the rate of mass transfer back to the bulk solution. An increase in Δp at the gel region of Figure 4.7 would give a temporary increase in permeate flux, which will in turn decrease to the maximum steady-state value as the gel layer becomes thicker. Since Equation 2 has failed to

predict linamarase flux data of Figure 4.7, due to chemical polarisation, the Langmuir adsorption isotherm is proposed as

$$v = \frac{v_m \Delta p}{1 + \beta \Delta p} \quad (5)$$

where v_m is the layer of molecular coverage and β is the Langmuir constant. Equation 5 can be rearranged linearly as

$$\frac{1}{v} = \frac{1}{v_m \Delta p} + \frac{\beta}{v_m} \quad (6)$$

A plot of $1/v$ versus $1/\Delta p$ is presented in Figure 4.8, where the values v_m and β are obtained as 10.9 and 7.2, respectively. These values are then used in Equation 5 to predict the linamarase flux, as also shown in Figure 4.8. It is interesting to observe the convincing agreement between the theory and the experiments with standard error of 0.011. This agreement of the model values and experimental data proposes therefore that the Langmuir adsorption isotherm is able to predict the fouling and chemical polarisation of the membrane during linamarase purification from cassava tissues. This proposition can be buttressed further: as the solute deposits on the pores and surface of the membrane, van der Waal forces are created between the molecules, thus resulting in the fouling and chemical polarisation. This is a form of adsorption otherwise referred to as physisorption, which was easily adsorbed as shown in Figure 4.6a, when the membrane was cleaned with water, as discussed in Section 4.5.

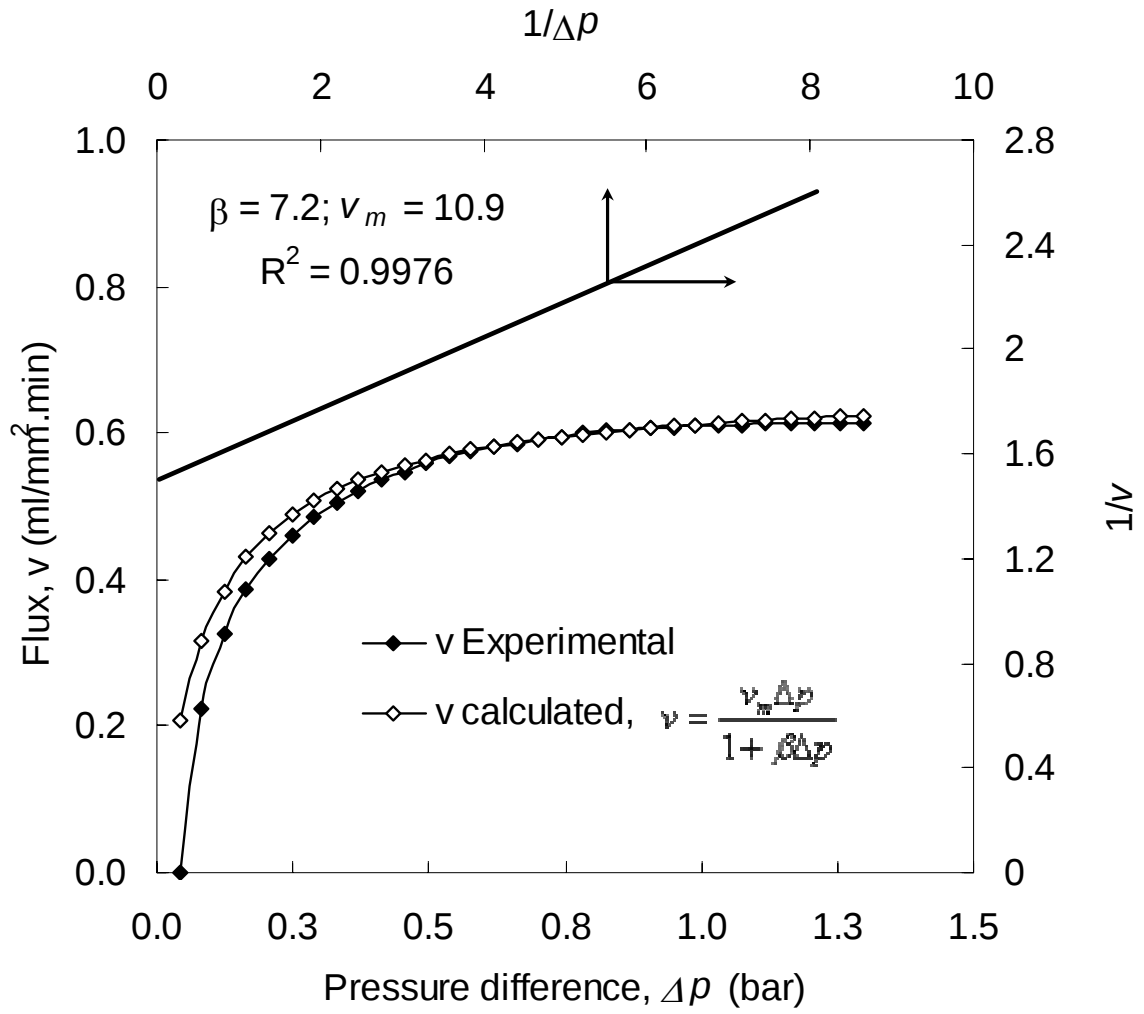


Figure 4.8: Prediction of membrane fouling and chemical polarization with adsorption isotherm which gave a standard error of 0.011.

CHAPTER FIVE

CONCLUSION AND RECOMENDATION

5.1 Conclusion

Freshly harvested and deveined leaves and stem were homogenised using a blender with 200 ml of 0.1 M sodium acetate buffer (pH 5.5). The green slurry-like mixture was preliminary filtered and centrifuged using a Beckman J2-21 model at 1000 rpm, while the clear supernatant was decanted. The solution was precipitated and allowed to stand for 16 hrs at 4 °C. The solution was again centrifuged at 1000 rpm for 1 hr and dissolved in a 25 ml 0.1 M sodium phosphate buffer (pH 6.0). This was subjected to further purification with the miniflex UF crossflow membrane. The membrane module contained a polyethersulfon membranes of different pore sizes (0.45, 0.2, 0.1 and 0.02 μm) which were used to isolate the cassava linamarase. The Experion system for proteomic and genomic expression analysis was used to characterise the enzyme linamarase purified.

The isolation experiments was started by recycling the grinded cassava petioles in aqueous solution across the membrane surface. This operation was carried out at a set pressure (1.5 bar) with varying membrane pore sizes, under ambient temperature. In this process, however, the enzyme (linamarase) was excluded based on molecular weight difference over the membrane pore size as retentate, while the solvent and solutes such as linamarin, lotaustralin, riboflavin and other lower molecular weight components that initially constitute the impurities of the enzyme, did pass through the membrane and collected as permeate. Thus, the membrane performance on linamarase purification was evaluated based on its flux. The effects of the varied parameters on the permeate flux were evaluated in three different types

of experiments, namely: effect of the transmembrane pressure on permeation flux; effect of time on the permeation flux by continuous recirculation of the permeate and retentate to the feed tank for 15 hours at fixed operating conditions of transmembrane pressure (1.5 bar) and room temperature (25°C); and the effect of water and methanol as washing solvents on the membrane after continuous use.

It was observed that the permeate and retentate from 0.45 and 0.2 μm membrane contained linamarase, while only the retentate of 0.1 μm membrane contained linamarase and that no permeate was collected in 0.1 and 0.02 μm membranes due to the fouling and clogging of the small membrane pores. At the same time, no linamarase was present in the retentates of the 0.02 μm membrane. It was therefore concluded that linamarase was finally purified by the 0.2 μm membrane. 0.45 μm membrane pore size showed decrease of permeation flux and transmembrane pressure with respect to time from initial 0.75 $\text{ml/mm}^2\cdot\text{min}$ at a transmembrane pressure of 1.2 bar, 0.1208 $\text{ml/mm}^2\cdot\text{min}$ in 3 ½ hours and then 0.004 $\text{ml/mm}^2\cdot\text{min}$ after 10 hours of continuous recirculation of the permeate to the feed tank. Interestingly, at 15 hours of the filtration process, filtration had stopped due to clogging and membrane fouling. In order to restore the membrane activity after its saturation that led to fouling, the membrane was washed to continue further with the isolation and purification process. Water was observed to be a better washing solvent than methanol during linamarase purification from cassava tissues, as the cassava tissues are more easily dissolved in water.

On closer look at the data of the linamarase flux, the experiments do not agree with Equation 2 derived from Hagen-Poiseuille equation, while water flux agrees well with Equation 2 in terms of the linear relationship between v , and Δp , where the data of linamarase flux is nonlinear. A convincing agreement between the theory and the experiments with standard

error of 0.011 was later observed when Langmuir adsorption isotherm was used to predict the fouling and chemical polarisation of the membrane during linamarase purification from cassava tissues. This proposition was buttressed further, as the solute deposits on the pores and surface of the membrane due to the van der Waal forces that are created between the molecules, thus resulting in fouling and chemical polarisation. This is a form of adsorption otherwise referred to as physisorption, which was easily desorbed when the membrane was cleaned with water

However, in this study, some limiting factors were encountered one of such factors is the less degree of control on the operating transmembrane pressure, which consequently revealed an undulating transmembrane pattern during the process of purification. The problem of instant hydrolysis of the substrate linamarin by linamarase to hydrogen cyanide (HCN) is another challenge in the purification of the enzyme since the acidic medium interferes with the practicality of the process.

5.2 Recommendations

1. Pertinently, due to its role in biological processes and its applications in biomedical and biotechnology, further work on the possibility of scaling up the process for economic production and purification of cassava linamarase should be encouraged.
2. Stability testing on linamarase should be conducted.
3. Techniques to increase the stability of linamarase enzyme to acidic conditions could be investigated.

4. The usefulness of cassava fermenting microorganisms could be further investigated for the production of other economically viable products such as acidulants and antimicrobial agents.
5. A biotechnological approach could be investigated for the treatment of odorous fermented cassava water and cassava root peels.
6. More studies should be conducted on the performance of the cross membrane, especially on the transmembrane pressure in order to gain high degree process control and efficiency on the purification of the enzyme with the membrane.

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APPENDIX 1

CALIBRATION CURVE RESULT

UV Spectrometer model; 4802 UV/VIS Unico

Wavelength; 510nm

Table I: Calibration curve of linamarin, absorbance read to two places of decimal

Linamarin Conc. Used (g/ml)	0	0.3	0.6	0.9	1.2	1.5	1.8	2.1
Absorbance	0	0.15	0.29	0.42	0.55	0.66	0.84	0.97

APPENDIX 2

KINETICS OF LINAMARIN ISOLATION BY ULTRAFILTRATION

Table II: Result of miniflex ultrafiltration in the isolation of linamarin in the form of crude cassava extract (CCE) at 1.5 bar with standard deviation for 3 experiments

BCE for bulk cassava extract; CCE for crude cassava extract.

Time (min)	1000g BCE.			Ave.	1500g BCE			Ave.	2000g BCE			Ave.
	CCE separated (ml)				CCE separated (ml)				CCE separated (ml)			
30	50	48	52	50	34	34	37	35	28	30	26	28
60	100	96	104	100	70	69	71	70	54	58	57	56
90	142	147	146	145	102	101	103	102	81	83	82	82
120	182	185	188	185	133	132	132	132	107	106	108	107
150	223	223	225	223	162	162	163	162	126	128	129	128
180	258	260	265	261	190	188	192	190	149	150	148	149
210	286	284	288	286	214	216	224	218	168	167	172	169
240	309	310	309	309	240	244	242	242	182	185	194	187
270	324	328	335	329	262	267	269	266	203	206	206	205
300	324	328	335	329	285	289	290	288	203	206	206	205
330	349	347	351	349	310	305	309	308	220	224	219	221
360	364	367	367	366	326	329	328	327	233	238	240	237
390	374	380	389	381	341	344	350	345	248	252	253	251
420	390	398	394	394	362	359	359	360	260	265	267	264
450	403	408	410	407	373	373	370	372	287	276	277	277
480	418	414	419	417	381	375	384	380	284	286	291	287
510	424	428	429	427	385	389	390	388	304	306	305	305
540	437	432	436	435	392	394	399	395	308	313	312	311
570	440	444	445	443	402	395	403	400	315	312	318	315
600	448	451	448	449	405	402	402	403	315	318	324	319

APPENDIX 2 CONTD

KINETICS OF LINAMARIN ISOLATION BY ULTRAFILTRATON

Table II: Result of miniflex ultrafiltration in the isolation of linamarin in the form of crude cassava extract (CCE) at 1.5 bar with standard deviation for 3 experiments

BCE for bulk cassava extract; CCE for crude cassava extract.

630	451	456	458	455	406	408	404	406	317	322	324	321
660	460	463	457	460	408	406	410	408	321	327	321	323
690	463	464	468	465	411	412	407	410	327	323	325	325
720	466	473	468	469	408	412	413	411				
750	470	472	474	472								
780	472	475	472	473								

APPENDIX 3

Table III: Optimum amount of enzyme determination with standard deviation for 3 experiments

Enzyme solution (ml)	Concentration of HCN released			Average
0	0	0	0	0
2	0.14	0.16	0.15	0.15
3	0.24	0.32	0.31	0.29
4	0.41	0.41	0.38	0.4
5	0.56	0.59	0.59	0.58
6	0.66	0.73	0.71	0.7
7	0.81	0.76	0.83	0.8
8	0.86	0.86	0.83	0.85
9	0.91	0.89	0.91	0.90
10	0.94	0.94	0.94	0.94
11	0.94	0.98	0.96	0.96
12	0.96	0.96	0.96	0.96
13	0.98	0.93	0.97	0.96

APPENDIX 4

Table IV: Linamarase purification with 0.45 μm

Pore Size	Time (min)	Vol. (ml) A	Vol (ml) B	Vol (ml) C	Flux (ml/mm ² . min) D	Flux (ml/mm ² . min) E	Flux (ml/mm ² . min) F	T.P (bar) X	T.P (bar) Y	T.P (bar) Z
0.45	30	54	48	44	0.75	0.666667	0.611111	1	0.8	1
	60	54	48	44	0.375	0.333333	0.305556	1	0.9	1
	90	53	46	42	0.24537	0.212963	0.194444	1	1	1
	120	53	44	41	0.184028	0.152778	0.142361	1	1	1
	150	49	44	40	0.136111	0.122222	0.111111	1	1	1
	180	47	43	38	0.108796	0.099537	0.087963	1	1	1
	210	46	42	36	0.09127	0.083333	0.071429	1	1	1
	240	43	42	35	0.074653	0.072917	0.060764	1	1	1
	270	41	41	33	0.063272	0.063272	0.050926	1	1	1
	300	39	39	31	0.054167	0.054167	0.043056	1	1	1
	330	39	37	30	0.049242	0.046717	0.037879	1	1	1
	360	39	35	28	0.045139	0.040509	0.032407	1	1	1
	390	36	33	26	0.038462	0.035256	0.027778	1	1	1
	420	28	32	24	0.027778	0.031746	0.02381	1	1	1
	450	23	28	23	0.023148	0.025926	0.021296	1	1	1
	480	19	24	20	0.016493	0.020833	0.017361	1	1	1
	510	16	15	15	0.013072	0.012255	0.012255	1	1.1	1
	540	13	13	13	0.010031	0.010031	0.010031	1	1.1	1
	570	8	10	10	0.005848	0.00731	0.00731	1	1.1	1
	600	6	3	3	0.004167	0.002083	0.002083	1	1.1	1
	630	5	2	2	0.003307	0.001323	0.001323	1	1.1	1
	660	3	2	2	0.001894	0.001263	0.001263	1	1.1	1
	690	2	2	2	0.001208	0.001208	0.001208	1	1.1	1
	720	2	2	2	0.001157	0.001157	0.001157	1	1.1	1.1
	750	2	2	2	0.001111	0.001111	0.001111	1.1	1.1	1.1
	780	2	2	2	0.001068	0.001068	0.001068	1.1	1.1	1.1
	810	2	2	2	0.001029	0.001029	0.001029	1.1	1.1	1.1
	840	2	2	2	0.000992	0.000992	0.000992	1.1	1.1	1.1
	870	0	0	0	0	0	0	1.1	1.1	1.1
	900	0	0	0	0	0	0	1.1	1.1	1.1

Where A = Volume collected before washing, B = Volume collected after washing with water.

C = Volume collected after washing with methanol, D = Flux before washing, E = Flux after washing with water, F = Flux after washing with methanol, X = Trans membrane pressure before washing, Y = Trans membrane pressure after washing with water, Z = Trans membrane pressure after washing with methanol.

APPENDIX 5

Table V: Linamarase purification with 0.2 μm

Pore Size (μm)	Time (min)	Vol. (ml) A	Vol (ml) B	Vol (ml) C	Flux (ml/mm ² . min) D	Flux (ml/mm ² . min) E	Flux (ml/mm ² . min) F	T.P (bar) X	T.P (bar) Y	T.P (bar) Z
0.2	30	44	13	10	0.611111	0.180556	0.138889	1	1.3	1.3
	60	44	13	10	0.305556	0.090278	0.069444	1	1.3	1.3
	90	42	12	9	0.194444	0.055556	0.041667	1	1.3	1.3
	120	41	11	8	0.142361	0.038194	0.027778	1	1.3	1.3
	150	40	11	8	0.111111	0.030556	0.022222	1	1.3	1.3
	180	38	10	7	0.087963	0.023148	0.016204	1	1.3	1.3
	210	36	9	7	0.071429	0.017857	0.013889	1	1.3	1.3
	240	35	9	7	0.060764	0.015625	0.012153	1	1.3	1.3
	270	33	8	6	0.050926	0.012346	0.009295	1	1.3	1.3
	300	31	8	6	0.043056	0.011111	0.008333	1	1.3	1.3
	330	30	7	5	0.037879	0.008838	0.006313	1	1.3	1.3
	360	28	6	5	0.032407	0.006944	0.005787	1	1.3	1.3
	390	26	6	5	0.027778	0.00641	0.005342	1	1.3	1.3
	420	24	6	5	0.02381	0.005952	0.00496	1	1.3	1.3
	450	23	5	4	0.021296	0.00463	0.003704	1	1.3	1.3
	480	20	5	4	0.017361	0.00434	0.003472	1	1.3	1.3
	510	15	4	3	0.012255	0.003268	0.002451	1	1.3	1.3
	540	13	4	3	0.010031	0.003086	0.002315	1	1.3	1.3
	570	10	4	3	0.00731	0.002924	0.002193	1	1.3	1.3
	600	3	3	3	0.002083	0.002083	0.002083	1	1.3	1.3
	630	2	3	2	0.001323	0.001984	0.001323	1	1.3	1.1
	660	2	3	2	0.001263	0.001894	0.001263	1	1.2	1.1
	690	2	2	2	0.001208	0.001208	0.001208	1	1.2	1.1
	720	2	2	2	0.001157	0.001157	0.001157	1.1	1.2	1.1
	750	2	2	2	0.001111	0.001111	0.001111	1.1	1.2	1.1
	780	2	2	2	0.001068	0.001068	0.001068	1.1	1.2	1.1
	810	2	2	2	0.001029	0.001029	0.001029	1.1	1.2	1.1
	840	2	2	0	0.000992	0	0	1.1	1.2	1.1
	870	0	0	0	0	0	0	1.1	1.2	1.1
	900	0	0	0	0	0	0	1.1	1.2	1.1

Where A = Volume collected before washing, B = Volume collected after washing with water.

C = Volume collected after washing with methanol, D = Flux before washing, E = Flux after washing with water, F = Flux after washing with methanol, X = Trans membrane pressure before washing, Y = Trans membrane pressure after washing with water, Z = Trans membrane pressure after washing with methanol

APPENDIX 6

Table VI: Linamarase purification with 0.1 μm

Pore Size (μm)	Time (min)	Permeate Flow (ml)	Permeate Volume (ml)	Flux ($\text{ml}/\text{mm}^2 \cdot \text{min}$)	Transmembrane Pressure (bar)
0.1	60	98	98	0.7	1
	120	98	196	0.7	1
	180	98	294	0.7	1.1
	240	97	391	0.7	1.1
	300	97	488	0.7	1.2
	360	96	584	0.7	1.2
	420	96	584	0.6	1.1
	480	96	584	0.5	1.1

APPENDIX 7

Table VII: Water permeation flux with 0.45 μm

Pore Size (μm)	Time (min)	Volume collected (ml)	Flux ($\text{ml}/\text{mm}^2 \cdot \text{min}$)	Transmembrane Pressure (bar)
0.45	2	50	10.42	1.5
	4	50	5.208	1.5
	6	48	3.333	1.5
	8	48	2.5	1.5
	10	48	2	1.5
	12	46	1.597	1.5
	14	45	1.339	1.5
	16	45	1.172	1.5
	18	45	1.042	1.5
	20	45	0.938	1.5

Table VIII: Water permeation flux with 0.2 μm

Pore Size (μm)	Time (min)	Volume collected (ml)	Flux ($\text{ml}/\text{mm}^2 \cdot \text{min}$)	Transmembrane Pressure (bar)
0.2	2	42	8.75	1.5
	4	42	4.375	1.5
	6	41	2.847	1.5
	8	41	2.135	1.5
	10	41	1.708	1.5
	12	40	1.389	1.5
	14	40	1.191	1.5
	16	40	1.042	1.5
	18	40	0.926	1.5
	20	40	0.833	1.5