

# **CHARACTERISATION OF BACTERIAL MYROSINASE**

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award of the degree of Doctor of philosophy of Imperial College  
London**

## **Declaration of originality**

I certify that the work and material in this thesis has not been previously submitted for a degree anywhere for any award. Where other sources of information have been used they have been acknowledged.

## **ACKNOWLEDGEMENTS**

Because he is the greatest and where my thanks should go first so thanks Allah (God). I am also very grateful to my supervisor Dr John Rossiter who introduced me to the myrosinase and glucosinolates and for his guidance throughout my PhD without his support this thesis would not have seen the light. My thanks also go to my colleagues in Rossiter's group (Linda, Huda, Numrah and the future nun Vin!) for the great atmosphere in the lab. I would like to thank my friends in Raddika and Buck groups for the great lab times especially at weekends! A special thanks to Janice Taylor and Jasmine Pham for their help. Last, but not least a big and special thanks to my family my wife Layla and my daughters Moneera, Safa, Marwa, Fatima and Khadeeja and for my son Ali for their love, support and patient. Thanks is due to the Libyan government for funding.

**Dedicated to the soul of my uncle Abdulssalam Albaser**

## Abstract

The degradation of glucosinolates (GLSs) are mediated by the enzyme commonly known as myrosinase ( $\beta$ -thioglucosidase glucohydrolase; EC 3.2.3.1). There has been limited work on bacterial myrosinases which is surprising since they must play a role in both soil and animal gut ecology. The only bacterial myrosinase that has been purified to homogeneity is that of *Enterobacter cloacae* (no 506) but no amino acid sequence is available and there is very limited information on the characteristics of these enzymes. In this study a bacterial myrosinase from a soil isolate *Citrobacter spp* was purified to homogeneity and characterised. A combination of ion exchange chromatography two steps, (Mono Q column followed by gel filtration Superdex 75) were used. The molecular mass of both denatured and native protein is approximately 66 kDa. The optimum temperature and pH of the crude extract is 25 °C and pH 6.0 respectively. The enzyme is slightly activated by ascorbate but inhibited by glucose. Metal ions  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $Fe^{3+}$  and  $Mg^{2+}$  also inhibited  $\beta$ -O-glucosidase activity with  $Fe^{3+}$  causing the largest loss of (76%) of activity followed by  $Mg^{2+}$ ,  $Zn^{2+}$  and  $Ca^{2+}$  while the metal ion chelator EDTA reduced enzyme activity by 70%. *In vivo* cultures of *Citrobacter* with sinigrin as a carbon source produced an as yet an unknown metabolite, while cell free extracts incubated with sinigrin produce 2-propenyl isothiocyanate. Sinigrin is completely degraded within 12 h of incubation of the *Citrobacter* in M9 medium either alone or in the presence of glucotropaeolin or gluconasturtiin. The latter two glucosinolates were metabolised differently and approximately 39% of glucotropaeolin and 20% gluconasturtiin remained in culture medium after 25 h of incubation. None of the tested  $\beta$  configuration substrates induced myrosinase. The enzyme hydrolysed sinigrin and glucoerucin at a faster rate than glucotropaeolin and glucoraphanin. The enzyme has both  $\beta$ -O-glucosidase and  $\beta$ -S-glucosidase activity with a greater affinity to aryl- $\beta$ -glucoside with a  $K_m$  of 0.0183 mM and a  $V_{max}$  of 0.6 nmole.L<sup>-1</sup>.min<sup>-1</sup>

while for sinigrin a  $K_m$  of 0.54 mM and a  $V_{max}$  of 3.1 nmole.L<sup>-1</sup>.min<sup>-1</sup>. The enzyme activity could not be recovered from SDS-PAGE gels but was detected and eluted from native PAGE. SDS-PAGE of the eluted protein showed fewer protein bands and was comparable to the ion exchange second run in terms of number of bands on SDS-PAGE gels. The *N*-terminal sequence of the pure *Citrobacter* myrosinase shows little similarity with  $\beta$ -glycosidases while internal peptide analysis revealed some similarity with other  $\beta$ -*O*-glucosidases. However, no homology with any known myrosinases was found. Peptide sequencing of bands of 71 and 72 kDa obtained by different purification techniques (likely the same proteins) show some similarity with  $\beta$ -glucosidase of *Citrobacter* 30\_2, while the peptide sequence of the secreted myrosinase band of  $\approx$ 57 kDa shows similarity with glycoside hydrolase, family 3 of *Ktedonobacter racemifer* DSM 44963. The genome sequence of *Citrobacter* is required in order to allow further study such as cloning.

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## **CHAPTER ONE INTRODUCTION**

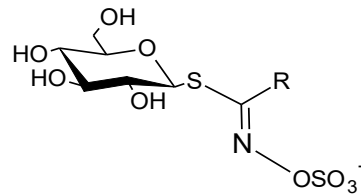
## Introduction

### 1.0 Plant metabolite overview

Life is a dynamic process which involves many constantly changing chemical reactions. These changes are catalyzed and regulated by enzymes. The physiological functions and the mechanisms of many enzymes systems are still not known. Many secondary plant metabolites are known and several such as cyanogenic glycosides, terpenoids, cardenolides and iridoid glycosides have been linked with plant defense system (Goyal et al., 2012). Plants of cruciferous vegetables possess secondary metabolites known as glucosinolates (GLS). They are  $\beta$ -thioglycosides which are found in the order Brassicales. GLSs are metabolised by thioglucoside glucohydrolase EC 3;2;3;1 (myrosinase) which also exists in the order Brassicales. The resulting products of the degradation process are thiocyanate, isothiocyanate (ITC) or nitrile (discussed later) and some of these products are linked with the plants defence system and are reported to be toxic to herbivores, fungi, viral, bacterial pathogens and nematodes. This system is known as the myrosinase GLS system (Bones and Rossiter, 1996; Kuśnierczyk et al., 2007).

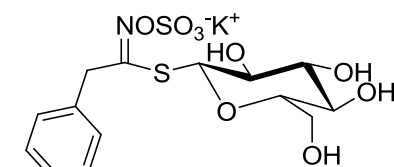
### 1.1 The chemical structure of glucosinolates

Naturally occurring GLS (Figure 1.1) are (*Z*)-*N*-hydroxyiminosulfate esters possessing a sulphur-linked  $\beta$ -D- glucopyranose moiety and an amino acid-derived side chain which can be derived from one of eight amino acids (Fahey et al., 2001) methionine, phenylalanine, tryptophan or branched chain amino acids (Halkier and Gershenzon, 2006).

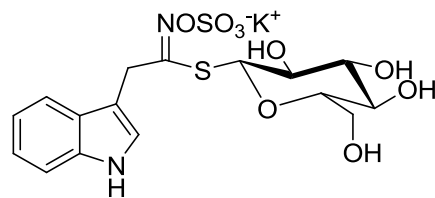


**Fig 1.1** Chemical structure of a glucosinolate

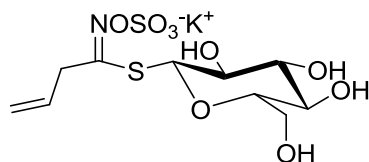
GLS can be classified by their precursor amino acid and the types of modification to the R group (Figure 1.2) into three categories (aliphatic, aromatic, or indolyl), the aliphatic GLS are those compounds derived from alanine, leucine, isoleucine, methionine or valine.



p-hydroxybenzylglucosinolate  
*phenylalanine derived*



indolylglucosinolate  
*tryptophan derived*

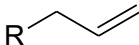
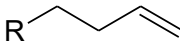
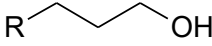
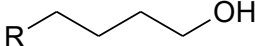
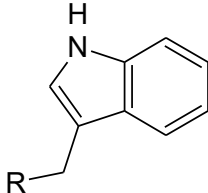
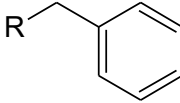
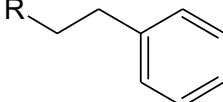


2-propenylglucosinolate  
*methionine derived*

**Fig 1.2** Examples of the classification of GLS according to R group of amino acids.



The aromatic GLS are derived from phenylalanine or tyrosine, and the indole GLS from tryptophan. The sulphate group of the GLS is strongly acidic and plants accumulate GLS by sequestering them as potassium salts in the plant vacuoles (Halkier and Gershenzon, 2006). More common GLS are shown in Figure 1.3

semisystematic name	trivial name	side chain
2-propenyl	sinigrin	
3-butenyl	gluconapin	
3-hydroxypropyl	-	
4-hydroxybutyl	-	
indol-3-ylmethyl	glucobrassicin	
benzyl	glucotropaeolin	
2-phenethyl	gluconasturtiin	

**Fig 1.3** The side chain structure of some typical GLS; R refers to the general structure of the GLS side chain.

## 1.2 Occurrence in Cruciferous plants

Cruciferous or *Brassica* vegetables (order Brassicales) belong to the family Cruciferae which are also known as Brassicaceae. The plants in this family have flowers with four equal sized petals in the shape of a crucifer cross while *Brassica* is a Latin term for cabbage. Species of this family are widely consumed by humans as cooked or fresh vegetables including *Brassica oleracea* var. *Italic* (broccoli); *Brassica oleracea* var. *Gemmifera* (Brussels sprouts), *Brassica oleracea* var. *Capitata* (cabbage); *Brassica oleracea* var. *Botrytis* (cauliflower); collard greens, kale, *Brassica oleracea* var. *Gongylodes* (kohlrabi); mustard, *Brassica napus* (rutabaga); *Brassica rapa* var. *rapa* (turnips); bok choy and Chinese cabbage (Higdon et al., 2007). The typical or strong flavour or pungent aromas of brassicas is largely due to sulphur containing compounds that are derived from GLS (Drewnowski and Gomez-Carneros, 2000). GLS are also known as mustard oil glycosides and are secondary metabolites, found among cruciferous vegetables and exclusively in dicotyledonous plant (Oerlemans et al., 2006). The first observation of the unique properties of GLS or mustard oil was recorded at the beginning of the 17<sup>th</sup> century as a result of attempts to understand the chemical origin of the sharp taste of mustard seeds (Fahey et al., 2001). The occurrence of GLS in plants for human consumption appears to be limited to cruciferous plants and the concentrations of GLS depends on variety, cultivation conditions, climate and agronomic practice (Jongen, 1996). The concentration of GLS in plants is highly variable and can be between 1% dry weight in some *Brassica* vegetables (Rosa et al., 1997) and 10% in some seeds (Josefsson, 1970). It is believed that most plant species contain a limited number of major GLS, although as many as 34 different GLS have been reported in *Arabidopsis thaliana* (Hogge, 1988; Kliebenstein et al., 2001). The distribution of GLS varies among plant parts with both type and concentration differences between roots, leaves, stems and seeds. The seeds or young sprouts of broccoli can contain 70-100  $\mu\text{mol}$  total GLS gram per fresh

weight, *B. oleracea* (which includes broccoli, cabbage, Brussels sprouts and kale) contain Glucobrassicin (3-indolylmethyl) and glucoiberin (3-methylsulfinylpropyl) and most contain substantial amounts of sinigrin (2-propenyl). The major GLS in kales and cabbage are 2-propenyl, glucobrassicin (3-indolylmethyl) and 3-methylsulfinylpropyl (Cartea and Velasco, 2008), glucoraphanin (4-methylsulfinylbutyl), 2-propenyl, progoitrin (2-hydroxy-3-butenyl), gluconapin (3-butenyl) and the indole GLS 3-indolylmethyl and neoglucobrassicin (1-methoxy-3-indolylmethyl). The common (Ambrosone et al., 2004) GLSs in broccoli (Kushad et al., 1999), 2-propenyl, 2-hydroxy-3-butenyl and 3-indolylmethyl are the main GLSs in Brussels sprouts, collard and cauliflower (VanEtten, 1976; Kushad et al., 1999). Each of these vegetables contains smaller amounts of other GLS (Cartea and Velasco, 2008). The predominant GLS in turnip roots are 2-hydroxy-3-butenyl and gluconasturtiin (2-phenylethyl) (Sones et al., 1984), while 3-butenyl and glucbrassicinapin (4-pentenyl) were identified as the most abundant GLS in edible parts of turnip greens (Kim et al., 2003; Padilla, 2007) and in turnip tops (Rosa et al., 1997). In *B. napus*, 4-pentenyl followed by 3-butenyl have been shown to be the most abundant GLS in a variety in of *B.rapa* cv Nabicol (Cartea et al., 2008) while in Swede 3-indolylmethyl, 2-hydroxy-3-butenyl and 2-phenylethyl were found to be the major GLS (Velasco et al., 2010). In 1830 sinalbin (4-hydroxybenzyl) was the first GLS to be isolated from the seed of *Sinapis alba* (white mustard), followed by 2-propenyl that was also isolated in the same year from *Brassica nigra* (black mustard). To date over 120 different GLS (Table 1.1) have been identified in plants, each plant species contains up to four different GLS in considerable amounts (Fahey et al., 2001).

Brassica (B) semisystematic name	Plant common name	GLS side chain
<i>B. oleracea var. italica</i>	broccoli	3-methylsulfinylpropyl 4-methylsulfinylbutyl indole-3-methyl
<i>B. oleracea var. capitata</i>	cabbage	1-methoxyindole-3-methyl 2-propenyl 3-methylsulfinylpropyl
<i>B. oleracea var. gemmifera</i>	Brussels sprouts	indolylmethyl 2-propenyl 3-methylsulfinylpropyl 3-butenyl 4-methylsulfinylbutyl 2-hydroxy-3-butenyl indol-3-methyl
<i>B. oleracea var. botrytis</i>	cauliflower	1-methoxyindole-3-methyl 2-propenyl 3-methylsulfinylpropyl 4-methylsulfinylbutyl indole-3-methyl
<i>B. oleracea var. acephala</i>	kale	1-methoxyindole-3-methyl 2-propenyl 3-methylsulfinylpropyl 3-butenyl 2-hydroxy-3-butenyl
<i>B. rapa</i>	rape, Chinese cabbage & turnip tops	indole-3-methyl 3-butenyl 4-pentenyl

**Table 1.1** Main cruciferous vegetables scientific, common name and side chain.

### 1.3 Isolation and extraction of GLS

#### 1.3.1 Extraction of GLS

GLS always coexist with plant myrosinase, inhibition of myrosinase activity is essential to ensure GLS are not hydrolysed by myrosinases (Moreno et al., 2006). For this reason extractions of GLS are usually conducted at temperatures of 65-100 °C, this temperature is close to boiling point of the solvent mixture used for extraction. The use of denaturing agent such as methanol in combination with high temperature is sufficient for myrosinase inactivation (Mithen et al., 2000; Moreno et al., 2006; Clarke, 2010). Most early descriptions of GLS isolation used paper or thin layer chromatography (Moreno et al., 2006). Combination of paper chromatography and high voltage electrophoresis has been used however with considerable complications and low yield (Fahey et al., 2001). Indole and aryl GLS have been isolated on acidic alumina as initial step, followed either by size exclusion chromatography on Sephadex G10 (Hanley et al., 1984) or ion exchange chromatography on DEAE-Sephadex A-25 (Hanley et al., 1983). Thies (1988) has reported a method for isolation of two GLS (2-propenyl and benzyl) in gram quantity; for other GLS crystallizing remained issue and hence only very few additional GLS have been crystallized (Thies, 1988). Extraction of GLS from plant parts is best achieved by the use of either methanol-water (7:1) or ethanol-water (1:1) mixtures (Clarke, 2010). Phosphate buffer (20 mM, pH 7) or water followed by boiling (20 min) has been found to be more effective than the use of alcohol solvent for 2-propenylGLS extraction from horseradish and black mustard (Stoin et al., 2007). Phosphoric acid (2%) has been used in extracting GLS from Brussels sprouts (Doorn He et al., 1998). If the aim is to remove GLS from oilseed protein then the use of a mixture of ammonia (10 %) in methanol containing water (5%) was used successfully to extract GLS of seeds at ratio of 6-7 (solvent to seed) and blending (2 min) followed by quiescent period (10-15 min), this has been found

sufficient in minimizing GLS content below detection limits (Rubin et al., 1986; Liu et al., 1995). It was reported that optimum conditions for extraction of glucoraphanin from the toxic weed *Cardaria draba* were ethanol (20%) in water (70 °C, pH 3 / 20 min) (Powell et al., 2005). Due to structure diversity and the plant matrices and the range of seed, it is recommended to use methanol (70%) for extraction unless validated and proved alternative solvent are required (Clarke, 2010).

### 1.3.2 Analytical standard of GLS

In aqueous extraction which may include lead acetate or barium acetate in order to precipitate free sulphate and protein (Brown et al., 2003), followed by centrifugation, the purification is carried on an anion exchanger column in which a resin of DEAE Sephadex A25 is used for enzymatic desulfation using sulphatase and analysis methods (Barillari et al., 2005), the elution from the resin is typically carried out by using potassium sulphate ( $K_2SO_4$ ) (0.5 M) followed by evaporation and dissolution in hot methanol in order to leave insoluble salts, finally the recrystallization step is from cold ethanol then drying over phosphorus pentoxide ( $P_2O_5$ ) (Clarke, 2010). The florisil solid phase is used in extraction in order to clean up intact GLS, this carried with application in methanol, dichloromethane and hexane mixture, followed by washing with dichloromethane and hexane mixture and elution with methanol ethyl acetate (Lee et al., 2006; Millán et al., 2009). The use of copolymeric anion exchanger of styrene divinylbenzene will result in an additional dimension for separation, elution of GLS in order of their hydrophobicity is now possible using inorganic anions of  $SO_4^{2-}$ ,  $NO_3^-$ ,  $ClO_3^-$  and  $ClO_4^-$  (Elfakir et al., 1994). Separation of gram quantity of chromatography and structurally similar GLS can be achieved by using high speed counter chromatography which is solely rely on partition coefficient of the solute between mobile and stationary phase, Fahey et al 2003 reported the separation of homologues

glucoraphanin and glucoiberin by partition coefficients, this was achieved by using 1-propanol acetonitrile – saturated aqueous ammonium sulphate–water (1:0.5:1.2:1) in biphasic system with the aqueous phase as the stationary phase while the organic phase as the mobile phase (Fahey et al., 2003).

### 1.3.3 Detection method of GLS

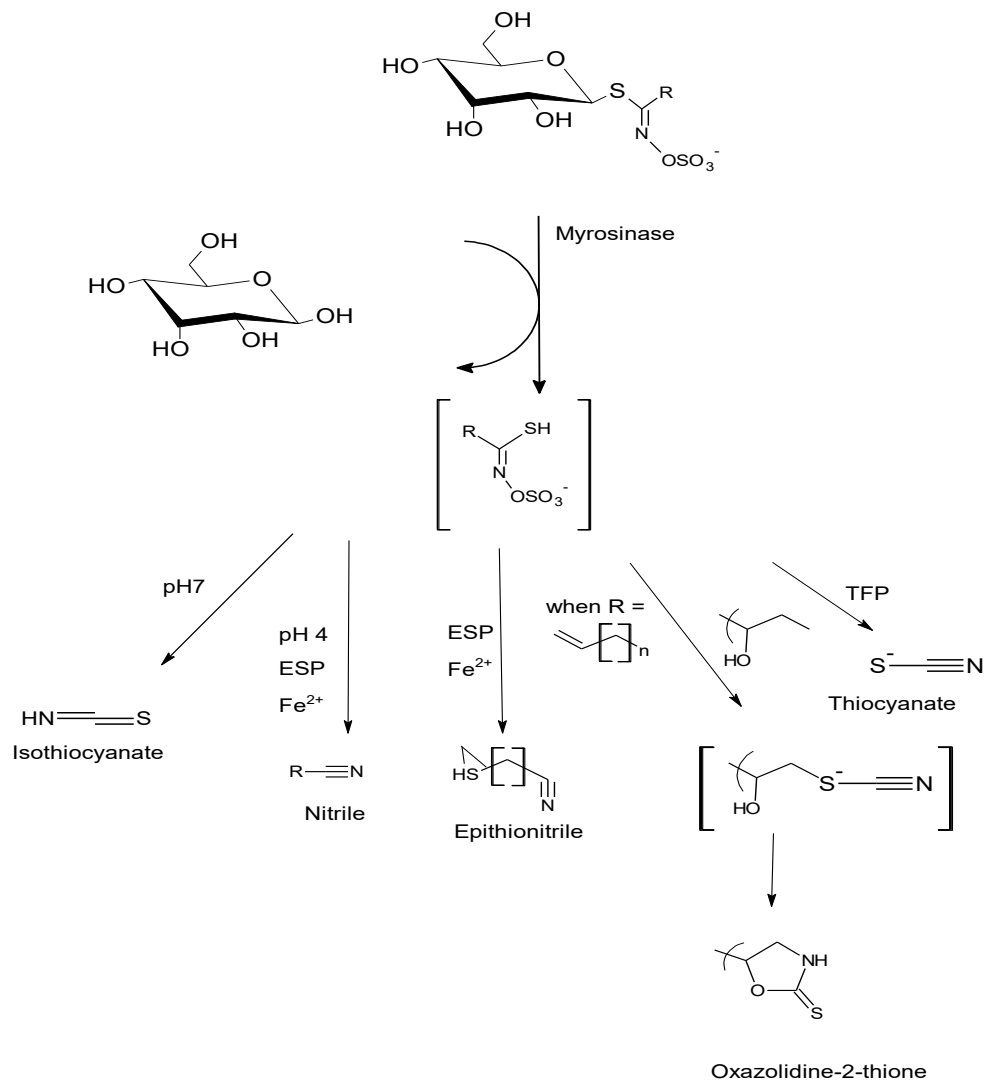
Several analytical methods have been developed; in general these methods can be divided into two either direct determination of intact GLS (total or individual GLS) or indirect measurement of the breakdown products (Lee et al., 2006; Moreno et al., 2006). Upon hydrolysis by myrosinase equimolar amount of glucose is yielded, and methods for the measurement of enzymatically released glucose can be used. These methods include hexokinase coupled to NADH production, glucose oxidase and peroxidase coupled to different coloured dyes such as dianisidine and quinoneimine (Mithen et al., 2000; Moreno et al., 2006; Clarke, 2010). Measurement of desulphoglucosinolates is a common alternative method for GLS analysis; however; this method requires more time due to additional sample processing including extraction, binding to Sephadex, enzymatic desulfation and in the final step elution of desulphoglucosinolates (Mellon et al., 2002). High performance liquid chromatography (HPLC) and gas chromatography (GC) have been used for direct determinations of GLS (Szmigielska and Schoenau, 2000). For individual GLS HPLC separation with UV detection or GC are the most frequently used methods (Thies, 1976; ISO, 1995), for determinations of total GLS in rapeseed and rapeseed meal two official methods; the European community and the ISO methods. The methods involve the use HPLC for individual desulfoglucosinolates followed by their summation (ISO, 1995) and the use of X-ray fluorescence for determination of total sulfur in the rapeseed sample that can be correlated with the total GLS (ISO, 1994). Other methods that have been adopted

for analytical procedure for total GLS rely on treatment of sample with myrosinase followed by determination of released glucose (Koshy et al., 1988) or rely on formation of coloured complexes of GLS with tetrachloropalladate (Thies, 1982) or thymolsulfuric acid (DeClercq and Daun, 1989) after sample preparation and cleanup. Near-infrared reflectance spectroscopy has also been employed and successfully tested for total GLS determinations in a range of *Brassica* seed samples (Velasco and Becker, 1998)

#### **1.4 The hydrolysis of GLS and potential health benefits**

GLSs can be hydrolysed (Figure 1.4) by myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) an enzyme that is present in all cruciferae.





**Fig 1.4** Hydrolysis of GLS by the myrosinase and their different hydrolysis products; ESP = Epithiospecifier protein; TFP = thiocyanate-forming protein (Halkier and Gershenzon, 2006).

Myrosinase is compartmentalised from GLS, located on the tonoplast of the vacuole of myrosin cells (Bones and Rossiter, 1996; Kissen and Bones, 2009). When cruciferous plants are eaten by humans the cells are broken down (damaged) by chewing resulting in the breakdown of tissue and thus the enzyme comes into contact with the GLS and hydrolysis can occur.

As a result of hydrolysis of GLS by myrosinase activity D-glucose moiety is released (Mithen et al., 2000; Al-Turki and Dick, 2003) together with sulphate and one or several bioactive breakdown compounds that include nitrile, ITC, thiocyanate or cyanoepithioalkane are formed. ITCs are known to be bioactive and possess anti-carcinogenic activity by maintaining cell homeostasis and suppressing signalling pathways associated with cell proliferation and inflammation (Rouzaud et al., 2004; Juge et al., 2007). The nature of the degradation product depends on factors such pH, substrate, availability of ferrous ions and the level and activity of specific protein factors such as the epithiospecifier protein (ESP). ITCs are the major products at physiological pH, whereas nitriles are formed at more acid pH (Halkier and Du, 1997; Bones and Rossiter, 2006) although in the plant it will depend on the presence of protein factors such as ESP

#### 1.4.1 Processing of brassica vegetables (Effect of heat treatment on myrosinase)

Treatment of brassica vegetables by domestic processing such as cooking or microwaving may reduce the activity of the plant myrosinase. It was found that myrosinase activity was reduced by 20% after cooking cauliflower (*Brassica oleracea*) for 10 min at 100 °C (Bradfield, 1987), while 0-2.5% of the original activity was detected by (McMillan et al., 1986). In Brussels sprouts after cooking for 9 min at 100 °C, (Bogdan and Lloyd, 1987) myrosinase was completely inactivated while for rapeseed meal (*Brassica napus*) 95°C for 10 min completely inactivated myrosinase. It has been shown that ESP (which redirects GLS degradation away from ITCs toward nitriles and epithionitriles) is thermally unstable as compared to plant myrosinase in broccoli (Traka and Mithen, 2009). It is likely that the majority of domestic cooking will denature both plant myrosinase and ESP leaving intact GLS (Traka and Mithen, 2009). Domestic processing of brassica vegetables results in a loss of GLS through blanching

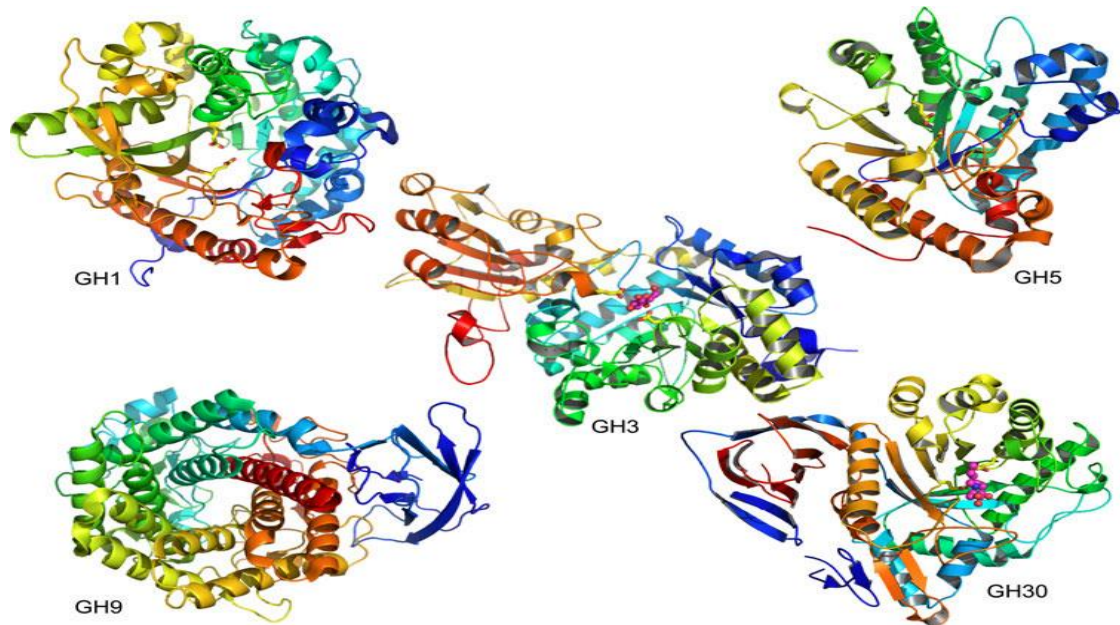
and GLS can be thermally degraded to give non-typical degradation products (Bones and Rossiter, 2006). Thus unless eating raw vegetables it is unlikely that individuals will have the benefit of the original concentration of these compounds. Importantly the consumption of raw vegetables may give rise to nitriles and epithionitriles through the action of ESP and nitrile specifier proteins (NSP) (Bones and Rossiter, 1996; Kissen and Bones, 2009). (NSP) in which case inactivation of myrosinase and associated proteins may be of benefit.

### 1.5 Classification of $\beta$ -glucosidase

Due to the nature of the abundance of nonreducing terminal  $\beta$ -linked D-glucosyl residues which has led to the allocation of the E.C. numbers of many enzymes which hydrolyse their glycosidic bond, the examples are amygdalin hydrolase (3.2.1.117), vicianin  $\beta$ -glucosidase (3.2.1.119), glucosyl ceramidases or glucocerebrosidases (3.2.1.45), glucan 1,4- $\beta$ -glucosidases (3.2.1.58) prunasin hydrolase (3.2.1.118), coniferin  $\beta$ -glucosidase (3.2.1.126), glucan 1,3- $\beta$ -glucosidases (3.2.1.74), raucassicine  $\beta$ -glucosidase (3.2.1.125) strictosidine  $\beta$ -glucosidase (3.2.1.105) and steryl- $\beta$ -glucosidase (3.2.1.104) as well as some other  $\beta$ -glucosidases which they exhibit an extra activities like  $\beta$ -D-mannosidase (3.2.1.25),  $\beta$ -D-galactosidase (3.2.1.23),  $\beta$ -D-fucosidase (3.2.1.38) and  $\beta$ -disaccharidase activities such as  $\beta$ -apiosyl- $\beta$ -D-glucosidase (3.2.1.161) (Ketudat Cairns and Esen, 2010). Glucosidases are a varied group of hydrolases that have been classified according to different criteria. There is no single well defined method for this classification (Bhatia et al., 2002). Basically two methods have been suggested; the first one on the bases of substrate specificity, while the second method on the amino acid sequence (Henrissat and Bairoch, 1996). In the former enzymes are classified into three categories; the first called aryl glucosidase which hydrolyse aryl glucosides; the second is known as the real (or true) cellobiases

where cellobiose is hydrolysed and glucose is released; the last category is called broad substrate specificity enzymes that hydrolyse a wide range of substrates. The last category contains most of  $\beta$ -glucosidases which have been characterised so far. Early classification was proposed according to the available sequence, this scheme grouped the enzyme into two types; Type I and Type II  $\beta$ -glucosidases (Beguin, 1990; Bhatia et al., 2002). Around 2000 glycosyl hydrolases are proposed (Herissat, 1991; Henrissat and Davies, 1997). So far 129 families are proposed in the frequently updated Carbohydrate Active enZYme (CAZY) Web site (<http://www.cazy.org/Glycoside-Hydrolases.html>).  $\beta$ -Glucosidases are placed in family 1 or 3 of glycosyl hydrolases Figure 1.5 ; Family 1 which include around sixty two  $\beta$ -glucosidases (from mammals, archaeobacteria, plants) in addition to 6-phosphoglycosidases and thioglucosidases. The exception is acid beta glucosidase (glucosylceramidase) that are placed in family 30. The majority of family 1 enzymes show significant beta galactosidase activity (Bhatia et al., 2002), the three dimensional structure is known for most members of family 1. These include cyanogenic  $\beta$ -glucosidase (also known as linamarase) which extracted from *Trifolium repens* (Tolley et al., 1993), the *Paenibacillus polymyxa* (formerly known *Bacillus polymyxa*)  $\beta$ -glucosidase (Sanz-Aparicio et al., 1998; González-Blasco et al., 2000). The sub-species *alkalophilus* of *Bacillus circulans* (Hakulinen et al., 2000), *Thermus nonproteolyticus* (Wang et al., 2003), *Lactococcus lactis* (Wiesmann et al., 1995), *Thermotoga maritime* (Zechel et al., 2003), Cel B from *Pyrococcus furiosus* (Kaper et al., 2000), *Sinapis alba* (Burmeister et al., 1997), the *Zea mays* (maize) ZmGlu1 and Zm-p60 (Czjzek et al., 2000; Zouhar et al., 2001), *Sorghum bicolor* (Verdoucq et al., 2004), *Triticum aestivum* (Sue et al., 2006), *Brevicoryne brassicae* (Husebye et al., 2005). The white-rot fungus (basidiomycete) *Phanerochaete chrysosporium* (Nijikken et al., 2007) and more recently from the anaerobe bacterium *Clostridium cellulovorans*, the fungus *Trichoderma reesei*, and from the termite *Neotermes koshunensis* (Jeng et al., 2011). The complex of ZMGLu1

with the non-hydrolysable inhibitor p-nitrophenyl- $\beta$ -D-thioglucoside was proposed (Czjzek et al., 2001).



**Fig 1.5** Structure of  $\beta$ -glucosidase family groups; GH= glucohydrolase; GH1= *ZeaMays*; GH3= *Hordeum vulgare* Exo I  $\beta$ -glucan glucohydrolase; GH5= *Candida albicans* exo- $\beta$ -(1,3)-glucanase exoglucanase; GH9= *Vibrio parahaemolyticus*, putative exoglucanase; GH30= *Homo sapiens*, acid  $\beta$ -glucosidase/glucoocerebrosidase. (Ketudat Cairns and Esen, 2010).

### 1.5.1 Microbial degradation of GLS

Many studies have shown that after consumption of cooked cruciferous vegetables (where there is no myrosinase activity) ITCs can be detected in urine but in small amounts, between 10% and 30% that would have occurred with functional plant myrosinase. The conversion of GLS to ITCs occurs probably due to the action of colonic microflora (Shapiro et al., 1998; Getahun and Chung, 1999; Conaway et al., 2000; Rouzaud et al., 2004). Antibiotic therapy reduces the gut microflora resulting in a reduction of ITCs (Shapiro et al., 1998). ITCs are detected in human tissues such as

blood and prostate as glutathione derivatives, providing another evidence for the importance of the gut microflora in the conversion of GLS to ITCs (Traka and Mithen, 2009). Greer and Deeney (1959) were the first to propose that the human intestinal microflora is able to hydrolyse GLS *in vivo*. Initially they first looked for myrosinase activity in the body fluids and tissues and eventually suggested that intestinal microflora may be able to produce myrosinase (Greer and Deeney, 1959). Later an *in vitro* myrosinase –like activity in rat and fowl (Marangos and Hill, 1974), faecal microflora was proven. The isolation of bacterial strains that have myrosinase like activity from faecal microflora were made by several authors from human (Oginsky et al., 1965; Tani et al., 1974) and from fowl (Miguchi et al., 1974) that were able to metabolise 2-hydroxy-3-butenyl or 2-propenyl *in vitro*. The link between hydrolysis of GLS in the gut was made by (Rabot et al., 1993). Subsequently, myrosinase activity has also been demonstrated in human gut microflora, (Greer and Deeney, 1959) and was assessed by detecting the presence of 5-vinyloxazolidine-2-thione (a progoitrin derivative) in the human urine after the ingestion of pure 2-hydroxy-3-butenyl, and in the culture media of human *Enterobacteriaceae* (Oginsky et al., 1965) strains which were incubated with the 2-hydroxy-3-butenyl and were able to convert it into goitrin *in vitro* (Oginsky et al., 1965; Elfoul et al., 2001; Krul et al., 2002). To date bacterial myrosinase like activity is considered to be under investigated (Nugon-Baudon and Rabot, 1994). The only characterised microbial myrosinase is from *Enterobacter cloacae* strain 506 (Tani et al., 1974) that has been purified to homogeneity, however; no functional genomics analysis has been reported (Traka and Mithen, 2009). Several papers reported the production of myrosinase from fungi like *Aspergillus sp.* (Ohtsuru et al., 1973; Sakorn et al., 1999; Rakariyatham, 2005). Like many other vegetables cruciferous vegetables contain a number of nutrients and phytochemicals that possess cancer chemopreventive properties that include fibre, carotenoids, folate and chlorophyll (Higdon et al., 2007). However GLS content of these vegetable set them apart from the others (Vallejo et al., 2003). Several epidemiological studies (Graham et

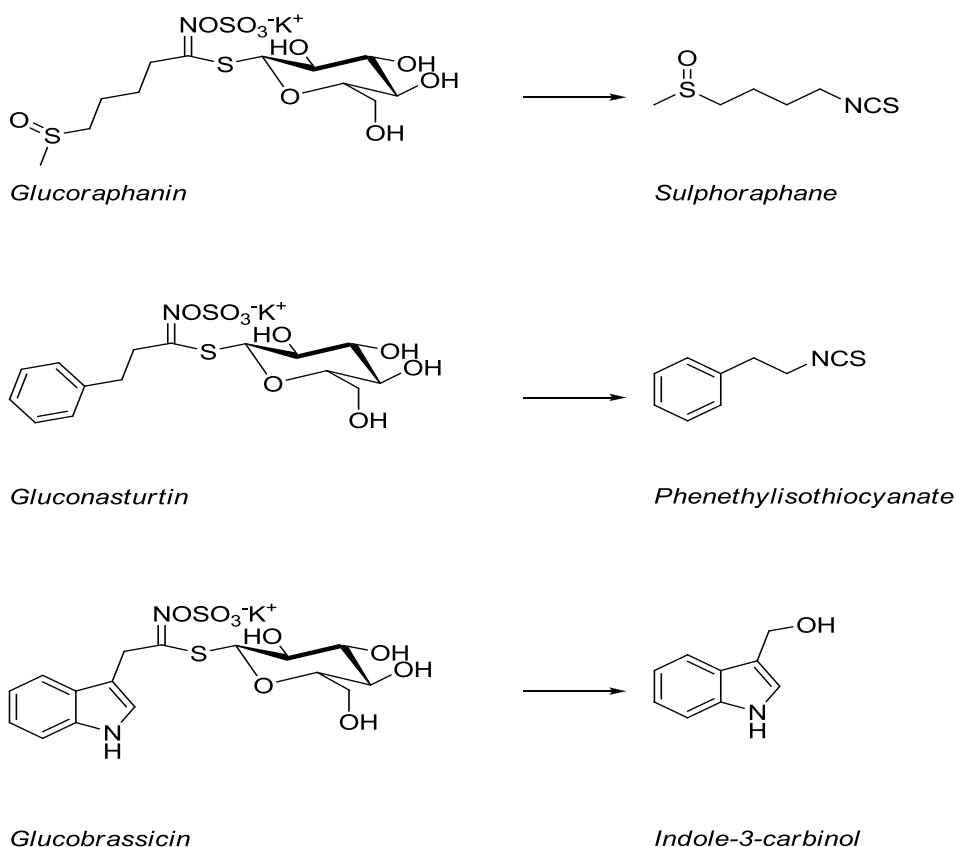
al., 1978; Haenszel, 1980) have linked the consumption of cruciferous vegetables with a lower risk of tumour formation in digestive tract of human (colon, stomach and rectum). Evidence from both prospective cohort studies and retrospective case-control studies (Traka and Mithen, 2009) proposed that there is an inverse link between consumption of cruciferous vegetables and the risk of bladder (Zhao, 2007), breast (Fowke et al., 2003; Ambrosone et al., 2004), stomach (Hansson et al., 1994), colorectal (Lin et al., 1998; Seow et al., 2002), lung (London et al., 2000; Spitz, 2000; Zhao, 2001; Wang, 2004) and prostate cancer (Cohen et al., 2000; Giovannucci et al., 2003; Kirsh et al., 2007) and myocardial infarction (Cornelis et al., 2007). It has been reported that ITCs are potent anticarcinogenic compounds *in vivo* and *in vitro* (Zhang and Talalay, 1998).

### 1.5.2 Biological effect of GLS breakdown products

Foods that physiologically possess active component provide health benefit beyond their nutritional basis. These foods are known as functional foods (Moreno et al., 2006). *Brassica* vegetables such as broccoli, parsnip, Brussels sprouts, Chinese cabbage, radish, horseradish, wasabi, white mustard, watercress and cauliflower contain the bioactive component GLS. Consumption or intake of these vegetables is more strongly associated with cancer protection than consumption of vegetables in general (Finley, 2005). Recently the use of natural products for cancer prevention has received considerable attention. GLS are among the most studied bioactive compounds in *Brassica* vegetables (Fenwick et al., 1983). After the hydrolysis of GLS by myrosinase the potential components such as ITCs and indole are produced (Finley, 2005; Moreno et al., 2006). Consistent data have shown that the ITCs (chemoprotective agents) derived from *Brassica* vegetables influence carcinogenesis during many steps of cancer including initiation and promotion phases of cancer development (Johnson,

2002). Accumulating evidence indicates that ITCs are involved in inhibition of phase I and induction of phase II metabolism enzymes (Traka and Mithen, 2009). Enzymes of phase I are monooxygenases which metabolize lipophilic procarcinogens and often convert them to epoxides which are highly carcinogenic (Johnson et al., 1994). The most studied ITCs and hydrolysis products (Figure 1.6) implicated in human nutrition are sulforaphane, phenyl ITC and indole-3-carbinol (Cartea and Velasco, 2008), and 2-propenyl ITC (Telang et al., 1997; Zhang and Talalay, 1998). ITCs such as phenyl ITC, benzyl ITC and sulforaphane modify the balance of phase I (such as the cytochrome P450 family) and phase II (such as glutathione-S-transferase family) xenobiotic metabolizing enzymes which are expressed in liver and in the colon. Phase II enzymes metabolize the products of phase I and form inactive water soluble compounds that are excreted in urine (Johnson, 2002) and may prompt the liver to produce detoxification enzyme (Phase II enzymes) that help neutralise cancer-causing substances (Wang, 2004; Hwang and Jeffery, 2005; Cornblatt et al., 2007). Sulforaphane serves as an indirect antioxidant and induces cell cycle arrest followed by apoptosis. It may prevent tumour growth by blocking the cell cycle and promoting apoptosis (Thornalley, 2002; Lund, 2003; Keum et al., 2004). Furthermore it has been shown to have a potential antibiotic effect against *Helicobacter pylori* which causes gastritis and stomach cancer (Fahey et al., 2002; Cartea and Velasco, 2008).





**Fig 1.6** The most studied GLS and their hydrolysis products reproduced from (Cartea and Velasco, 2008); A aliphatic, B aromatic, C indole GLS.

A recent study using NMR (Chu, 2009) compared the metabolism of 2-propenylGLS by *Lactobacillus agilis* R16 and *Citrobacter sp.* (used in this project) has shown two different mechanisms. ITCs were generated *in vivo* when *L. Agilis* was grown on a medium containing 2-propenylGLS as a substrate. However, no active myrosinase could be detected from lysed cells, In stark contrast the preliminary 1D  $^1\text{H}$  NMR (D<sub>2</sub>O) analysis showed that *Citrobacter sp.* degraded 2-propenylGSL within 28 h into unknown metabolites as the spectrum was not comparable to allylcyanide (nitrile), allylamine or ITC standards which have been previously reported (Combourieu et al., 2001; Chu, 2009). Inoculation of germ free rats with *Bacteroides thetaiomicron* (II8) caused a considerable increase in the excretion of GLS metabolites and a correspondingly lower faecal excretion of intact 2-propenylGLS (Johnson, 2002). The production of 2-propenyl ITC from 2-propenylGLS was investigated in a dynamic *in*

*vitro* large intestinal model inoculated with a complex microflora of human origin. Analysis showed that both 2-propenyl ITC in the lumen and dialysis fluid of the model has shown that 2-propenyl ITC peaks after 9 and 12 h after the addition of 2-propenylGLS. The amount of 2-propenyl ITC was equivalent to 10-30% of administered 2-propenyl GLS, and it was suggested AITC may be converted into other unknown metabolites (Oginsky et al., 1965; Elfoul et al., 2001; Krul et al., 2002).

#### 1.5.2.1 Antimicrobial effects

Due to their biological activity GLS degradation products ITC became of interest, many of these products have biocidal activity against a wide of different organisms such as bacteria, fungi, plants and insects (Vaughn and Berhow, 2005). In early 1900s; the antimicrobial effects of the extracts of cruciferous plant were reported however interest in their use as food preservatives has only grown recently (Delaquis and Sholberg, 1997). Attention has been focused particularly on 2-propenyl ITC that was isolated from horseradish (*Armoracia lapathifolia*), it constitutes of 90% volatile essential oils, it has shown consistent antimicrobial activity in both vapour and liquid forms (Kyung and Fleming, 1997; Shofran et al., 1998; Ward et al., 1998). This poorly water soluble compound is more potent against microorganisms in the gaseous state, this is indicated by lower values for 50% inhibitory concentration, interestingly pathogenic bacteria is strongly inhibited while lactic acid bacteria is largely unaffected (Ward et al., 1998). The bactericidal activities of 2-propenyl ITC was tested against a variety of bacteria and it appeared to be effective during all growth stages and not only in exponential growth phase (Lin, Preston, et al., 2000). This compound was also been reported to have bacteriostatic and bactericidal agents in the preservation of precooked roast beef and lettuce and its under investigation for use against pathogens to control

contamination of prepacked salads as these can still contain bacteria despite washing with chlorinated water (Ward et al., 1998; Lin, Kim, et al., 2000)

Several authors reported that isothiocyanates possess antimicrobial and biocidal activity against pathogenic microbes (bacteria and fungi), soil-borne pests such as nematodes and many insects (Bennett and Wallsgrave, 1994; Vig et al., 2009). Many literature reports have proposed the antimicrobial activity of GLs (Lazzeri et al., 1993; Brown and Morra, 1996, 1997; Sarwar et al., 1998; Smolinska et al., 2003), however, there is a lack of knowledge of the mechanism of this process (Vig et al., 2009). It has been proposed that the hydrolytic products may inactivate several intracellular enzymes of the pathogens, this may be done by oxidative breakdown of the bridges between thiol groups (S-S-) that are found in enzymes, or by obstruction of ATP synthesis in the bacterial cell by the action of oxidative phosphorylation in the mitochondria (Zsolnai, 1966; Kojima and Ogawa, 1971). Mucete et al. 2006 investigated the antibacterial activity of ITCs extracted from horseradish (*Armoracia rusticana* Lam - Fam. *Cruciferae*) roots against six microorganisms, these were *Escherichia coli*, *Candida albicans*, *Bacillus subtilis*, *Staphylococcus aureus*, *Agrobacterium tumefaciens* and *Rhizopus nigricans*. They found that all tested microbes were sensitive to horseradish ITCs and the study recommended the utilization of these compounds in food and medicine domain (Mucete et al., 2006). The minimum inhibitory concentration effect of degraded 2-propenylGLS products 2-propenyl ITC, 2-propenyl cyanide, 1-cyano-2,3-epithiopropene, and propenyl thiocyanate were tested against 9 bacterial species and 8 yeast species, it was found that 2-propenyl cyanide at 1,000 ppm did not inhibit any of the bacteria or yeasts tested. 2-propenyl ITC showed inhibitory effects in the range of 50-1000 ppm for bacteria and 1-4 ppm for nonxerotolerant yeasts and against xerotolerant yeasts at 50 ppm (Shofran et al., 1998). Sulforaphane of broccoli sprouts was found to effectively inhibit *Helicobacter pylori*, and also killed multiple strains including those that are known to be antibiotic resistant. Furthermore administration of sulforaphane for five days eradicated this bacteria from 8 out of 11 xenografts of

human gastric tissue implanted in immune compromised mice (Fahey et al., 2002; Haristoy et al., 2003). *In vitro* antibacterial activity of GLS and their enzymatic hydrolysis product was evaluated against different gram reactions bacteria isolated from the human intestinal tract; the gram positive were *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus saprophyticus* while the gram negative were *Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Enterobacter hormaechei*, *E. coli* (two strains), *Hafnia alvei*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Stenotrophomonas maltophilia*, the study found that both DL-sulforaphane (SFN) and benzyl ITC (BITC) had significant antimicrobial effects on both gram positive and gram negative tested pathogens. The study suggested the use of these compound as an alternative or with combination the commercial antibiotics in order to control infectious diseases (Aires et al., 2009). Gram positive bacteria are more susceptible to ITCs than gram negative bacteria (Smelt et al., 1989). Benzyl ITCs as an antibiotic have been used to treat infections of urinary and respiratory tracts (Mennicke et al., 1988). Some microbes may degrade the inhibitory compounds to avoid toxicity (Vig et al., 2009). Benzyl ITCs were identified as the main antibacterial component found in *Salvadora persica* root chewing sticks; it is more effective in killing gram negative periodontal pathogens bacteria such as *Aggregatibacter actinomycetemcomitans* HK 1519 and *Porphyromonas gingivalis* (ATCC 33277) and other pathogenic gram negative bacteria such as *Haemophilus influenzae*, *Salmonella enterica* and *Pseudomonas aeruginosa*. The study concluded that the root extracts of *Salvadora persica* might be useful as an adjunct in treatment of periodontal diseases (Sofrata et al., 2011).

## 1.6 Beta Glucosidase ( $\beta$ -S-glucosidases and $\beta$ -O-glucosidase)

### 1.6.1 Occurrence and importance

The  $\beta$ -glucosidases ( $\beta$ -D-glucopyranoside glucohydrolases, (E.C. 3.2.1.21) are enzymes which hydrolyze glycosidic linkages in aryl and alkyl  $\beta$ -glycosides (e.g. methyl- $\beta$ -D-glucoside, p-nitrophenyl- $\beta$ -D-glucoside) ,disaccharides and short chain oligosaccharides (e.g. cellobiose) to release non reducing terminal glucosyl residues. The affinity of these enzymes towards its substrate(s) is dependent on the nature of the enzyme source (Woodward and Wiseman, 1982). Many of these enzymes have shown also synthetic activity via reverse hydrolysis or transglycosylation (Crout and Vic, 1998). The enzyme occurs ubiquitously in all living organisms; in plants, insect, mammals (including human) and microorganisms (bacteria, fungi, archaea) and some viruses also encode glucosidases. They perform different functions in these organisms (Woodward and Wiseman, 1982; Ketudat Cairns and Esen, 2010; Naumoff, 2011). The widely distributed enzyme is vital for several biological processes like biomass conversion (cellulolysis) by microbes and insects, and also have the potential for biotechnological applications (Langston et al., 2006) and catabolism of exogenous glucosides and glycolipids in animals (Ketudat Cairns and Esen, 2010). Microbial  $\beta$ -glucosidase is key enzyme in industrial flavour improvement to release aromatic compounds from glucosidic precursors that are present in fruits and in the fermented products (Gueguen et al., 1996). They can be used also in improvement of the bitter taste of citrus fruit that formed due to naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside). This glucosidic compound is hydrolyzed by  $\alpha$ -rhamnosidase and  $\beta$ -glucosidase. The liberation of the flavouring compound terpenol, benzyl and phenylethyl alcohols from their precursor glucosides is due to the presence of  $\beta$ -glucosidase in yeast (*Debaryomyces hansenii*) (Rosi et al., 1994). The release of

cyanides from cyanogenic glucosides is linked with plant and insect  $\beta$ -glucosidases which form part of a defence process in these systems. Plant myrosinase is also involved in phytohormone activation (Smith and Van Staden, 1978; Schliemann W, 1984; Matsuzaki and Koiwai, 1986; Wiese and Grambow, 1986; Brzobohaty et al., 1993; Ketudat Cairns and Esen, 2010), seed development and metabolism of pigment and catabolism of cell wall (Ketudat Cairns and Esen, 2010). The lack of a human membrane bound lysosomal acid glucosidase is linked with Gaucher's disease, this making cells unable to hydrolyse glycosylceramides and as a result enlargement of organs such as spleen, liver and lymph nodes occurred due to accumulation of glycosylceramides in lysosomes of reticuloendothelial cells (Winkelmann, 1972). Patients with such disease administered intravenous injection of pure glucosidase from human placenta (Esen, 1993). The hydrolysis of phenolic and phytoestrogen (flavonoid and isoflavonoid) which occur naturally in vegetables, fruits, tea and soybeans is due to  $\beta$ -glucosidase activity, the released aglycone moiety has potent biological activity and is used as an anticancer agent. They are also used in food industry and in general biomedical research (Bhatia et al., 2002). The hydrolysis of phloridzin and the liberation of the aglycone moiety, which is precursor of melanin which known as dark hair colour promoters and in reduction of skin cancer (Bhatia et al., 2002). Isoflavones are diphenol like compounds, their structure and function are similar to estrogen of the human, estradiol, but are much less effective than estradiol (Setchell, 1998; Setchell and Cassidy, 1999). Due to this resemblance isoflavones were suggested to have protective effects against many types of hormone dependent diseases (Uzzan and Abuza, 2004). In the intestinal tract the bacterial  $\beta$ -glucosidase play a role in hydrolysing the non absorbed  $\beta$ -glucoside form of isoflavone such as those found in soy proteins which need to be hydrolysed for bioavailability and further metabolism (Setchell et al., 2002). Following the release of aglycones a further metabolism of genistein and dadzein takes place (Donkor and Shah, 2008). The absence of glycosides in the plasma has been confirmed (Setchell et al., 2002) and the study

concluded that isoflavone glycoside is not absorbed intact but hydrolysed by enzymes mainly  $\beta$ -glucosidase released by intestinal microflora. This finding was in agreement with other research groups (Slavin et al., 1998) reporting that glycosides are only hydrolysed by the action of intestinal enzymes and mainly  $\beta$ -glucosidase (from *Escherichia coli* and *Clostridia*) into aglycones which are readily absorbed in the intestine. Two strains of *E. coli* were isolated from human intestine and their ability to hydrolyse isoflavone glycoside genistin and daidzin were confirmed (Hur et al., 2000).

#### 1.6.2 Plant $\beta$ -thioglucosidase glucohydrolases (myrosinases, EC 3.2.3.1)

The first observation of myrosinase activity was in 1840 in mustard seeds (Pihakaski and Pihakaski, 1978), It occurs in all Brassicaceae (Cruciferae) species examined and has also been found in Capparaceae, Caricaceae, Resedaceae, Salvodoraceae, Bretschneideraceae, Tovariaceae, Drypetes (Euphorbiaceae), Gyrostemonaceae, Akaniaceae, Bataceae, Limnanthaceae, Moringaceae, Pentadiplantdraceae and Tropaeolaceae (Rodman, 1991). As mentioned earlier plant  $\beta$ -thioglucosidase belongs to the family 1, the unique class of glycosidases which catalyze the hydrolysis of S-linked glucosides (anionic 1-thio- $\beta$ -D-glucoside N-hydroxysulphates), constituting GLS that occur mainly in the *Brassicales* (Sang et al., 1984; Rask et al., 2000; Fahey et al., 2001; Zhang et al., 2008). In Cruciferae myrosinase appears always to be accompanied by GLS (one or more GLS), it acts on thioglucosides such as GLS (e.g., 2-propenyl) releasing glucose and sulphate and produces ITCs, nitrile, thiocyanates amine, epithionitrile or oxazolidine-2-thione (Pihakaski and Pihakaski, 1978; Bones, 1990; Rask et al., 2000). Myrosinases are present in multiple forms and more than 25 myrosinase isoenzymes have been predicted in *Brassica napus* (oilseed rape). Isolation and cloning of cDNA encoding myrosinase has enabled more accurate studies of these isoenzymes and their distribution in tissues (Falk et al., 1992, 1995;

Xue et al., 1992, 1995; Chadchawan et al., 1993; Lenman et al., 1993; Machlin et al., 1993; Thangstad et al., 1993; Xue and Rask, 1995). Studies on sequence homology of myrosinases of oilseed rape and *Sinapis alba* (white mustard) showed that Brassicaceae myrosinase is encoded by a gene family comprising at least three subfamilies, denoted MA (Myr1), MB (Myr2) and MC (Rask et al., 2000). Six myrosinase genes have been identified in *Arabidopsis thaliana* among which TGG1, TGG2, TGG4, and TGG5 are functional, while TGG3 and TGG6 are predicted to be pseudogenes due to multiple frame-shift mutations in the coding region (Zhang et al., 2002; Wang et al., 2009). Myrosinase is the only known  $\beta$ -glucosidase which has been found to have many activities, including cell wall lignification (Dharmawardhana et al., 1995; Escamilla-Treviño et al., 2006), degradation of cell wall in endosperm during germination (Leah et al., 1995), signalling, phytohormones activation (hydrolysis of glucosides of cytokinins, abscisic acid, gibberellins and auxins) (Nowacki and Bandurski, 1980; Schliemann W, 1984; Wiese and Grambow, 1986; Brzobohaty et al., 1993). It is also important in plant defence against some pathogens due to the release of thiocyanates, coumarins, hydroxamic acids and cyanide from glucoside (Nisius, 1988; Poulton, 1990; Jones et al., 2001; Halkier and Gershenzon, 2006; Suzuki et al., 2006). Different locations of plant glucosylhydrolase have been reported these include cell wall, vacuole, cytoplasm, endoplasmic reticulum and peroxisomes (Minic, 2008). Plant myrosinase has been well investigated and received much attention in terms of its characterisation (Bones and Rossiter, 1996). It is an abundant protein in some brassica seeds and can be easily extracted and partially purified by simple ammonium sulphate fractionation followed by ion-exchange (Bones and Rossiter, 2006). Early work has shown that ascorbate can activate the enzyme by acting as a catalytic base (Burmeister et al., 2000; Bones and Rossiter, 2006). 1mM L-ascorbate increased the activity of the mustard enzyme (2-propenylGLS was used as substrate) >25 fold but the activity did not increase when *p*-nitrophenyl- $\beta$ -D-glucoside was used (Botti et al., 1995). The kinetic properties differ widely species to species (James and Rossiter, 1991), and



large variations have also been reported in the specific activity of various cruciferous plant myrosinases (Fahey et al., 2001). These range from 0.3 to 10.5  $\mu\text{mol}/\text{min}/\text{mg}$  protein in twelve partially purified enzyme of cruciferous plants (Andrew et al., 1984). Myrosinase is a dimeric protein with a range of molecular weights from 62-75 kDa (Radojicic et al., 2008). The molecular weight of *Brassica napus* and *Sinapis alba* is 120,000-150,000. Only four substrates (2-propenylGLS, *p*-nitrophenyl- $\beta$ -D-glucoside, *O*-nitrophenyl- $\beta$ -D-glucoside and benzylGLS) out of 29 tested glycosides were hydrolysed by *Lepidium sativum* myrosinase but no activity was detected from methyl or phenyl  $\beta$ -glucosides.  $\alpha$ -Glucosides were also inactive. The hydrolytic rate of the tested GLS substrates was similar regardless of the nature of the side chain (Botti et al., 1995).

### 1.6.3 Human $\beta$ -glucosidase

Amongst the  $\beta$ -glucosidases, this includes the family 1 lactase-phloridzin hydrolase and cytoplasmic  $\beta$ -glucosidases, GH 30 including bile acid  $\beta$ -glucosidase and human acid  $\beta$ -glucosidase. These enzymes may play role in the glycolipids metabolism and utikusation of dietary glucosides. Another group of GH family 1 is thought to play a role in cell signalling (Ketudat Cairns and Esen, 2010). So far five proteins of family 1 are known in human; klotho ( $\alpha$  and  $\beta$ ) and Klotho lactase phloridzin hydrolase related protein. The latter is known to have broad specificity and was known as cytoplasmic  $\beta$ -glucosidase and recently was given additional name the Klotho related protein (Hayashi et al., 2007). It has been found in high levels in hepatocytes and the brush border of the epithelial cells and has been shown to metabolise plant dietary flavonoids (Ketudat Cairns and Esen, 2010). In addition to cytoplasmic  $\beta$ -glucosidase and lactase-phloridzin hydrolase, the latter has two activities as  $\beta$ -glucosidase toward exogenous glucoside such as phloridzin, and  $\beta$ -galactosidase activity toward lactose. This enzyme

is found in intestines and involved in food digestion (Tribolo et al., 2007). Two different  $\beta$ -glucosidases have been characterised; the cytosolic which found mainly in the liver kidney and intestines. The lysosomal  $\beta$ -glucosidase (Beutler, 1992). The natural substrates of the acid  $\beta$ -glucosidase in human are sphingosylglucoside and glucosylceramide (Esen, 1993).

#### 1.6.4 Aphid myrosinase

The only insect glycoside hydrolase gene was identified in *Drosophila melanogaster*. Several insects adapted glucoside hydrolase from the plants they fed on for digestive (such as  $\beta$ -glucosidase of insect larvae) or protection purposes (Zagrobelny et al., 2008). Myrosinase was isolated from *Brevicoryne brassicae* (cabbage aphid) which feeds on cabbage (Jones et al., 2001). Insect myrosinase does not require ascorbate for activity unlike most plant myrosinases (Bones and Rossiter, 2006). It has been reported that aphid myrosinase may have a dual function acting both as an oxidase and thioglucosidase (Frédéric et al., 2002). However further investigation is required as there is nothing in the structure of this protein to indicate any oxidative catalytic properties (Husebye et al., 2005).

#### 1.6.5 Microbial $\beta$ - glucosidase and myrosinase

The conversion of cellulose ( $\beta$ -glucan) the most abundant biological macromolecule in the world is dependent on three enzymes; endo- $\beta$ -glucanases such as cellulase which hydrolyses cellulose to cellobiose; the second enzyme is exo- $\beta$ -glucanases such as cellobiosidases; the third enzyme is  $\beta$ -glucosidase which hydrolyses cellobiose into two glucose molecules (Ketudat Cairns and Esen, 2010). Bacterial and fungal  $\beta$ -

glucosidases are mainly a part of cellulase enzyme system; its duty is to hydrolyse short chain oligosaccharides and cellobiose (which is formed as a result of the synergistic action of endoglucanases and cellobiohydrolases) into glucose (Bhatia et al., 2002). The produced glucose may inhibit the  $\beta$ -glucosidase, the activity decrease as the length of glucose chain increases and as a result cellobiose accumulates thus cellulase will be inhibited; therefore the hydrolysis of cellulose will be at reduced levels (Bisaria and Mishra, 1989; Kubicek et al., 1993). The hydrolysis of genistin and diadzin to genistein and diadzein respectively with liberation of glucose, was confirmed to be due to a  $\beta$ -glucosidase enzyme of *Lactobacillus casei* subspecies *rhamnosus* and as a result the bitter and astringent isoflavonoid glucosides are reduced from the cooked soybean syrup (Matsuda et al., 1994). Intestinal conversion of non absorbed isoflavones into bioactive forms is linked with action of bacterial  $\beta$ -glucosidase, the reactions which presumably take place in colon include reduction, dehydroxylation, demethylation and C-ring cleavage. Types of intestinal bacteria involved in such conversion is not well known; however, strains of *Bifidobacterium* (Tochikura et al., 1986; Tsangalis et al., 2002; Hsu et al., 2005) are the only mostly studied for  $\beta$ -glucosidase production which lead to transformation of isoflavones to bioactive and bioavailable forms (Tsangalis et al., 2002, 2004; Wei et al., 2007). The reduction of viscosity is due to gellan lyase and the extracellular  $\beta$ -glucosidase activity which are produced by *Bacillus* sp and they have been shown to catalyse the cleavage of glycosylrhamnosyl glucose (trisaccharides). The glucose and rhamnosyl-glucose are released (Bhatia et al., 2002). Brabban and Edwards 1994 reported the isolation of eight different environmental microbes of which were able of growing on M9 medium contained 2-propenylGLS as the only carbon source. Among these isolates seven gram positive bacteria; two species are belongs to the genus *Streptomyces*, four species belong to genus *Bacillus*, one isolate belongs to *Staphylococcus* in addition to unknown fungus. These organisms were also able to degrade rapemeal GLS. Yeast and bacterial  $\beta$ -glucosidase are mainly intracellular (Brabban and Edwards, 1994).

Among of the earliest purified bacterial  $\beta$ -glucosidase is that of *Agrobacterium faecalis* which was characterised and expressed in *E.coli* (Day and Withers, 1986; Wakarchuk et al., 1986). It is a dimer of 50 kDa monomers with high specificity for cellobiose (Trimbur et al., 1992). The  $\beta$ -glucosidase of the anaerobic bacteria *Clostridium thermocellum* has been characterised, it was suggested that the enzyme is located in the periplasmic space; the enzyme has optimum pH at 6.0 and temperature at 65 °C. The enzyme is stable for 7h at 60 °C; the  $K_m$  values of *p*-nitrophenyl- $\beta$ -D-glucoside and cellobiose are 2.8 and 83 mM respectively (Cooney CL, Wang DIC, Wang SD, Gordon J, 1978).  $\beta$ -glucosidase of *Acetivibrio cellulolyticus* which was secreted in the culture medium, the molecular weight of this enzyme was estimated to be 81000 kDa (Saddler and Khan, 1980). *Bacteroides succinogenes* produces  $\beta$ -glucosidase with  $K_m$  4.3 mM of cellobiose at optimum pH at 6.8 (Groleau D, 1981), the aryl- $\beta$ -glucosidase of *Erwinia herbicola*, this enzyme is degrading the glucoside (phloridzin) of the plant (such as pear and apple trees) which the bacteria invade and as a result the aglycone which is toxic to the bacteria is released. The enzyme was found to have better affinity ( $K_m$ ) toward phloridzin (0.135 mM) than *p*NPG but no activity towards arbutin or salicin (Garibaldi and Gibbins, 1975). Many bacterial strains are able to degrade GLS and those partly characterised include *Escherichia coli* (Oginsky et al., 1965; Rabot et al., 1993), *Bacteroides thetaiotaomicron* (II8) (Rabot et al., 1995) which is isolated from human faeces by Rabot et al., 1995 and characterised by Elfoul et al., 2001, *Bacteroides vulgatus* (Rabot et al., 1993), *Bifidobacterium longum* (Cheng et al., 2004), *Enterobacter cloacae* (Tani et al., 1974), and *Lactobacillus agilis* (Palop et al., 1995), *Bacillus cereus* (Huber et al., 1983). The three strains of *Bifidobacterium* (*B. pseudocatenulatum*, *B. adolescentis* and *B. longum*) (Cheng et al., 2004) shown ability to digest 2-propenylGLS and glucotropaeolin (benzylGLS *in vitro* within 24-48h of cultivation but declined at medium of pH from (7.1 to 5.2). Myrosinase activity from sonicated cell free extracts of *B. adolescentis* increased in the presence of 0.5 mM ascorbic acid suggesting that the addition of ascorbate may be an activator of the

bacterial myrosinase. The study suggested that the three species of *Bifidobacterium* could be involved in digestive degradation of GLS in the human intestinal tract (Cheng et al., 2004).

#### 1.6.6 Myrosinase activity in soil

Soil sample extracts from a rapeseed field showed enzyme activity, but there was no detectable activity from a pasture soil. The highest activity was obtained from soil which was collected directly from rapeseed rows; this activity was estimated to be equivalent to a myrosinase concentration of 20 µg per kilogram of soil, a lower activity was found in soil sampled between rapeseed rows (5 µg/Kg soil). Extracellular myrosinase activity was produced from *Rhizopus* sp. that is present in the rhizosphere of *Rorippa sylvestris* (Mizutani, 1999). Extracellular myrosinase was found in soil indicating that GLS hydrolysis occurs in the rhizosphere of *Brassica* spp and possibly impacts as an allelochemical towards organisms within the rhizosphere. The presence of extracellular enzyme in the soil maybe linked to rhizosphere microorganisms such as *Rhizopus* spp. in addition to cruciferous plant residues. Various organic compounds exude from plant roots and these substances are thought to influence rhizospheric microflora (Clark, 1940; Papavizas and Davey, 1961). They contain primary metabolites such as saccharides and amino acids which are nutritious for microorganisms (Rovira, 1956). The inhibitory effect of root radish and cabbage extracts of nonhost plants on arbuscular mycorrhizal fungi in alfalfa plants grown in sand-vermiculite pots has reported by (Ocampo et al., 1986). This study also found that the extract of cabbage roots considerably decreased the spore germination of *Glomus mosseae* spores (Ishimoto et al., 2000). PCR was used (Marilley et al., 1998) to amplify 16S rDNA genes that represent the bacterial communities in the rhizosphere, the study concluded that plant roots create a suitable environment for the

microbial population. *Bacillus subtilis* and *B. mycooides* survive during extreme winter temperature in the soil where temperature range is from 0 to 5 °C (Pandey and Palni, 1998).

Myrosinase activity was evaluated in soil extracts by using gas chromatography to monitor 2-propenylGLS hydrolysis products 2-propenyl ITC (Borek et al., 1996). Rakariyatham (2005) reported overproducing activity myrosinase from filamentous fungi and the organism isolated from soil. The study which included one hundred and fifty of soil samples that potentially containing GLS degrading activity used 2-propenylGLS barium sulphate agar technique for testing myrosinase activity, 28 new isolated strains with positive opaque zone. Myrosinase activity was detected in all these new isolates. The study concluded with a newly soil isolate *Aspergillus* sp. NR46F13 which degraded GLS and produced 3.19 U mL<sup>-1</sup> of myrosinase in 48 h of cultivation (Rakariyatham, 2005). Two non pathogenic strains of *Fusarium* (Ls-F-in-4-1 and Rs-F-in-11) which were isolated from the surface-disinfested roots of the Brassicaceae plants (*Lepidium sativum* and *Rorippa sylvestris*) induced the resistance of the *Lepidium sativum* seedlings against *Pythium ultimum* (Ishimoto et al., 2004).

### 1.7 Inhibition and substrates and optimal condition for $\beta$ -glucosidases

Transition state sugar analogues, free aglycones and substrate analogues glycosides are reported to inhibit  $\beta$ -glucosidase. In addition to fluroglucosides which are considered as slowly hydrolysed substrates metal ions in general do not inhibit the enzyme except Ag<sup>+</sup>, Hg<sup>2+</sup>, Fe<sup>3+</sup> and Cu<sup>2+</sup> which were reported to inhibit myrosinase. Sulfhydryl directed reagents were reported to inhibit most plant myrosinases (Botti et al., 1995).  $\beta$ - Glucosidases show a range of pH and stability and *this* depends on amino acid sequence and source of enzyme; pH 4-7.5 is the optimum for most  $\beta$ -

glucosidases and found to be stable at range of pH from 4 to 9; the enzyme can be stored either at 0 or 4 °C at pH7-8 after removal of proteases. Many  $\beta$ -glucosidases are not denatured by ionic detergent like SDS and this facilitates extraction with buffers that contain up to 3% SDS; and also allow zymogram development when samples are applied to SDS-PAGE without heating. The enzymes differ greatly in optima temperature and thermostability; the activity of mesophilic  $\beta$ -glucosidases is at 30-65 °C, however, generally the activity may decrease or be lost above 55-70 °C. An exception of this is the  $\beta$ -glucosidase which was purified from thermophilic archaeobacteria *Pyrococcus furiosus* which may have optimum temperature above 100 °C (Kengen et al., 1993). This thermostability is linked with the increased number of proline residues and an increase in internal water, binding to more subunits in the quaternary structure when compared with different mesophilic glucosidases (Chi et al., 1999; Ketudat Cairns and Esen, 2010). The enzyme assay usually run at 30-40 °C (Ketudat Cairns and Esen, 2010).

### 1.8 The genus *Citrobacter* overview

The genus *Citrobacter* was proposed in 1932 by Werkman and Gillen for the citrate utilising (Werkman and Gillen, 1932) gram negative, rod shaped non spore forming, typically mobile facultative anaerobes belonging to the family *Enterobacteriaceae*. They utilise citrate as a sole carbon source, ferment glucose and as a result acid and gas are produced. They are catalase and methyl red positive while oxidase and Voges-Proskauer tests are negative and they do not decarboxylate lysine. Member of this genus may or may not ferment lactose. In taxonomic terms the genus *Citrobacter* is most closely related to *Salmonella* and *Escherichia*. They occur in faeces of human, animals, food, water, sewage and soil. The genus so far contains 11 recognised species *C. freundii*, *C. amalonaticus*, *C. braakii*, *C. farmeri*, *C. gilleni*, *C. koseri*, *C.*

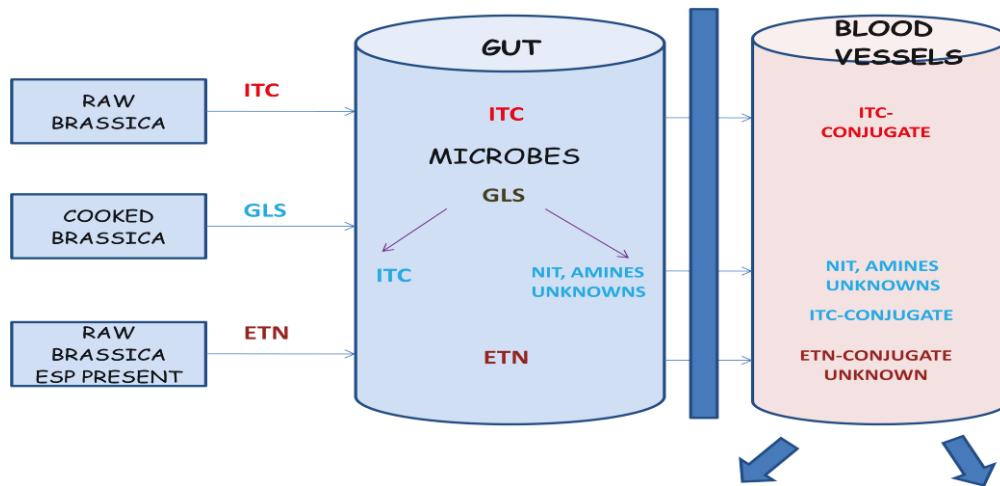
*murlinae*, *C. rodentium*, *C. sedlakii*, *C. werkmanii* and *C. youngae* (Frederiksen, 2005). Infections in neonates and immunocompromised hosts have been linked with various species of *Citrobacter*. Neonates and young infants are among two groups which are at high risk for clinical disease due to *Citrobacter koseri* and as a result they develop meningitis and sepsis, this will lead to abscesses in the central nervous system. The other group is persons at any age that are immunocompromised or debilitated (Doran, 1999). *C. freundii* is found in humans (often found in urine, sputum, throat, blood and wound swabs), birds, animals, mammals, fish, reptiles and amphibians, it also found in food, soil, water and sewage. *C. amalonaticus* is found in faeces of animals and human (as an opportunistic pathogen) and in water, soil and sewage. *C. braakii* is found in human stools and isolated from animals, *C. farmeri* is found in human urine, blood, wounds and stools. *C. gillanii* is found in environment and in human stools, blood and urine. *C. koseri* is found in the faeces of animal and human, and in water, soil, sewage and food, also clinically isolated from human urine, nose, throat wound swabs and sputum; it is known to cause fatal neonatal meningitis that is often complicated with cerebral abscesses, some cases occur in small outbreaks. *C. murlinae* found in human stool, urine wound, and blood and in food. *C. rodentium* occur in rodents only, this include the strain which is known to produce transmissible murine colonic hyperplasia. *C. sedlakii* found in human blood, stools and wounds. *C. werkmanii* occur in soil, human blood and stools. *C. youngae* found in human stools, wounds and urine, it also isolated from food and from animals (Frederiksen, 2005).

### **1.9 General scheme of glucosinolate metabolism**

From point of view that cooking of cruciferous vegetables inactivates both plant myrosinase and ESP and as a result intact GLS can reach the large intestine where they can be degraded by the gut bacteria. The ability to hydrolyse GLS is widely



distributed among the human intestine bacteria (Oginsky et al., 1965; Elfoul et al., 2001; Krul et al., 2002). Figure 7 summarises the Potential metabolism of GLS.



**Fig 1.7** The general scheme of metabolism of raw and cooked brassica. ESP; epithiospecifier protein, ETN; epithionitrile, NIT; nitrile

### 1.10 Hypothesis

*Citrobacter* (unpublished data of a new isolate from soil) and gut bacteria (see microbial myrosinase) contain a myrosinase- like enzyme that contributes to the metabolic processing of GLS.

### 1.11 The aim of the project

From point of view that many epidemiological studies continue to provide evidence linking consumption of cruciferous vegetables with lower incidences of cancers, then it is of major importance to investigate bacterial myrosinases. The main experimental goals of this work are:-

- i. Extract and purify gram quantities of GLS based on (Thies 1988).
- ii. Purify the myrosinase from *Citrobacter*
- iii. Investigate growth & induction of *Citrobacter* myrosinase.
- iv. Carry out a full characterisation of the active enzyme in terms of its substrate specificity for different GLS and other naturally occurring secondary metabolite beta glycosides.

**CHAPTER TWO**  
**GROWTH CHARACTERISTICS OF AN ISOLATED *CITROBACTER* CULTURED**  
**ON A SINIGRIN MEDIUM**

## 2.0 Introduction

The detoxification of glucosinolates degradation products by the action of microbes potentially improves the protein availability and quality of animal feed (Brabban and Edwards, 1994). Different mechanisms have been proposed for detoxification of seed meal which includes removing myrosinase from seed by ablation with Barnase (degrades RNA) (Borgen et al., 2010; Ahuja et al., 2011), and heat treatment for the inactivation of plant myrosinase. However, toxic effects can still occur even after inactivation of plant myrosinase by the microbial population of ruminants. Therefore detoxification by a myrosinase-producing microbe might be an interesting alternative (Palop et al., 1995). Soil contains a diverse population of microorganisms including bacteria which exist in any natural environment or ecosystem. Plant and microbial myrosinase activity is found in soil cropped with *Brassica napus* via roots disruption, decomposition and exudation. Myrosinase activity is also found in extracts of soil samples from rapeseed field (Borek et al., 1996). So far bacterial myrosinase has been found to be an inducible enzyme occurring in the presence of the substrate in the growth medium (Tani et al., 1974). Due to the cost of the glucosinolate substrate sinigrin several authors reported the use of a mustard extract in the culture medium. Tani et al (1974) reported the use of a mixture of mustard extract (6%) and the substrate sinigrin (0.01%) to induce bacterial myrosinase of *Enterobacter cloacae* no. 506, the induction rate reached the similar levels of the induction with sinigrin (0.1%) alone (Tani et al., 1974). The use of mustard extracts alone gave poor induction (Tani et al., 1974) while Reese et al (1958) reported that the addition of sinigrin or mustard flour to culture medium of *Aspergillus sydowi* increased the yield of myrosinase several fold (Reese et al., 1958). The use of minimal salts (M9 without  $\text{CaCl}_2$ ) plus progoitrin (0.08%) was not suitable for isolation and induction of bacterial myrosinase; however growth occurred when progoitrin was replaced with glucose (0.05%). The use of nutrient broth plus progoitrin (0.075%) allowed the induction of bacterial myrosinase of *E. coli* (Oginsky et al., 1965). Microorganisms

differ in their capability to use substrates as nutrient sources. Brabban & Edwards (1994) isolated microorganisms (bacteria and fungi) from different environments including Mud (petrochemically contaminated soil) which were capable of degrading sinigrin (0.1%) as a carbon sole in M9 medium. Some of these isolates reduced the level of glucosinolates to zero (Brabban and Edwards, 1994). Palop et al. (1995) used 3 mM of sinigrin in a modified version of MRS medium for screening *Lactobacilli* for sinigrin degradation. Cheng et al. (2004) reported the use 0.5 mM of sinigrin in GAM medium. Generally speaking a 10 mM concentration three fold higher concentrations than the reported of the other microbes such as of *Enterobacter cloacae* no. 506. In this present work, a new isolate of the genus *Citrobacter* has been found that can utilise sinigrin as a sole carbon source and has a soluble myrosinase activity (John Rossiter, Imperial College London, unpublished data). The soil isolate belongs to the family *Enterobacteriaceae* alongside *Enterobacter cloacae* no. 506 (Tani et al., 1974) which has previously shown to have myrosinase like activity. Further details of isolation & identification of *Citrobacter* are given in section (2.2.2.1).

## 2.1 The aim of this section

Microbial myrosinase is induced by incubation with GLS such as sinigrin and due to the high cost of commercially available sinigrin an investigation was carried out to assess other glucosides especially for large scale purification of the *Citrobacter* myrosinase. The aims of this Chapter are listed below.

- Purification of sinigrin based on the method described by Thies (1988).
- Culture optimization for induction of *Citrobacter* myrosinase
- Investigation of inducers for *Citrobacter* myrosinase

## 2.2 Method

### 2.2.1 Isolation and purification of Sinigrin

The isolation of sinigrin was performed according to the method described by Thies (1988). Boiling methanol (550 ml) was added to defatted *Brassica juncea* powder (100 g) in a beaker and boiled for 10 min and vacuum filtered (the process was repeated once more). The methanol extract was evaporated to near dryness using a vacuum rotary evaporator. The residue was dissolved in deionised water (200 ml) and dichloromethane (DCM 200 ml) added (in order to remove lipids), and gently shaken. The mixture was left to settle before the DCM layer (lower layer) was discarded (this step was repeated once more). The aqueous phase was evaporated to near dryness and methanol (400 mL) added and warmed while mixing (this aids sinigrin solubility in methanol), followed by addition of a small amount of charcoal heating and then filtered under vacuum. The extract was then passed through a florisil (10 g) column overlaid with a small amount of sand and methanol (30 mL) was added followed by the extract and the eluate evaporated to dryness and finally dried under vacuum over phosphorus pentoxide ( $P_2O_5$ ). This process yielded 0.8 g of crude sinigrin. Alternatively a commercial source was used which was purchased from Apin Chemicals, UK.

### 2.2.2. Isolation of *Citrobacter* sp with myrosinase activity (John Rossiter, Imperial College London, unpublished data)

Previous work on bacterial myrosinases has done little more than demonstrate GLS hydrolysis taking place in some bacteria including *Enterobacter cloacae* furthermore the enzyme was not isolated or characterized in any way. Myrosinase activity is

found in soil (Al-Turki and Dick, 2003) and in soil extracts of *Brassica napus* field (Borek et al., 1996). Soil bacteria may be responsible for myrosinase activity in soil and for this reason for searching a *Brassica napus* field (Wye, UK) for bacteria that may inhabit this environment (soil). Soil sample (10-20 g) was added into minimal salts (M9) that contained sinigrin as a sole carbon source and then incubated for several days until growth occurred after which inoculated on fresh M9 plus sinigrin this step was repeated 5 times. After the final enrichment stage the culture was streaked out onto nutrient agar plates and incubated overnight at 30°C. There were two distinct types of colonies and each was further subcultured to give pure colonies. Five colonies were selected from each plate and inoculated into M9 containing sinigrin. A distinctive smell of sulfur was associated with the growth of the bacteria. Identification of the bacteria was based on 16S rRNA gene sequencing and has been classified by Dr Arjan Narbad, Norwich institute of Food research, (Norwich, UK) as a member of the genus *Citrobacter* (Rossiter unpublished data). The organism was checked for purity and was grown and maintained on nutrient agar (slopes and plates) and kept at 4°C fridge for frequent use and subcultured at monthly intervals. For long term maintenance of cultures a glycerol stock of *Citrobacter* was made, the *Citrobacter* (850 µL) was mixed with sterile glycerol (150 µL of 100%) and mixed well then kept at -80 °C.

### 2.2.3 Routine culture medium

A modified version (glucose and calcium chloride CaCl<sub>2</sub> were omitted) of the classical Difco M9 minimal salts solution was used as a basal medium that contains the following per litre Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O (12.8 g), KH<sub>2</sub>PO<sub>4</sub> (3.1 g), NaCl (0.5 g), NH<sub>4</sub>Cl (1.0 g), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.5 g) (the latter was sterilised by filtration using 0.2µm

Minisart filter). The pH of the medium was adjusted to 7.0 and then autoclaved at 121°C for 15 min, after cooling MgSO<sub>4</sub>.7H<sub>2</sub>O was added aseptically to the medium.

#### 2.2.4 Optimal sinigrin concentration for the growth of *Citrobacter*

Triplicates of different concentrations of filter sterilised sinigrin (1, 2.5, 5, 7.5, 10 and 12.5 mM) were mixed with sterilised M9 media (10 mL), and a fresh culture of the *Citrobacter* was inoculated into each tube prior to incubation overnight at 30 °C. The O.D was measured at 600 nm using spectrophotometer.

#### 2.2.5 Cell lysis (Cell free extract)

##### 2.2.5.1 Cell preparation:-

Inoculum from overnight cultures in nutrient broth (200µL containing approximately 10<sup>5</sup> cells) was used to seed M9 medium (15 mL) supplemented with sinigrin (750 µL, 10mM) or glucose (8.3 mM). The cultures were harvested (Eppendorf centrifuge 5810 R, Hamburg Germany) (3200 x g 30 min, 4°C) after 24h at 30 °C, yielding approximately 0.45 g wet weight. The supernatant was discarded and the pellet was resuspended, washed with buffer (CPB) (10 mL, 20mM, pH 6.0) and centrifuged (10 min, 3200 x g). The supernatant was decanted and the cell pellet gently resuspended in CPB (5 mL, 20mM, pH 6.0) and protease inhibitor cocktail (25 µL) (Melford Labs Ltd, UK) was added to prevent protein degradation after lysing cells. The suspension was kept on ice until lysed.



#### 2.2.5.2 Bacterial cell disruption

##### 2.2.5.2.1 *French press*

A one shot cell disruption system (Constant Systems Ltd UK) was used to break up bacterial cells. The chamber was pre-chilled at 4 °C, and a bacterial cell suspension (5 mL) was added to the chamber and pressurised to 30 KPSI (2000 bar). Three mL of the recovered lysate was centrifuged (Eppendorf centrifuge 5415R Hamburg Germany, 16100 x g, 4 °C, 10 min) and the supernatant harvested and the pellet reconstituted with CPB (3 mL, 20mM, pH6.0) and kept at 4°C until required. Sodium azide (2.5µL, 10%) was added to the recovered supernatant to inhibit bacterial growth.

##### 2.2.5.2.2 *Ultra-sonication*

The cell suspension (5-10 mL) was subjected to ultrasonication (Sonics Vibracell) with 40% power (2 seconds on and 2 seconds) off over a period of 10 min in total. Cells debris was removed by centrifugation (16100 x g, 4 °C, 10 min).

#### 2.2.6 Quantification of sinigrin by high performance liquid chromatography (HPLC)

Glucosinolates were analysed using a technique that removes the sulphate group to give desulpho-glucosinolates which are easily separated by reverse phase liquid chromatography. HPLC analysis was performed using a an Agilent 1200 series liquid chromatography system (Agilent Technologies, USA), SynergiHydro column (Phenomenex, USA) with UV detection (Waters 486 tunable absorbance detector, USA) set at 229 nm was used to quantify the sinigrin in the extracted sample. DEAE A-25 Sephadex (10 g) (Sigma Aldrich USA) was added to acetate buffer (450 mL 1M, pH 5.0) and left overnight to equilibrate. The buffer was decanted and replaced

with water mixed and decanted (Xs 3). Finally the Sephadex was resuspended in ethanol (20%) and decanted (Xs 2). DEAE-A25 Sephadex (1mL of a 50% suspension) was added to a Bio-Rad minicolumn and washed with deionised water (1mL, Xs 2). Serial dilutions of sample were made (1/10, 1/100 and 1/1000). The standard tube contained 1.8 mg Sinigrin (sigma, UK) in 1 mL Milli Q H<sub>2</sub>O. 200 µL of each (standard, sample) was pipetted onto to the ion exchange column before washing twice with 1 mL of Milli Q H<sub>2</sub>O. The columns were then rinsed twice with 500 µL of 0.02 M acetate buffer pH 5.0, before adding 75 µL of partially purified sulfatase enzyme. The columns were left overnight at room temperature. Samples were eluted by washing three times with 500 µL of Milli Q H<sub>2</sub>O (1.5 mL of each sample was collected in 15 mL Falcon tube), then 1 mL of each sample was transferred into HPLC glass vials. The desulphoglucosinolates were analysed on a Synergy Hydro column (150 mm x 20 mm, Phenomenex, USA) and 2 % acetonitrile [B] versus Milli Q water [A] mobile phase (Table 2.1). Sample elutes were analysed with flow rate 0.2 mL/ min and detected at 229 nm by a UV detector (Waters 486 tunable absorbance detector, USA). The total running time for each sample was 36 min.

Time (min)	%B (CH <sub>3</sub> CN)	%A (H <sub>2</sub> O)
0	2	98
15	25	75
17	70	30
19	70	30
21	2	98
36	2	98

**Table 2.1** HPLC gradients; the sample (10 µL) was injected onto HPLC with flow rate 0.2 mL/min using gradient [A] acetonitrile versus [B] Milli Q H<sub>2</sub>O. The total running time was 36 min.

### 2.2.7 Protein determination

For protein determination Sigma Bradford reagent (B 6916 sigma, UK) was used and the reagent calibrated against bovine serum albumin (BSA) at different concentrations. The principle of this assay is based on the formation of a complex between the dye (Brilliant Blue G) and protein in a solution. The protein and dye complex causes a shift in the absorption maximum of the dye (from 465 to 595 nm). The amount of absorbance is proportional to protein present. BSA (10 mg) was dissolved in Milli Q water (10 mL) to make a stock solution. This solution was diluted 1 in 10 and used to construct a standard curve. Reagent volumes and concentrations are shown in the (Appendix A).

Samples were mixed and incubated for 10 min at room temperature and the absorbance measured at 595nm. For the estimation of myrosinase different volumes of the enzyme were added to Milli Q H<sub>2</sub>O to give a total volume of 500 µL before 500 µL of the reagent (Bradford) was added and mixed. The mixture (1 mL) was incubated at room temperature for 10 min and the absorbance measured at 595 nm. A calibration graph was constructed to allow estimation of the protein concentrations in samples (Appendix A).

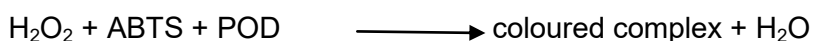
### 2.2.8 Protein concentration

Protein samples from bacterial cell free extracts and purification processes were generally desalted and concentrated to increase the concentration of protein. Protein samples were concentrated using ultra filtration membrane tube NMWL 10.000 (4 mL UFC801024, 0.5 mL UFC501024, Amicon ultra, Millipore, USA) by

centrifugation at 2500 x g for 10-20 min or over a period of time to give the desired concentration.

### 2.2.9 Detection of $\beta$ -S-glucosidase (myrosinase) activity with the GOD-Perid reagent

The God-Perid method is a clinical test for determining blood sugar level where the released glucose is measured (Werner et al., 1970). The principle of this method is based on glucose oxidation to gluconate by the action of glucose oxidase enzymes and formation of hydrogen peroxide ( $H_2O_2$ ) which oxidizes a chromogenic (2,2'-Azino-bis (3-Ethylbenzthiazoline-6-Sulfonic Acid) (ABTS) substance to a coloured product under the action of peroxidase (POD).



GOD = glucose oxidase; POD= peroxidase.

The intensity of absorbance of thus formed stained product is proportional to the concentration of glucose in the sample.

On hydrolysis by myrosinase one mole of glucosinolate potentially gives one mole of glucose. Thus an accepted measure of myrosinase activity is the amount of glucose produced. Thus the GOD PERID method provides a convenient assay for myrosinase. From a standard graph of glucose V colour formed at 420 nm the amount of glucose produced by the myrosinase reaction can be read off. The specific activity is determined as  $\mu\text{mol}$  of glucose/min/mg of protein.

#### 2.2.9.1 Preparation of GOD-Perid reagent

ABTS (2-2-azino-bis (3-ethylbenzo-thiazolin-6-sulfonic acid) (250 mg, Sigma A 1888), glucose oxidase (12.7mg, 157.5 U/mg, Sigma G7141) and peroxidase (4.7 mg, 148U/mg, Sigma P8125) were dissolved in Tris (Sigma T1503) buffer (3 g Tris, pH 7.2) to a volume of 250 mL. The reagent was mixed well and wrapped with aluminium foil and then was kept in the fridge at 4°C.

#### 2.2.9.2 Glucose assay

The glucose stock solution was prepared by dissolving glucose (0.1g) in Milli Q water (100 mL) and was diluted 1/10 (1 mL of this solution was added to 9 mL of Milli Q water). GOD-Perid reagent (1 mL) was added to each tube making the total volume 1300 µL, then the tubes were incubated (15 min) at water bath (37 °C), the absorbance was measured at 420 nm using a spectrophotometer (Appendix A).

#### 2.2.9.3 Esculin reagent

Esculin hydrate (Sigma E8250) (0.2%) was dissolved in CPB (0.2 M, pH 6.0), and gently warmed until completely dissolved. In a separate tube FeCl<sub>3</sub> (BHD 28379) (1% was dissolved in Milli Q H<sub>2</sub>O) was added to the esculin solution. The reagent was wrapped with aluminium foil and kept at room temperature.

#### 2.2.9.4 *p*-Nitrophenol standard

Glucosidase catalyses the hydrolysis of *p*-nitrophenyl- $\beta$ -D-glycoside to *p*-nitrophenol and glucose. Therefore the enzyme activity can be determined by measuring any of the two products but *p*-nitrophenol is more convenient to measure as it produces a yellow colour. The amount of yellow-orange colour can be translated into the amount of released *p*-nitrophenol. Under alkaline conditions the *p*-nitrophenolate anion absorbs light at 400-450 nm. A calibration graph is shown in appendix A.

#### 2.2.9.5 Myrosinase assay

A total volume of 300  $\mu$ L of enzyme, substrate (60  $\mu$ L of 10 mM of glucosinolate or *p*-nitrophenyl- $\beta$ -D-glucopyranoside (*p*NPG) or esculin) and CPB (20 mM, pH 6.0) was incubated at 25 °C for 2 h or less. For glucose release from GLS the reaction was stopped by boiling the mixture for 5 min and cooled, followed by the addition of 1 mL of GOD-Perid reagent and incubated 15 min at 37 °C. For the *p*-nitrophenol assay the reaction was stopped by addition of cold Na<sub>2</sub>CO<sub>3</sub> (1 mL of 100 mM). The resultant colour of both sinigrin and *p*-nitrophenol was measured at 420 nm. The released product (glucose or *p*-nitrophenol) was calculated from standard. Formation of black or dark brown colour is considered a positive result of esculetin formation.

## 2.2.10 Induction by $\alpha$ , $\beta$ substrates and enrichment medium

### 2.2.10.1 Growth conditions

*Citrobacter* was grown aerobically (30 °C) overnight on nutrient broth then inoculated into M9 containing the test substrate (10 mM) as a sole carbon source. Tested substrates were D (+) glucose (sigma G8270), D(+) trehalose (Sigma T9531),  $\beta$ -D-(+) cellobiose (Sigma C7252), methyl- $\beta$ -D-glucopyranoside (Sigma M0779), methyl- $\alpha$ -D-glucopyranoside (Sigma M9376), esculin hydrate (Sigma E8250), octyl- $\beta$ -D-1-thioglucopyranoside (Sigma O6004), p-nitrophenyl- $\beta$ -D-glucopyranoside (Sigma N7006) and sinigrin (Apin 03918S). The culture was then incubated (30°C) aerobically overnight. The cell density was measured by spectrophotometer at 600 nm. In a separate experiment cellobiose was used at different concentrations (1, 2.5, 5, 7.5, 10, 15 mM) and a constant glucosinolate (sinigrin) concentration (2mM) (triplicate) in M9 media (10 mL). The media then inoculated with an overnight culture of *Citrobacter* grown on NB and incubated aerobically at 30 °C for 24 h, the OD at 600 nm was measured using a spectrophotometer. This experiment was repeated but with different concentrations of sinigrin (3, 4, 5, 6 and 7 mM) and constant cellobiose concentration (7.5 mM). The induction of *Citrobacter* myrosinase was investigated using the enrichment medium nutrient broth (NB).

### 2.2.11 Detection of isothiocyanates (ITC) by Gas Chromatography (GC)

Gas chromatography with mass spectrometry (GC-MS, Hewlett Packard 6890 series) with the Hewlett Packard 5973 mass selective detector (HP, UK) was used to determine the amounts of ITC in samples. Samples were centrifuged in an

Eppendorf centrifuge at 13200 rpm for 2 min. A portion of the supernatant (100 µL) was extracted and diluted five times with deionised H<sub>2</sub>O. ITCs were then extracted with dichloromethane (2 Xs 0.7 ml) and the extracts combined and dried over magnesium sulphate (500 mg) and centrifuged in an Eppendorf centrifuge at 13200 rpm for 2 min. The dried extract was concentrated using a sample concentrator (TECHINE, USA) and nitrogen gas to a volume of 250 µL. The sample was transferred to GC-MS sample vials containing 250 µL inserts for GC-MS analysis. Analysis of each fraction (5 µL) was carried out on a HP-5MS 5% Phenylmethylsiloxane (30m X 250 mm) column. The GC-MS temperature was programmed at the range of 50 °C to 280°C (26 min total run). The injection splitter was set at 20:1 and helium was used as a carrier gas. The compounds were identified by using mass fragmentation ions.

#### 2.2.11.1 Glucosinolates substrate specificity

Different glucosinolates (sinigrin, erucin, glucotropaeolin and raphanin) were examined for their substrate specificity. Details of myrosinase assay are given in section 2.2.9.5.

#### 2.2.11.2 Analysis of glucosinolate degradation in culture medium

Different glucosinolates were used in this experiment; sinigrin (10 mM) was added to M9 medium (24 mL); while for glucotropaeolin and gluconasturtiin (2 mM) of each was mixed with 6 mM sinigrin then inoculated with overnight culture of *Citrobacter* (500 µL). An aliquot (1 mL) was taken from fresh inoculate at the time zero before incubating at 30 °C. For the sinigrin culture samples were taken after 2, 4, 6, 8, 10,

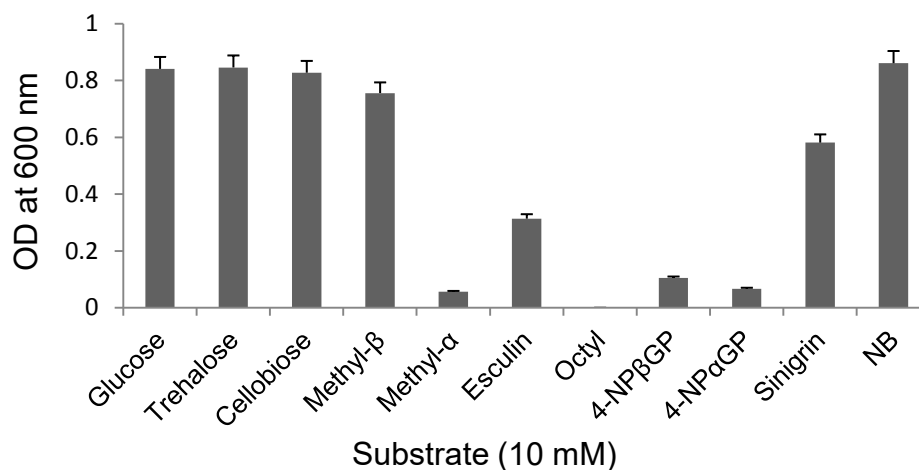


12 and 24 h. The OD of each sample was measured at 600 nm before centrifugation (16100 rpm, 3 min) in order to separate the bacterial pellet from glucosinolates and degradation products in the supernatant. All samples were immediately stored in freezer at -20 °C to quench any  $\beta$ -glucosidase activity. Barium acetate solution (100  $\mu$ L) was added to each sample and incubated at room temperature (2 min) in order to precipitate impurities proteins. For the rest of the procedure see (2.2.6). For GLS quantification by HPLC internal sinigrin standard was made (Appendix A)

## 2.3 Results

### 2.3.1 Substrate utilisation

In order to investigate alternative myrosinase inducers other than sinigrin the *Citrobacter* was screened for ability to grow with various  $\alpha$  and  $\beta$  sugar substrates. Among those tested were trehalose, cellobiose and glucosidic substrates with an  $\alpha$  and  $\beta$  configuration. Myrosinase expression was also investigated using nutrient broth (NB). Figure 2.1 shows that the highest optical density obtained was with NB with OD (0.861) followed by trehalose as a substrate and glucose respectively. Trehalose, glucose, cellobiose and methyl- $\beta$ -D-glucopyranoside gave the highest ODs (0.846, 0.841, 0.828 and 0.756 respectively) with sinigrin giving a lower OD (0.581) while esculin gave an OD of (0.314). All of the remaining substrates were only able to produce minimal bacterial growth. *Citrobacter* grew poorly on methyl- $\alpha$ -D-glucopyranoside and 4-nitrophenyl-D-glucoside ( $\alpha$  &  $\beta$ ) and failed to grow on octyl- $\beta$ -1-thioglucopyranoside. According to obtained OD, four substrates were selected in order to determine if the myrosinase is induced (trehalose, cellobiose, methyl- $\beta$ -D-glucopyranoside and esculin). Apart from trehalose the other three substrates were reported to induce  $\beta$ -glucosidase (Woodward and Wiseman, 1982).

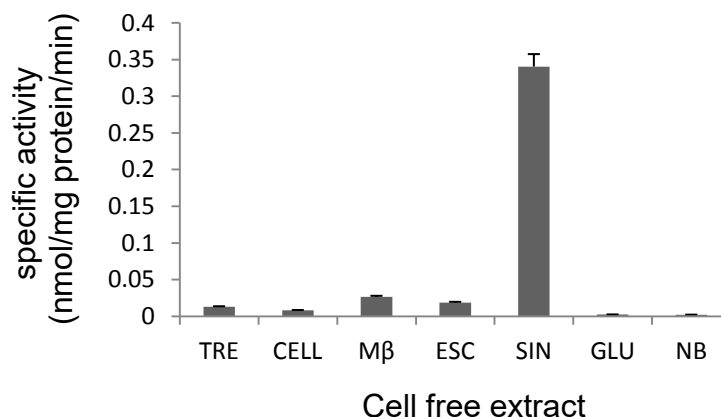


**Fig 2.1** Substrate utilisation. *Citrobacter* was grown overnight on NB only and M9 medium (5 mL) supplemented with the substrate (10 mM) and incubated aerobically (30 °C). Octyl = octyl  $\beta$ -D-1-thioglucopyranoside. NB= nutrient broth. This experiment was repeated twice and samples analysed in triplicate. The OD was measured at 600 nm.

### 2.3.2 Comparison of enzyme activity

The crude protein extracts from *Citrobacter* grown on the different substrates were subjected to a myrosinase activity assay. In addition to NB; substrates giving an OD  $\geq 0.3$  were selected, (glucose, trehalose, cellobiose, methyl- $\beta$ -D-glucopyranoside and esculin in addition to sinigrin). With the exception of sinigrin no appreciable myrosinase activity was found with NB or with the tested substrates. This finding suggests that the inducer sinigrin cannot be replaced by any of these substrates including those resembling sinigrin in having an  $\beta$ -O-glucosidic linkage. Increasing the concentration of esculin and methyl- $\beta$ -D-glucopyranoside to 20 mM did not yield a higher OD compared with the used concentration (10 mM).

The specific activity of each of these substrates were plotted in terms of myrosinase activity (Figure 2.2)



**Fig 2.2** *Citrobacter* myrosinase induction: TRE = trehalose; CEL = cellobiose; M $\beta$  = methyl- $\beta$ -D-glucopyranoside; ESC = esculin; SIN = sinigrin; GLU= glucose; NB= nutrient broth. Cell free extracts of these substrates were assayed against sinigrin (0.2  $\mu$ M) and buffer CPB (20 mM, pH 6.0).

Myrosinase activity was not detected in the supernatant of the growth medium supplemented with cellobiose *Citrobacter* likely uses another  $\beta$ - glucosidase specific for cellobiose. To investigate this, the cell free extract of each substrate was examined against the same substrate that was used in for the induction e.g. trehalose cell free extracts were assayed against trehalose (10 mM). For each substrate a sinigrin induced myrosinase extract was used as a control.

Carbon source for <i>Citrobacter</i> growth (10 mM)	Enzyme substrate	Specific activity (nmole/mg protein/min)			
		Trehalase	Cellobiase	Methyl- $\beta$ -D- glucosidase	<i>Esculinase</i>
Trehalose	Trehalose	0.11 (0.001)*	NA	NA	NA
Sinigrin	"	0.16 (0.01)*	NA	NA	NA
Cellobiose	Cellobiose	NA	0	NA	NA
Sinigrin	"	NA	0	NA	NA
Methyl- $\beta$ -D- glucopyranoside	Methyl- $\beta$ -D- glucopyranoside	NA	NA	0	NA
Sinigrin	"	NA	NA	0	NA
Esculin	Esculin	NA	NA	NA	0
Sinigrin	"	NA	NA	NA	0

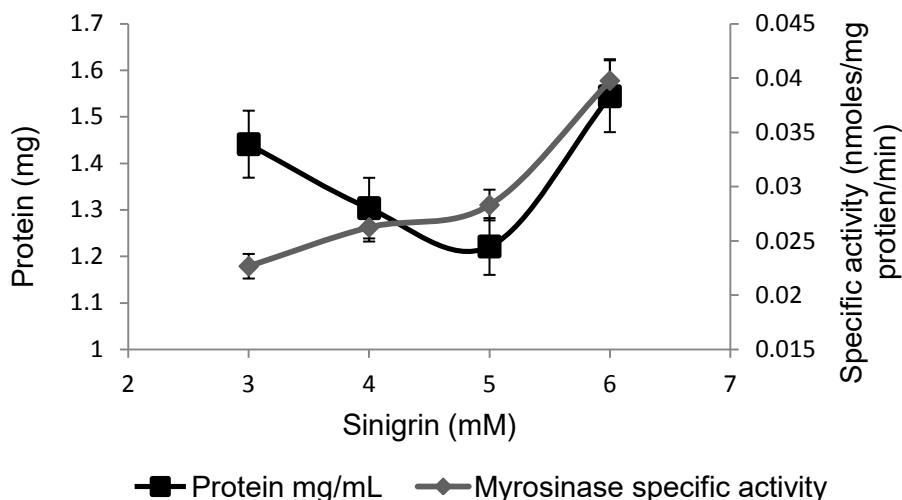
**Table 2.2** Substrate utilisation. Cell free extract of *Citrobacter* grown with each substrate was tested against the used growth substrate (10 mM). Crude protein extract (1  $\mu$ g) was mixed with CPB (20 mM, pH 6.0) and incubated (25°C, 60 min). Sinigrin extract was used for comparison. This experiment was carried out in triplicate. NA= not applicable. \* = standard deviation

The above data (Table 2.2) shows that only the cell free extract of cells grown on trehalose hydrolysed the substrate trehalose with a specific activity 0.11 nmole/mg protein/min while the sinigrin induced cell free extract gave a specific activity of 0.16 nmole/mg protein/min. Other substrates did not show any activity with crude protein extracts or from the control (sinigrin extract).

The possibility that lower concentrations of sinigrin might be sufficient to induce myrosinase activity was tested by using a combination of cellobiose and sinigrin. This would have the advantage of reducing the costs incurred by using high concentrations of sinigrin. Tables (2.3) and Figure (2.3) shows the results of the growth experiment of *Citrobacter* grown on M9 containing different concentrations of cellobiose and sinigrin.

Cellobiose ( mM)	Sinigrin (mM)	Mean OD At 600 nm
7.5	3	0.368
7.5	4	0.514
7.5	5	0.574
7.5	6	0.652

**Table 2.3** The OD (optical density) of *Citrobacter* culture grown (24 h, at 30 °C) on M9 medium containing cellobiose and sinigrin. The concentration of sinigrin was varied while cellobiose concentration was constant (7.5 mM).

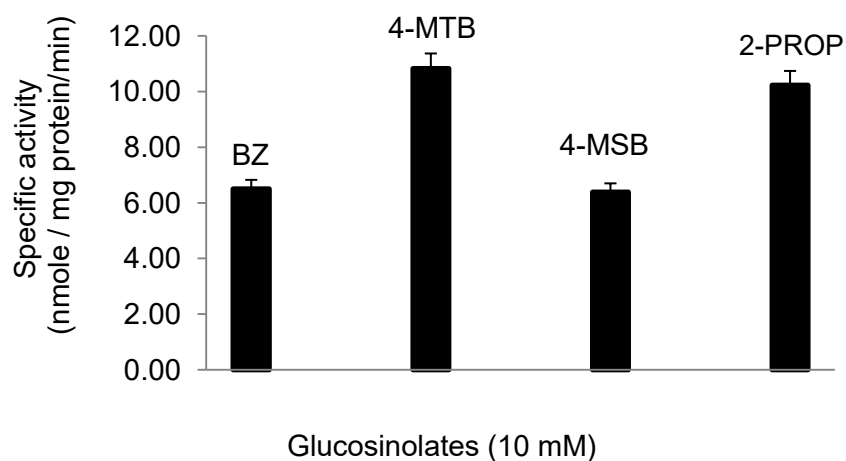


**Fig 2.3** Determination of optimal myrosinase activity. The media contained 7.5 mM cellobiose and varying concentrations of sinigrin. The enzyme activity was measured by mixing sinigrin (0.2  $\mu$ M) and buffer (CPB 20 mM, pH 6.0) the enzyme was added prior incubation (37  $^{\circ}$ C, 10 min), the reaction was stopped by boiling (5 min). The protein concentration was determined using the Bradford method.

From Figure 2.3 it can be seen that the OD increased as the concentration of sinigrin increased increasing the concentration of sinigrin results in an increase in myrosinase activity. The protein concentration also increased in the presence of cellobiose. However, although the myrosinase activity was increased it did not reach the same level as in *Citrobacter* cultures grown only with sinigrin (0.2 nmole/ min / mg protein) as a carbon source. It has been noticed that the amount of protein decreased (0.2 mg) when the amount of sinigrin increased to 5 mM.

### 2.3.3 Substrate specificity

The enzyme degraded all the tested GLS Figure (2.4), the most degradable being erucin (4MTB) with a specific activity 10.83 nmole/min/mg protein followed by sinigrin (2-propenyl) 10.23 then glucotropaeolin (BZ) 6.50 and lastly glucoraphanin (4-MSB) 6.38.

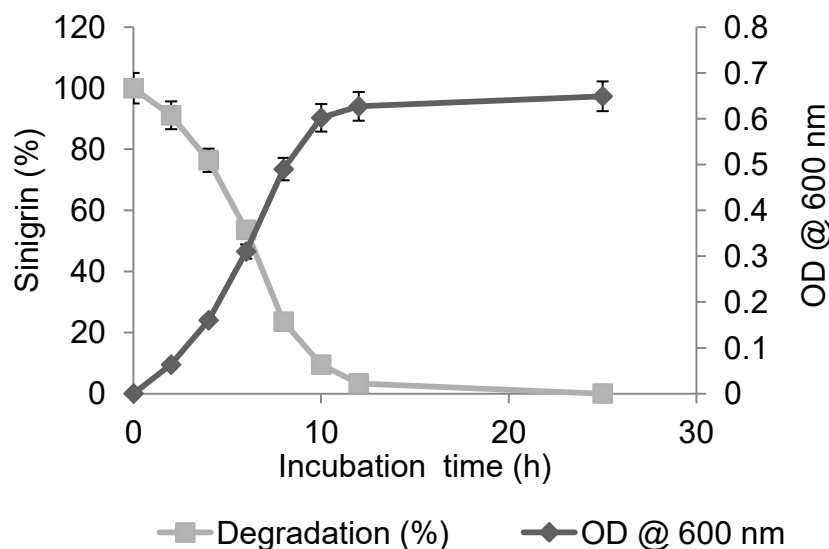


**Fig 2.4** Study on the *Citrobacter* myrosinase substrate specificity (nmole /min/mg protein), BZ= benzyl (glucotropaeolin); 4-MTB= 4-methylthiobutyl (glucoerucin); 4-MSB = 4-methylsulphinylbutyl (glucoraphanin); 2-propenyl (sinigrin).

#### 2.3.4 Glucosinolate degradation

When grown on M9 containing sinigrin *Citrobacter* reached maximum growth density (OD) of ~ 0.65 (Figure 2.5) after 12 h of incubation.

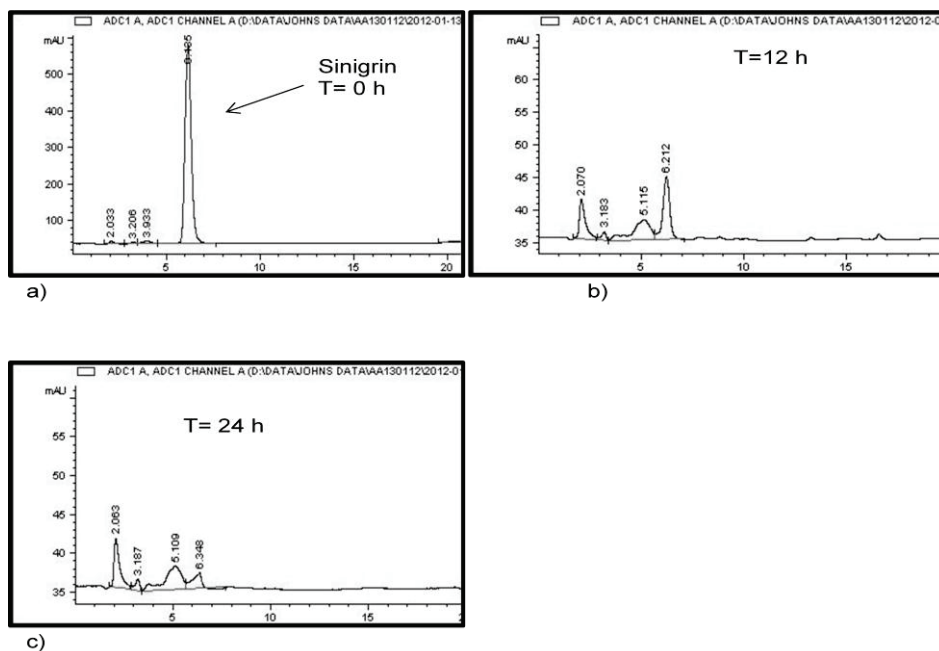




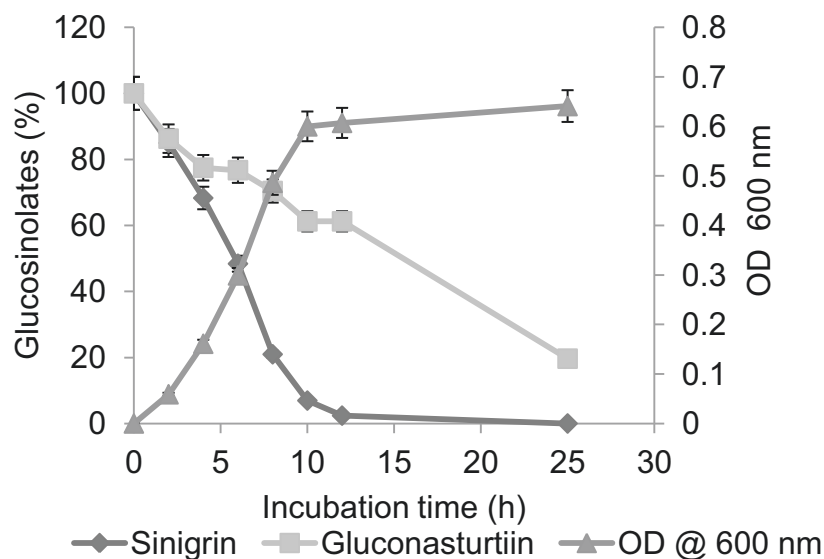
**Fig 2.5** *Citrobacter* growth and time course degradation of sinigrin. *Citrobacter* was grown on M9 with sinigrin (10 mM) carbon source, the OD significantly increased after 6-12 h of cultivation. There was no increase in the OD after 12-24 h of incubation.

The HPLC data (Figure 2.6) shows that most of sinigrin was utilised before 12 hrs of incubation and almost disappeared at 25 h of incubation. Sinigrin grown *Citrobacter* cells utilise most of the substrate within 12 hrs and the growth curve virtually mirrors that of sinigrin utilisation (Figure 2.5). Previous work has shown (J.Rossiter unpublished) that *Citrobacter* grows only slowly with glucotropaeolin and gluconasturtiin. Thus in order to improve the metabolism of these two glucosinolates each was supplemented with sinigrin to ensure induction of the myrosinase and other metabolic processes. Concentrations of sinigrin were 6 mM and the other glucosinolates 2 mM. Representative HPLC chromatograms are shown in Figures 2.7 and 2.8 while the extent of metabolism and growth are shown in Figures 2.9 and 2.10. The growth curves (Figure 2.9 and 2.10) for each experiment were similar to that of *Citrobacter* grown on sinigrin (Figure 2.5) with M9 medium. However, it is clear that *Citrobacter* prefers sinigrin as a substrate as both glucotropaeolin and gluconasturtiin are metabolised much more slowly. It is also evident that gluconasturtiin is a better

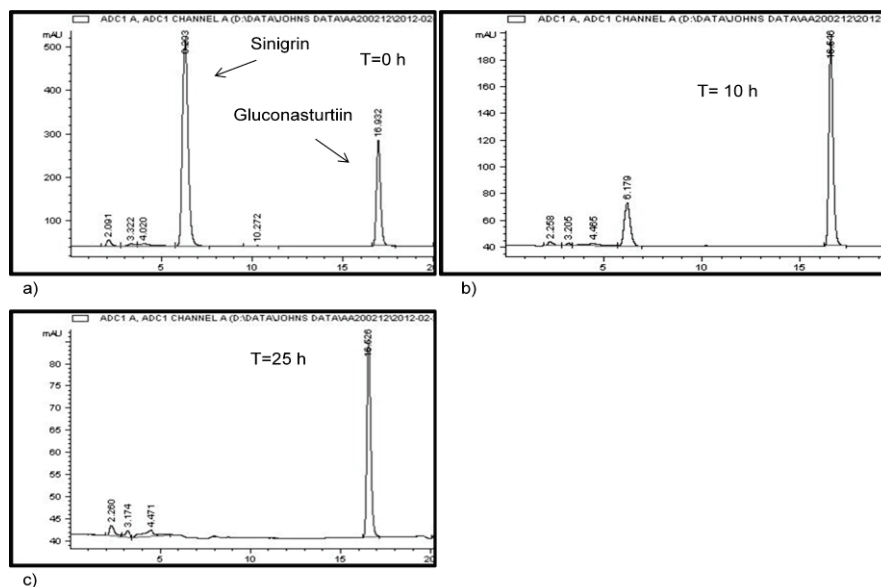
substrate than glucotropaeolin for *Citrobacter* growth. Thus at the end of the fermentation i.e. 25 hrs 40% of glucotropaeolin remains and 20 % gluconasturtiin.



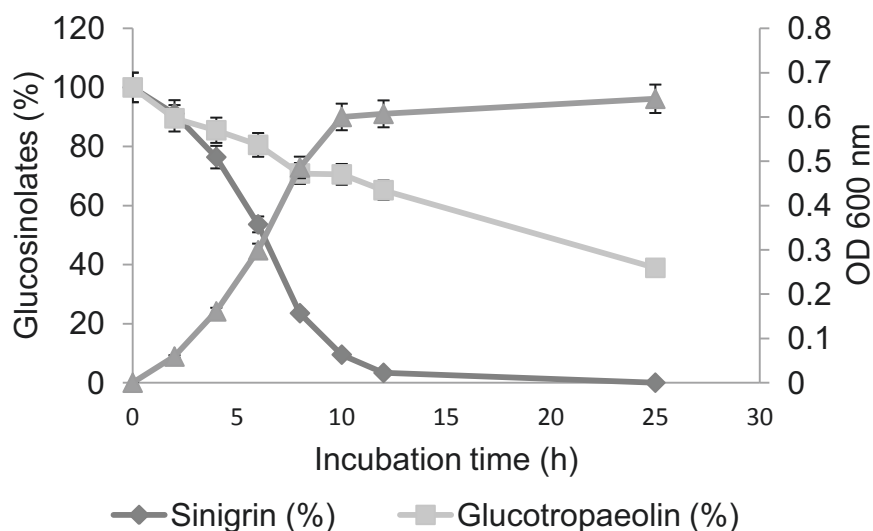
**Fig 2.6** HPLC chromatogram. Degradation of sinigrin (6 mM). An aliquote (1 mL) of each time point was taken and assayed for the presence of each sinigrin using HPLC. a) incubation time = 0 h; b) 10 h; c) 25 h.



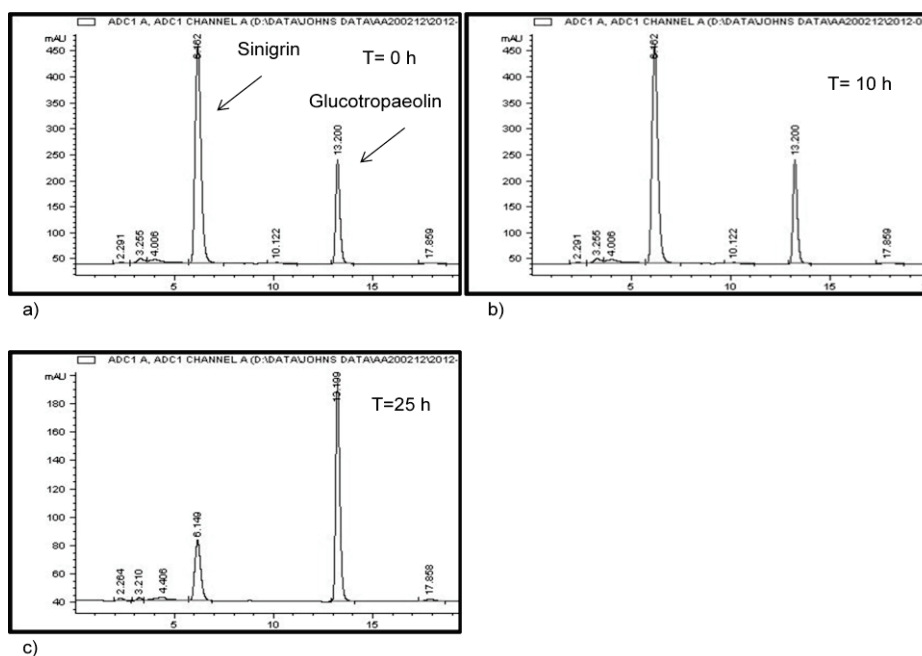
**Fig 2.7** *Citrobacter* growth and degradation time course curve. A mixture of sinigrin (6 mM) and gluconasturtiin (2 mM). The OD was measured (600 nm) at each time point (0-25 h).



**Fig 2.8** Representative HPLC chromatograms of glucosinolate metabolism. Degradation of sinigrin (6 mM) & gluconasturtiin (2 mM). An aliquot (1 mL) of each time point was assayed for the presence of each GLS substrate using HPLC. a) incubation time = 0 h; b) 10 h; c) 25 h.



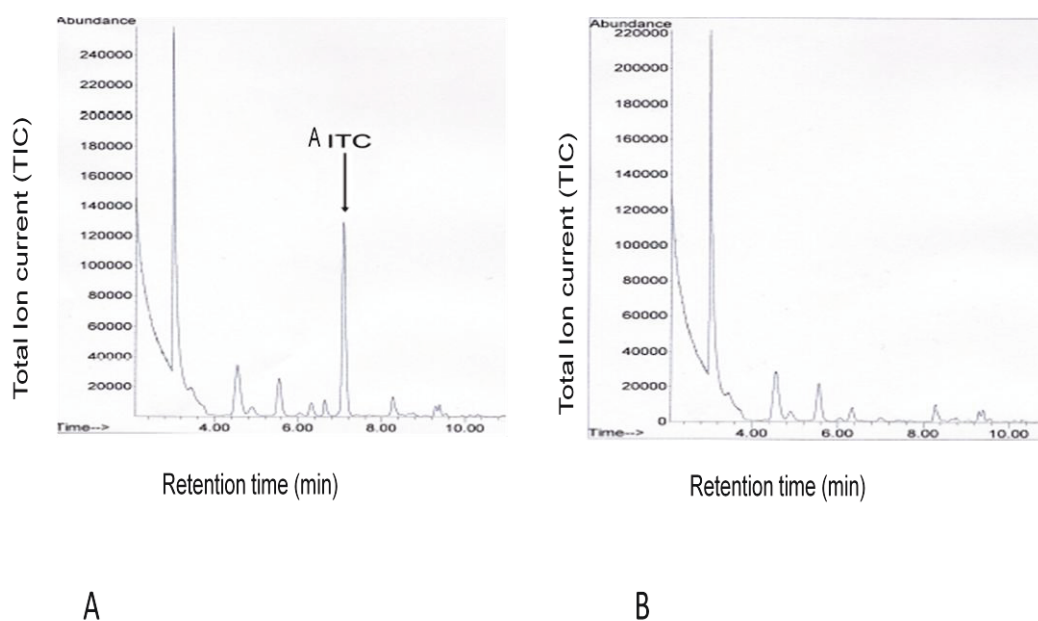
**Fig 2.9** Glucosinolate growth & degradation time course. *Citrobacter* was grown on M9 with sinigrin (6 mM) and glucotropaeolin (2 mM) as the carbon source. The amount of sinigrin significantly decreased after 10 h of cultivation and almost disappeared after 12-25 h while 60% of glucotropaeolin was consumed after 25 h.



**Fig 2.10** Representative HPLC chromatogram of glucosinolate metabolism. Degradation of sinigrin (6 mM) & glucotropaeolin (2 mM). An aliquot (1 mL) at the indicated time point was assayed for the presence of each GLS substrate using HPLC. a) incubation time = 0 h; b) 10 h; c) 25 h.

### 2.3.5 Glucosinolate degradation assay

The profile of glucosinolate hydrolysis products were analysed by GC-MS. The determination of ion mass spectra in the chromatogram indicated the identity of compounds whereas the total ion current indicated the relative abundance of the compound in the samples. Isothiocyanate production was confirmed by examining the mass spectrum of the isothiocyanate (a molecular ion of 99 and abundant fragmentation ion at 72). The following GC chromatogram (Figure 2.11) shows that the protein extract of *Citrobacter* (myrosinase) degraded sinigrin *in vitro*. The ITC (allylisothiocyanate) was detected after 7 min while *in vivo* only trace amounts of ITC were produced.



**Fig 2.11** GC chromatogram peaks of cell-free extracts of *Citrobacter sp.* incubated with sinigrin. (A) Detection of sinigrin hydrolysis product allylisothiocyanate (AITC) after 7min (B trace amounts were observed *in vivo*). The isothiocyanate was confirmed by examining the mass spectrum of the isothiocyanate (a molecular ion of 99 and abundant fragmentation ion at 72).

## 2.4 Discussion

Sinigrin isolation was conducted according to the method described by Thies (1988). Typically this yielded 0.7 g of crystalline sinigrin (from 60 g of defatted seed) which while sufficient for biochemical analysis/assays it is limiting for large scale cultures i.e. > 100 ml. The process is labour intensive particularly the seed defatting process. Thus alternative substrates (trehalose, cellobiose, methyl- $\beta$ -D-glucopyranoside, esculin) were investigated as inducers of myrosinase. In addition sinigrin was extracted with boiling water directly from ground seed. The disadvantage of using this method is the presence of impurities such as sugars and the presence of coloured material.

A relatively cheap commercially available source of sinigrin was available (Apin Chemicals) and was used for most of the large scale culture experiments (Chapter 4). Previous work has also utilised sinigrin for induction and purification of bacterial *Enterobacter cloacae* no. 506 myrosinase (Tani et al., 1974). *Citrobacter* cells can grow reasonably well on M9 medium supplemented with 0.4% (10 mM) sinigrin. This is close to concentration used to cultivate *Bacteroides thetaiotaomicron* II8 (Elfoul et al., 2001); however, the latter was grown on highly nutritious broth (brain-heart infusion broth) supplemented with yeast extract and haemin in addition to sinigrin.

Unlike the plant sweet almond glucosidase (Golden and Byers, 2009) and *Sphingobacterium* sp. strain OTG1 (Meulenbeld and Hartmans, 2001) which are known to hydrolyse octyl  $\beta$ -D-1-thioglucopyranoside, *Citrobacter* myrosinase did not hydrolyse this compound *in vitro*. *Citrobacter* also failed to grow on M9 supplemented with this substrate as a sole source of carbon. This is probably due to the octyl group which is

long and may prevent the molecule from reaching the myrosinase active site. In addition it lacks a sulphate group which may be essential for substrate recognition.

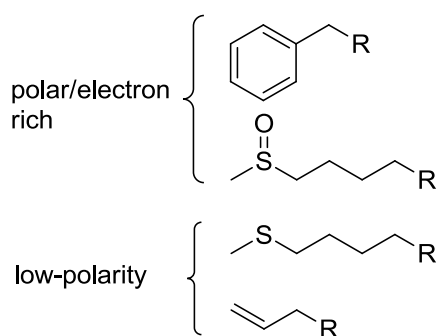
*Citrobacter* cells grow well on cellobiose (OD >0.8) and methyl- $\beta$ -D-glucopyranoside (OD >0.7) as a carbon source but lower growth was observed for esculin (OD >0.3). Bacterial  $\beta$ -glucosidases are often incorporated into large complexes known as cellosomes that contain polysaccharide degrading endoglucanases and proteins (for carbohydrate binding) to localize the cellosome and to attach it to the cellulose surface and the cell membrane. Fungal  $\beta$ -glucosidases are secreted enzymes while the cellular locations of bacterial  $\beta$ -glucosidases are less clear (Nisizawa et al., 1971; Wood and McCrae, 1982). Some authors have reported that some  $\beta$ -glucosidases are cell bound while others reported the equal distribution between intracellular and extracellular compartments (Lequerica et al., 1992). In this study cellobiase and other beta glucosidase activity that responsible for degrading methyl- $\beta$ -D-glucopyranoside could not be detected in cell free protein extracts of *Citrobacter*. This is in contrast to *Paenibacillus polymyxa* formerly *Bacillus polymyxa*, reclassified by (Ash et al., 1993) that possesses extracellular cellobiase and intracellular cellobiose phosphorylase (González-Candelas et al., 1989). It's more likely that *Citrobacter* produces a membrane bound  $\beta$ -glucosidase that doesn't exist in cell free protein extracts and hydrolyses the  $\beta$ -glucosidic bond within cellobiose and methyl- $\beta$ -D-glucopyranoside molecules. To prove this assumption the cell debris following lysis should have been assayed for such activity. A membrane bound  $\beta$ -glucosidase has been purified from the mould *Physarum polycephalum* (Hayase et al., 2008). Myrosinase activity could not be detected in the cell free extract of these two substrates indicating that neither cellobiose nor methyl- $\beta$ -D-glucopyranoside can be used in induction of *Citrobacter* myrosinase. A similar results was found for the partially purified  $\beta$ -glucosidase of *Erwinia herbicola* Y46 (Garibaldi and Gibbins, 1975). Poor growth was

recorded from *pNPG* ( $\alpha$  &  $\beta$  forms) and methyl- $\alpha$ -D-glucopyranoside, for the *pNPG* ( $\beta$  form) the colour of the medium (M9 plus *pNPG*) changed from colourless to yellowish indicating that the enzyme myrosinase is expressed by this substrate and hydrolysed it to minimum levels. There was; however, no increase in cell density after longer incubation (24 h) possibly due to the toxic effect of released phenol (yellow colour) that inhibited growth of *Citrobacter*. The highest myrosinase expression was seen in the sinigrin culture. In comparison to sinigrin all tested substrates failed to induce *Citrobacter* myrosinase to or similar levels possibly due to the lack of sulphate or thio group that exists in the GLS substrate. A mixture of sinigrin and cellobiose was also investigated for *Citrobacter* myrosinase induction, *Citrobacter* cells multiply faster in the presence of cellobiose (7.5 mM) and sinigrin (varied concentration) in the culture medium and myrosinase activity is only increased as the sinigrin concentration increased. It is evident that *Citrobacter* myrosinase is induced by sinigrin but not by cellobiose despite the obvious increase in OD (Figure 2.3). This is supported by the induction experiment (Fig. 2.2) which has shown that sinigrin cannot be replaced by any of the tested substrates including cellobiose. There was a small decline in protein concentration which may be due to proteolysis or could be consequence of error. Perhaps one should run a time course to monitor disappearance and degradation of both cellobiose and sinigrin in the growth medium by HPLC, this may give a further explanation. Interestingly sinigrin seems to induce not only myrosinase in *Citrobacter* but also trehalase with higher specific activity (0.16 nmole/mg protein/ min) compared with cells grown on trehalose (0.11 nmole/mg protein/ min) induced cells (Table 2.2). Induction of *Citrobacter* myrosinase by using a different culture medium other than M9 was not investigated. The use of enrichment medium (NB) did not yield an increase in myrosinase expression (Figure 2.2)



The metabolism of sinigrin by *Citrobacter* both *in vivo* and *in vitro* has been studied; the product ITC was clearly produced by cell free protein extracts but hardly produced by the resting cell and it is likely that the ITC is being further metabolised. The absence of ITC in the resting cells experiment doesn't mean that it has not been released; indeed Palop et al. (1995) suggested that ITC can be further metabolised (Palop et al., 1995). Chu (2009) used NMR to establish the metabolism of sinigrin *in vivo* for both *Citrobacter* and *Lactobacillus agilis*. It was clear from that work that each bacterium metabolises sinigrin in a very different way with *Lactobacillus agilis* producing ITCs while *Citrobacter* produced an unknown metabolite. The study concluded that the sinigrin is metabolized within 28 h and converted into an unknown product which was not comparable to allyl cyanide (nitrile), allylamine or isothiocyanate standards.

Substrate specificity studies were carried out with four different glucosinolates glucoerucin, glucotropaeolin, glucoraphanin and sinigrin. It would appear that myrosinase catalyses the hydrolysis of sinigrin and glucoerucin at a faster rate than glucotropaeolin and glucoraphanin (Figure 2.4). These differences must be due to the functionality of the side chain (Figure 2.12) which most likely impact on the binding of these molecules to the active site.



**Fig 2.12** Side chain of glucosinolate used in substrate specificity.

*Citrobacter* metabolised the whole amount of sinigrin (10 mM) which was the only carbon source in M9 medium during 12 h of cultivation; however, when the supplemented amount of sinigrin was reduced to 6 mM in the presence of glucotropaeolin (2 mM) sinigrin was completely metabolised while approximately 39% of glucotropaeolin was available in the medium; however, there was no further growth (Figure 2.9 & 2.10). Unlike the three *Bifidobacteria* species which were reported to digest these two glucosinolates after 24-48 h of cultivation these substrates were added separately (Cheng et al., 2004). The mixture of gluconasturtiin (2 mM) and sinigrin (6 mM) in M9 medium indicated that sinigrin completely metabolised after 25 h cultivation while 20% of gluconasturtiin was available. This indicates that *Citrobacter* prefers sinigrin over gluconasturtiin and glucotropaeolin respectively. Ideally one would have done this experiment by cultivating *Citrobacter* on a medium contains a single GLS as sole source of carbon source but previous data obtained by Dr Rossiter showed that growth was poor on these substrates. Thus while the *Citrobacter* myrosinase is very efficient in metabolising sinigrin it does seem that the nature of the side chain is very important in determining metabolism. From figure 2.4 it can be seen that the specific activity of *Citrobacter* myrosinase is affected by the nature of the side chain and it appears to confirm the substrate specificity in the *in vivo* culture, at least with the glucotropaeolin.

## **CHAPTER THREE**

### ***CITROBACTER* $\beta$ -O-GLUCOSIDASE AND MYROSINASE CHARACTERISATION**

### 3.0 Introduction

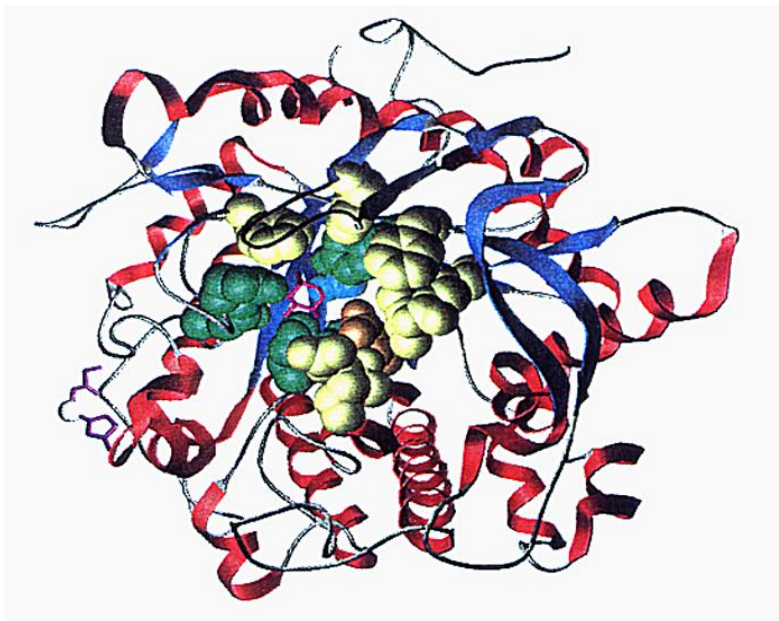
#### Beta Glucosidase

As discussed in Chapter 1  $\beta$ -glucosidases represent a major group of enzymes among glycoside hydrolases. Plant  $\beta$ -glucosidases catalyse the hydrolysis of substrates with a  $\beta$ -O-glucosidic linkage and this type of enzyme is placed in glucohydrolase (GH) family 1 ( $\beta$ -D-glucoside glucohydrolase, EC 3.2.1.21). Other types of plant  $\beta$ -glucosidase hydrolyse S-linked  $\beta$ -glycosidic bonds (e.g. glucosinolates) and are placed in GH family 1 ( $\beta$ -D-thioglucoside glucohydrolase, EC 3.2.3.1) and known by the trivial name myrosinase (Patchett et al., 1987; Esen, 1993; Botti et al., 1995; Czjzek et al., 2000).

#### 3.1 Myrosinase properties

Myrosinase was first discovered in mustard seed in 1840 (Bussy, 1840) and is also found in the intestinal bacteria *Enterobacter cloacae* (Tani et al., 1974) and *Paracolobactrum aerogenoides* (Oginsky et al., 1965), in the fungi *Aspergillus sydowi* (Reese et al., 1958) and *Aspergillus niger* (Ohtsuru et al., 1973), in the cruciferous aphids *Brevicoryne brassicae* and *Lipaphis erisimi* and in mammalian tissues (Bones and Rossiter, 1996). Based on amino acid sequence similarities with  $\beta$ -O-glucosidases myrosinases belong to GH family 1 along with a number of  $\beta$ -O-glycosidases. Members of GH1 represent 18 different substrate specificities (Burmeister et al., 1997). Plant myrosinase is a glycoprotein and usually exists as a dimer with subunit molecular masses of 60–75 kDa. A number of plant myrosinases have been isolated from different Brassicaceae species, mainly *Lepidium sativum* (garden cress) seedlings, *Sinapis alba* seeds (white mustard) and *B. napus* seeds (rapeseed). White mustard and rapeseed myrosinases are dimeric proteins with molecular weights between 120-150 kDa. Cress myrosinase was examined against 29 different glucoside substrates

and only two GLS substrates 2-propenyl (sinigrin) and benzyl (glucotropaeolin) were hydrolysed both at equal rates while the rate for the chromogenic glucoside (*p*NPG) was much less (Durham and Poulton, 1990). The  $K_m$  values for cress myrosinase with sinigrin, glucotropaeolin and *p*NPG are 300  $\mu$ M, 295  $\mu$ M 2.0 mM respectively. White mustard myrosinase hydrolysed sinigrin and has a  $k_m$  of 0.17 mM. The sulphate group is a prerequisite for *S. alba* myrosinase activity and it does not hydrolyse desulfated glucosinolate. Myrosinase isoenzymes have a broad pH optima ranging from pH 5.0-7.0 (Björkman and Lönnnerdal, 1973; Durham and Poulton, 1990). Plant myrosinases are strongly activated by L-ascorbic acid, however, higher concentrations inhibit the enzyme (Bones and Rossiter, 1996). The optimum ascorbate concentration for *B. napus* myrosinase is 0.3 - 0.5 mM (Bones and Slupphaug, 1989). The enzyme activity is only increased in the presence of sinigrin as a substrate. The hydrolysis rate of *p*NPG is not increased in the presence of ascorbate. Most plant myrosinases are inhibited by -SH directed reagents implying that a cysteine residue is essential for catalysis (Botti et al., 1995). The use of X-ray crystallography for the three dimensional structure of *S. alba* myrosinase (Figure 3.1) (Burmeister et al., 1997) has facilitated an understanding of the mechanism of plant myrosinase. The crystallised myrosinase belongs to the MA subfamily (Xue et al., 1992). The myrosinase is a dimer consisting of two identical subunits, each with more than 499 amino acid residues linked by a zinc ion ( $Zn^{2+}$ ). Each subunit has three disulfide bridges that possibly contribute to myrosinase stability. A  $(\beta/\alpha)_8$  barrel structure is present in myrosinase and this is a common feature of the  $\beta$ -O-glycosidases (GH1). The 3D structure shows a substantial number of hydrogen bonds and salt bridges between neutral and charged atoms. This has been linked with thermostability and stability against denaturation under high conditions of salt. The dimer has a large amount of carbohydrate 13 kDa which is probably required for maintaining solubility and molecular stability in the dehydrated seed environment. The study has reported that myrosinase is very soluble and can be precipitated at 66% of saturated ammonium sulphate (Burmeister et al., 1997).



**Fig 3.1** 3D structure of *Sinapis alba* myrosinase (adapted from Rask et al 2000 based on Burmeister et al. 1997). Blue is a secondary structure element  $\beta$  sheet,  $\alpha$  helices are shown in red, green shows residues involved in glucose-ring recognition. Aglycone recognizing residues are in yellow.

The  $(\beta/\alpha)_8$  barrel structure forms a hydrophobic substrate pocket which fits the side chain of glucosinolates. The two conserved arginine residues in the myrosinase active site are important for positioning and correct binding for the sulphate group of the substrate. This site probably specifies myrosinase as an S-glycosidase. Myrosinase belongs to the GH1 family it and shows structural and sequence similarity with the O- $\beta$ -glucosidases of this family. Similar properties are found in the  $\beta$ -glucosidase linamarase from *Triticum repens*.

Plant myrosinase contains a glutamic residue (Glu 409) which acts as a catalytic nucleophile and attacks the sugar ring (C-1) of the substrate to form a glycosyl enzyme-intermediate. This is followed by the release of the aglycone which autoarranges to give an isothiocyanate and loss of sulphate. The sulphate group and glucose are involved in recognition, the vital interactions between myrosinase and substrate are established by all hydroxyl groups in the sugar moiety. The aglycone is

an excellent leaving group; in the deglycosylation step the myrosinase mechanism is different from other  $\beta$ -O-glucosidases. This is due to replacement of a glutamate residue with glutamine (Gln 187) (Burmeister et al., 1997). Reports of previous work suggested that ascorbate created an allosteric effect with myrosinase, however, based on elegant work by Burmeister et al., 1997 it was shown that ascorbate acts as a catalytic base (Bones and Rossiter, 2006). In contrast to plant myrosinase, aphid myrosinase is not activated with ascorbate. It is more like a  $\beta$ -O-glucosidase than  $\beta$ -S-glucosidase (myrosinase) (Jones et al., 2001; Bones and Rossiter, 2006) yet has a similar  $K_m$  to that of ascorbate-activated plant myrosinase. Bacterial species of several different genera produce  $\beta$ -S-glucosidase which have been characterised to varying degrees. *Enterobacter cloacae* no. 506 is the only bacterium reported to possess myrosinase activity that has been purified to homogeneity. The general characteristics of *Enterobacter cloacae* no. 506 myrosinase have also been investigated. These included the effects of pH, temperature, metal ion, ascorbate and inhibitors (Tani et al., 1974). Although the myrosinase was purified no sequencing data was available limiting our knowledge of the structural characteristics of this enzyme.

### 3.2 Hypothesis

*Citrobacter* sp. contains both  $\beta$ -S-glucosidase and  $\beta$ -O-glucosidase activity (Chapter 2) and is active towards glucosinolates.

### 3.3 The aim of this section

- Characterise *Citrobacter*  $\beta$ -S-glucosidases and compare with previous data (of plant, fungus & aphid myrosinases) in terms of pH, temperature and effect of ascorbic acid.
- Investigate the kinetics in terms of substrate specificity for *Citrobacter*  $\beta$ -glucosidase using a  $\beta$ -S-thioglucoside substrate (sinigrin), a  $\beta$ -O-glucoside substrate (salicin) and a synthetic aryl  $\beta$ -O-glucoside (pNPG).
- Examine the activity of *Citrobacter* enzyme with different disaccharide substrates.



### 3.4 Methods

All *Citrobacter*  $\beta$ -glucosidase used in this section was obtained from a crude extract. The *Citrobacter* crude protein extract was prepared as previously described (Chapter 2) and desalted on an Econo-Pac 10DG Desalting Columns (BIORAD, USA) against citrate phosphate buffer (CPB) (20 mM, pH 6.0) before being concentrated using a spun column ultra filtration tube (10,000 NMWL, 4 mL UFC801024, Amicon ultra, Millipore, USA). Protein concentration was measured by the Bradford method. Myrosinase activity was assayed by incubating the desalted crude extract with glucosinolate (sinigrin) or phenethyl glucosinolate (gluconasturtiin) and the released glucose measured with the GOD Perid reagent (Chapter 2) while the activity of  $\beta$ -O-glucosidase was assayed by incubation of the crude protein extract with pNPG (Appendix A). All future reference to a crude protein extract refers to a desalted bacterial lysate.

#### 3.4.1 pH Optimisation for *Citrobacter* $\beta$ -glucosidase activity

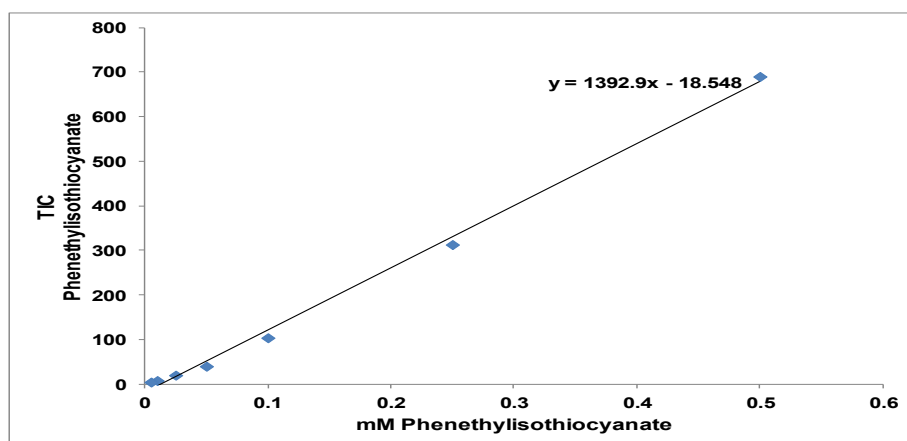
CPB (pH range of 3.6-7.6, 20 mM) was used to determine the optimum pH for *Citrobacter* myrosinase. The crude protein extract (5  $\mu$ g) was added to a mixture of sinigrin (0.2  $\mu$ M) in CPB to a final volume of 300  $\mu$ L at specific pH values (3.6, 4.0, 4.6, 5.0, 5.6, 6.0, 6.6, 6.8, 7.0 and 7.6) and incubated at 37 °C for 2 h. The activity was stopped by boiling the mixture for 5 min and after cooling glucose was assayed by the GOD Perid method (Appendix A).

### 3.4.2 The optimum temperature of *Citrobacter myrosinase*

The effect of temperature on the activity *Citrobacter* myrosinase was measured at different temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 ° C) by mixing a total volume (300  $\mu$ L) of the crude protein extract (1 $\mu$ g), sinigrin in a final concentration (0.2  $\mu$ M) and CPB (20 mM, pH 6.0) was incubated at the specified temperature for 30 min Glucose was assayed by the GOD Perid method (Appendix A).

### 3.4.3 Effect of ascorbate on *Citrobacter myrosinase*

A mixture of the crude protein extract (16  $\mu$ g) and CPB (20 mM, pH 6.0.) was incubated (30 °C, 1h) with ascorbate concentrations in the range 0.025-20 mM and gluconasturtiin glucosinolate (0.5 mM) to give a total volume of 300  $\mu$ L. ITC was extracted with DCM (see chapter 2). ITCs were detected by GC-MS (Chapter 2) and calculated from the standard graph Figure 3.2.



**Fig 3.2** Calibration graph for phenethyl isothiocyanate. TIC is the total ion current.

### **3.5 Activity & inhibition study of *Citrobacter* $\beta$ -glucosidase**

#### **3.5.1 Effect of glucose on $\beta$ -O-glucosidase activity**

The crude protein extract (1  $\mu$ g) to final volume 300  $\mu$ L was added to various concentrations of glucose (0, 2, 4, 6, 8, and 10 mM) in CPB (20 mM, pH 6.0) with *p*NPG (0.2  $\mu$ M) and incubated (25°C, 30 min). The reaction was terminated by the addition of Na<sub>2</sub>CO<sub>3</sub> (1 mL, 100 mM). The formed *p*-nitrophenol (yellow – orange colour) was measured at 420 nm using a spectrophotometer (Chapter 2).

#### **3.5.2 *Citrobacter* $\beta$ -O-glucosidase activity with disaccharides and sugars**

Sugars (trehalose, maltose, rhamnose, sucrose, lactose, cellobiose and galactose) (0.2  $\mu$ M) were added to CPB (20 mM, pH 6.0) together with crude protein extract (1  $\mu$ g) in a total volume of 300  $\mu$ L and incubated (25 °C, 120 min). The reaction was stopped by boiling (5 min). The released glucose was measured using the GOD Perid method (Chapter 2).

#### **3.5.3 Effect of metal ions & EDTA on $\beta$ -O-glucosidase activity towards *p*NPG**

The crude protein extract (3 $\mu$ g) was pre-incubated (25 °C, 30 min) with EDTA or metal ions (ZnSO<sub>4</sub>, CaCl<sub>2</sub>, MgCl<sub>2</sub> and FeCl<sub>3</sub>) (0.06  $\mu$ M) before the addition of *p*NPG (0.6  $\mu$ M) and the buffer (CPB 20 mM, pH 6.0.) in a total volume of 300  $\mu$ L then incubated (25 °C, 60 min). The reaction was terminated by the addition of Na<sub>2</sub>CO<sub>3</sub> (1 mL, 100 mM). The

formed yellow – orange colour was measured at 420 nm using a spectrophotometer (Chapter 2).

### **3.6 Determination of $K_m$ & $V_{max}$ for sinigrin, salicin and *p*NPG**

#### **3.6.1 Sinigrin & salicin**

Varying concentrations (0.1- 5 mM) of sinigrin and salicin (Appendix B) were incubated (25 °C, 10 min) with crude protein extract (2.1  $\mu$ g) and CPB (20 mM, pH 6) in a total volume 300  $\mu$ L. The reaction was stopped by boiling (5 min) and cooled before the GOD Perid reagent (1 mL) was added and incubated (15 min, 37 °C) before the absorbance measurement (420 nm) was recorded (Appendix A).

#### **3.6.2 *p*-Nitrophenyl- $\beta$ -D-glucopyranosiduronic acid (*p*NPG)**

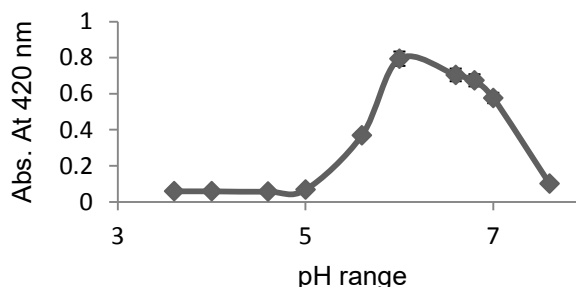
A stock solution of *p*NPG was prepared at a concentration of 30 mM (Appendix B). From this stock solution aliquots were mixed with the CPB (20 mM, pH 6.0) and the crude protein extract (1.11  $\mu$ g) to a final volume of 300  $\mu$ L and incubated (25 °C, 1h). The reaction was stopped by addition of  $\text{Na}_2\text{CO}_3$  (1 mL, of 100 mM). The formed yellow-orange colour was measured on a spectrophotometer (420 nm).

### 3.7 Results

Since the substrate used to assay the *Citrobacter*  $\beta$ -glucosidase in the following experiments was a  $\beta$ -S-glucoside (glucosinolate) all obtained data in these experiment represent myrosinase activity. This includes pH and temperature optima, effect of ascorbate and the  $k_m$  value of sinigrin. The other experiments represent the activity of *Citrobacter*  $\beta$ -O-glucosidase although it is possible that both activities arise from the same enzyme.

#### 3.7.1 Optimum pH for *Citrobacter* myrosinase

The optimum pH for *Citrobacter*  $\beta$ -glucosidase activity was determined over a range of pH using CPB. The absorbance at 420 nm of the GOD Perid reaction was used as a measure of activity (Chapter 2). The shape of the pH profile was not symmetrical and suggests a plateau of activity from pH 6-7. Maximum activity was at pH 6 and dropped off markedly after pH 7 and was inactive at a pH <5 (Figure 3.3).

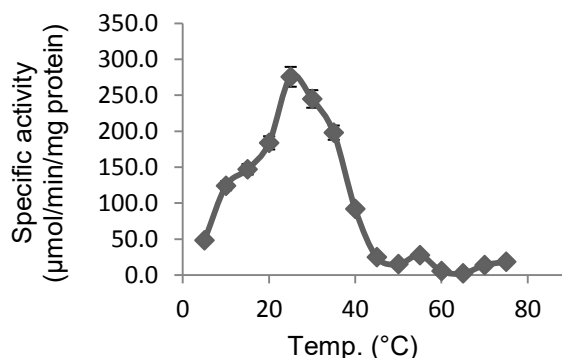


**Fig 3.3** The pH optimisation of *Citrobacter*  $\beta$ -glucosidase, the crude protein extract (5  $\mu$ g) was mixed with sinigrin (0.2  $\mu$ M) and buffer then incubated (25  $^{\circ}$ C, 30 min). The reaction was stopped by boiling (5 min) then the GOD Perid reagent (1 mL) was added and incubated (37  $^{\circ}$ C, 15 min). The highest activity was indicated by the absorbance reading at 420 nm.

Several structural features may contribute to the stability of myrosinase at its optimum pH. These include the presence of numerous salt bridges, the reduced exposed surface due to dimerisation of the myrosinase and three disulfide bonds (Burmeister et al., 1997). Myrosinase optimum pH is dependent on its origin. Broccoli myrosinase has an optimum pH of 6.5-7.0 and this value corresponds to the pH found in fresh broccoli juice (Ludikhuyze et al., 2000). Many bacteria produce  $\beta$ -O-glucosidases with optimum pHs similar to that of the *Citrobacter* myrosinase such as the extremely thermophilic anaerobe Wai21W.2 (Patchett et al., 1987); however, this  $\beta$ -O-glucosidase has not been reported to possess myrosinase like activity. Generally bacterial  $\beta$ -O-glucosidases have optimum pH values in the range 5.5-7.0 (LI et al., 1998) and are higher than the fungal  $\beta$ -glucosidases (Patchett et al., 1987). The pH of optimum activity of *Citrobacter* myrosinase (pH 6) is comparable with plant myrosinase of Broccoli (pH 6.5-7.0).

### 3.7.2 Determination of optimal temperature for *Citrobacter* myrosinase

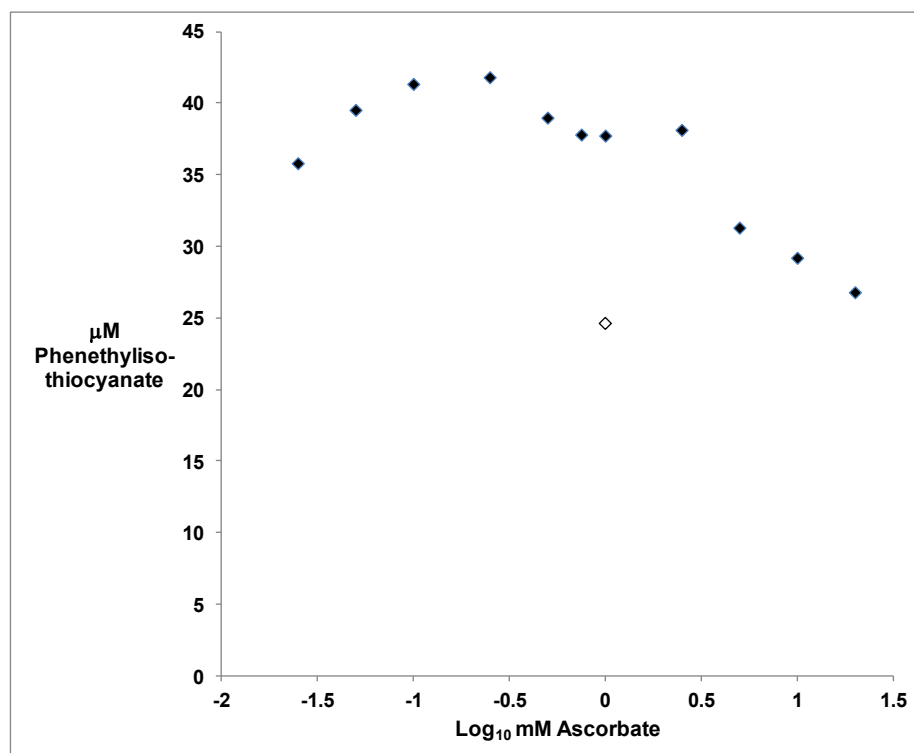
In addition to conformational changes in myrosinase induced by changes in pH, activity is also dependent on other factors such as temperature which may be related to the environment of the organism (Ludikhuyze et al., 2000). In general higher temperature creates more collisions among the molecules and therefore increases the rate of a reaction. More collisions increase the possibility that substrate will collide with the active site of the enzyme, hence increasing the rate of an enzyme-catalyzed reaction. The affinity of the enzyme for the substrate will also be important in determining the reaction rate. The rate of chemical reactions therefore increases with temperature but then decreases as enzymes denature and the structure of the protein becomes random. The *Citrobacter* myrosinase was found to be active over a range of temperatures from 5-40°C (Figure 3.4); however, the optimal activity was 25 °C.



**Fig 3.4** Determination of optimum temperature for *Citrobacter's* myrosinase, sinigrin (0.2  $\mu$ M) was mixed with the buffer (CPB, 20 mM, pH 6.0) and preincubated (30 min) at the examined temperature then the crude protein extract (2.5  $\mu$ g) was added, the reaction was stopped by boiling (5 min) and GOD Perid reagent (1 mL) was added and incubated (37  $^{\circ}$ C, 15 min). The highest activity was recorded at 25  $^{\circ}$ C.

### 3.7.3 Effect of ascorbic acid on *Citrobacter* myrosinase

The purpose of this assay was to determine if ascorbate activates bacterial myrosinase. The reason for this is that plant myrosinases are strongly activated by ascorbate (Burmeister et al., 2000) and there have been reports of ascorbate activation of a microbial myrosinase (Cheng et al., 2004).  $\beta$ -O-glucosidases are not known to require ascorbate (Ketudat Cairns and Esen, 2010). From Figure 3.5 there appears to be very a slight activation of the *Citrobacter* myrosinase which peaks at 0.25 mM. The data suggest that this marginal increase in activity may be a feature of *Citrobacter* myrosinase or may just reflect changes in tertiary structure as a consequence of increasing solute (ascorbate) concentration. The ascorbate assay was determined by GC-MS as the GOD Perid reaction is inhibited by ascorbate.



**Fig 3.5** The determination of the ascorbate optimal concentration for *Citrobacter* myrosinase using gluconasturtiin glucosinolate (0.5 mM) as a substrate. The enzyme crude protein extract (16  $\mu$ g) reaction mixture was incubated at 30°C for 1 hr. The concentration of substrate was 0.5 mM and ascorbate concentrations are expressed as the  $\log_{10}$  over a range of 0.025 – 20 mM. The unfilled diamond represents the control reaction with no ascorbate. Gluconasturtiin isothiocyanate was determined by GC-MS (Chapter 2).

Ascorbate is known to stimulate the activity of plant myrosinases (Burmeister et al., 2000) and the mechanism of this reaction was determined using X-ray structural analysis of *Sinapis alba* myrosinase. It was found that ascorbate acts as a surrogate catalytic base for the enzyme. The myrosinase inhibitor 2-fluoroglucotropaelin-2-deoxybenzylglucosinolate (Cottaz et al., 1997) was used to trap the glutamate residue (Glu 409). Earlier (Ohtsuru and Hata, 1979) had suggested that ascorbate caused an allosteric effect on the enzyme activity. The activation factor for *Citrobacter* at 0.25 mM ascorbate was (maximum  $\mu$ M PITC/control  $\mu$ M PITC) was 1.6 fold and this value was much lower than that of *Bifidobacter* sp. myrosinase with an activation factor of 6 fold

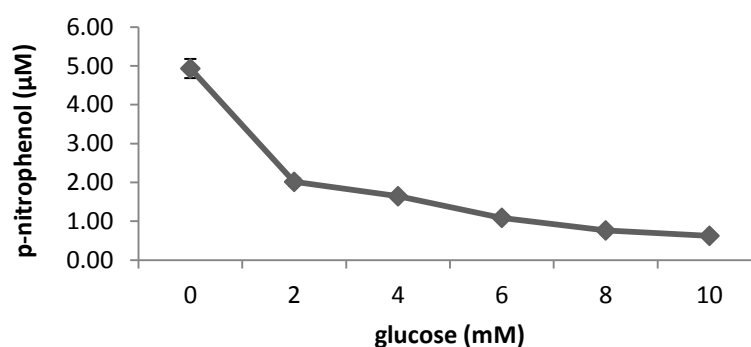


(Cheng et al., 2004) and not comparable with plant myrosinase of *Brassica napus* (40 fold) (Björkman and Lönnnerdal, 1973).

#### 3.7.4 Inhibition of *Citrobacter* $\beta$ -O-glucosidase by glucose

Glucose has been shown to inhibit the action of many microbial  $\beta$ -glucosidases (Saha and Bothast, 1996). As the GOD Perid reagent/assay measures glucose it was decided to use *p*NPG as a substrate.

The pre-incubation of *Citrobacter*  $\beta$ -O-glucosidase with different glucose concentrations decreased the amount of *p*-nitrophenol released from *p*NPG. Although glucosidase activity was detected at all glucose concentrations, at 2mM, the activity was reduced to approximately 50% of the control and was reduced by 90% at 10 mM of glucose (Figure 3.6).



**Fig 3.6** Glucose effect on *Citrobacter*  $\beta$ -glucosidase. The crude protein extract (1  $\mu$ g) was mixed with CPB (20 mM, pH 6.0) and glucose and incubated (25  $^{\circ}$ C, 30 min) after which *p*NPG (60  $\mu$ L of 10 mM) was added and incubated (25 $^{\circ}$ C, 30 min). The reaction was stopped by addition of Na<sub>2</sub>CO<sub>3</sub> (1 mL of 0.1 M). The released *p*-nitrophenol was measured (420 nm).

3.7.5 *Citrobacter* enzymic activity with sugars

The crude protein extract of *Citrobacter* hydrolysed  $\beta$ -D (+) trehalose which is made up of two glucose units linked by  $\alpha$ -glucoside bond but had no activity towards maltose (4-0- $\alpha$ -D-glucopyranosyl- $\alpha$ -glucose), sucrose (glucose 1 $\alpha$ → $\beta$  2 fructose), lactose (galactose 1  $\beta$  →4 glucose) cellobiose (composed of two glucose linked by  $\beta$ -glucoside) and galactose (Table 3.1).

Sugar	Enzyme activity
Trehalose {glu( $\alpha$ 1- $\alpha$ 1) glu}	+
Maltose {glu( $\alpha$ 1-4)glu}	-
Sucrose {glu( $\alpha$ 1- $\beta$ 2) fru}	-
Lactose {gal( $\beta$ 1-4)glu}	-
Cellobiose {glu( $\beta$ 1-4)glu}	-
Galactose	-

**Table 3.1** Activity on disaccharides and other sugar derivatives. The crude protein extract (1  $\mu$ g) was mixed with the sugar (final concentration of 0.2  $\mu$ M) and CPB (20 mM, pH 6.0) and incubated (25 °C, 120 min), GOD Perid reagent (1 mL) was used for detection of glucose using spectrophotometer at 420 nm.

3.7.6 Effect of metal ions and EDTA on *Citrobacter*  $\beta$ -glucosidase

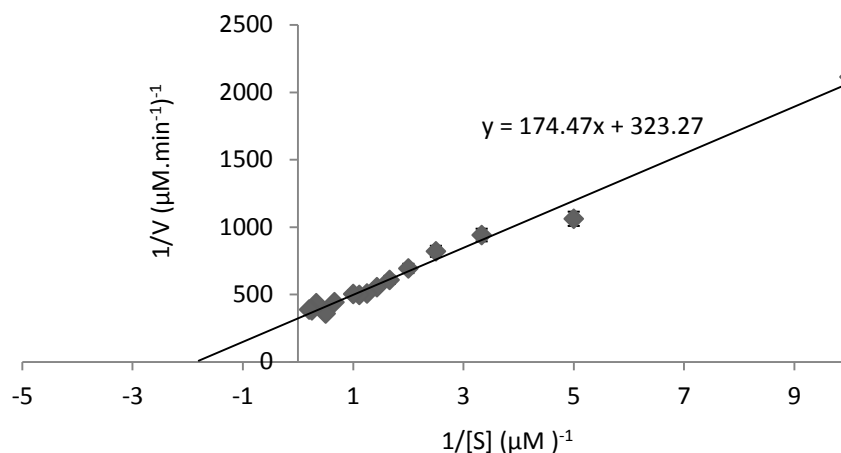
The divalent ions Zn<sup>2+</sup>, Ca<sup>2+</sup>, Fe<sup>3+</sup> and Mg<sup>2+</sup> inhibited the enzyme reaction to varying degrees with Fe<sup>3+</sup> causing the largest loss (76%) of activity followed by Mg<sup>2+</sup>, Zn<sup>2+</sup> and Ca<sup>2+</sup> while the metal ion chelator EDTA was also inhibitory (70%) (Table 3.2).

Metal salt	Relative activity (%)
0	100
ZnSO <sub>4</sub>	35
EDTA*	30
CaCl <sub>2</sub>	51
MgCl <sub>2</sub>	32
FeCl <sub>3</sub>	24

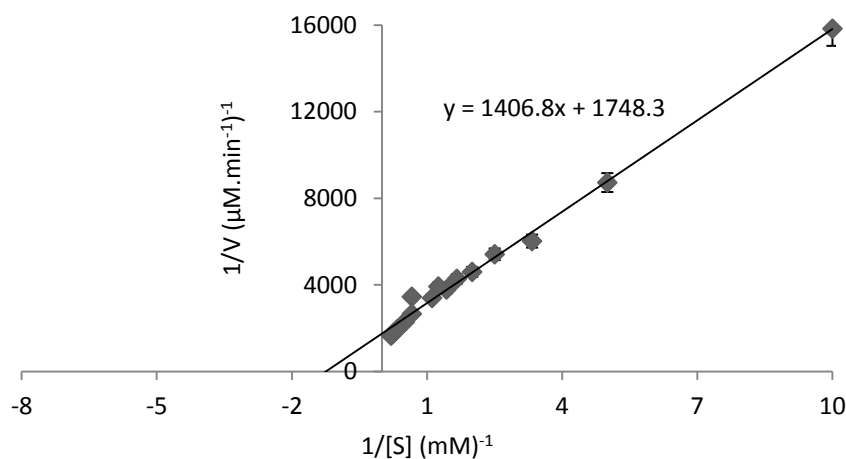
**Table 3.2** The effect of metal ion\* (EDTA non metal). All ions were added as chloride salts except Zn<sup>2+</sup> which was added as sulfate salt. The crude protein extract (2  $\mu$ g) in CPB (20 mM, pH6.0) was preincubated (30 min) with ions or EDTA (2  $\mu$ M) before the addition of the substrate *p*NPG (60  $\mu$ L). The reaction was stopped by addition of Na<sub>2</sub>CO<sub>3</sub> (1mL of 0.1M). The released *p*-nitrophenol was measured on a spectrophotometer at 420 nm.

### 3.7.7 Determination of $K_m$ and $V_{max}$ values of *Citrobacter* $\beta$ -glucosidase

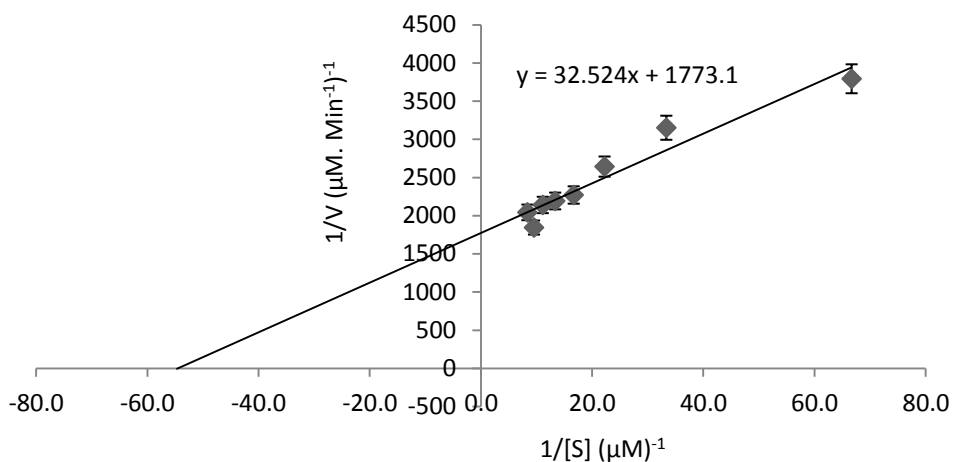
$K_m$  values are most useful in analyzing the ability of an enzyme to bind substrate. The apparent  $K_m$  and maximum velocity ( $V_{max}$ ) values for the *Citrobacter* myrosinase were calculated from X and Y intercept of the Lineweaver-Burk plots, where the reciprocal substrate concentration  $1/S$  ( $\mu$ M)<sup>-1</sup> was plotted against the reciprocal initial reaction rate  $1/V$  ( $\mu$ M.min<sup>-1</sup>)<sup>-1</sup>. The  $K_m$  were found to be 0.54 and 0.8 mM respectively for sinigrin and for salicin. For *p*NPG it was found to be 0.0183 mM indicating that the enzyme has a greater affinity for the aryl  $\beta$ -O-D-glucoside substrate than for sinigrin and salicin while the  $V_{max}$  were found to be 3.1, 0.6, and 0.526 nmole.L<sup>-1</sup>.min<sup>-1</sup> for sinigrin, *p*NPG and salicin respectively Figures (3.7, 3.8 and 3.9). Several studies on  $K_m$  values (sinigrin was used as a substrate) of different myrosinases showed different  $K_m$  values which for white mustard myrosinase is 0.42 mM (Björkman and Lönnnerdal, 1973) and for aphid myrosinase (cabbage aphid, *Brevicoryne brassicae*) is 0.613 mM (Jones et al., 2001). A higher  $K_m$  value was reported as 3.3-3.6 mM for fungal myrosinase toward sinigrin (Ohtsuru et al., 1969).



**Fig 3.7** Lineweaver-Burk for *Citrobacter* myrosinase at various sinigrin concentrations. The crude protein extract (2.1 μg) was incubated (25 °C, 10 min) with sinigrin and buffer (CPB 50 mM, pH 6.0). The released glucose was measured by GOD Perid method. The  $K_m$  value was found to be 0.54 mM;  $V_{max}$ , 3.1 nmol.L<sup>-1</sup>.min<sup>-1</sup>.



**Fig 3.8** Lineweaver-Burk plot for *Citrobacter* myrosinase at various salicin concentrations ( $y = 1406.8x + 1748.3$ ). The protein crude extract (2.1 μg) was incubated (25 °C, 10 min) with salicin and buffer (CPB 50 mM, pH 6.0). The released glucose was measured by GOD Perid method. The  $K_m$  value has been found to be 0.8 mM;  $V_{max}$ , 0.6 nmol.L<sup>-1</sup>.min<sup>-1</sup>.



**Fig 3.9** Lineweaver-Burk for *Citrobacter* myrosinase at various *p*NPG concentrations. The protein crude extract (1.11 μg) was incubated (60 min at 25 °C) with *p*NPG and buffer (CPB 20 mM, pH 6.0). The released *p*-nitrophenol was measured by at 420 nm using spectrophotometer. The  $K_m$  is 0.0183 mM;  $V_{max}$ , 0.526 nmol.L<sup>-1</sup>.min<sup>-1</sup>.

Fungal myrosinase such as *Aspergillus sydowi* has a greater affinity towards *p*NPG ( $K_m$ , 1 mM) than to sinigrin ( $K_m$ , 3.6 mM) while for *Aspergillus niger* a  $K_m$  of 1.5 mM towards *p*NPG and 3.3 mM towards sinigrin is reported (Ohtsuru et al., 1969).

### 3.6 Discussion

All *Citrobacter*  $\beta$ -glucosidase assays in this chapter were carried out using desalted cell free extracts obtained by using a French Press. Details are given in the purification Chapter 4. The crude protein extract may contain other  $\beta$ -glucosidases that possess similar optimum conditions to that of *Citrobacter* myrosinase; this includes pH value (pH 6.0) and temperature (25 °C) which were used in this study to assay *Citrobacter* myrosinase and hence may influence some data. Although all bacterial cell preparations were grown on sinigrin which is the specific inducer for *Citrobacter* myrosinase, the induction of other  $\beta$ -glucosidases cannot be ruled out (Chapter 2).

Ideally a pure *Citrobacter* myrosinase should be used for such characterisation but the cost of the commercial sinigrin required for large scale purification meant it was not feasible. Several authors reported the use of a bacterial crude protein extract for myrosinase investigations; Palop et al., (1995) tested the crude lysate of *Lactobacillus agilis* strain R16, Cheng et al., (2004) tested the crude lysate of three species of *Bifidobacteria*. The only bacterial myrosinase that has been characterised in a pure form is that of *Enterobacter cloacae* no. 506 (Tani et al., 1974).

In contrast to gram positive bacteria; *Citrobacter* is a gram negative bacterium that possess a thin cell wall (Frederiksen, 2005). The myrosinase/ $\beta$ -glucosidase obtained is a soluble enzyme that might be present in the periplasmic space which is the region between the inner (cytoplasmic) and outer membrane of gram negative bacteria. By contrast myrosinase like activity could not be detected in the cell free extract of *Lactobacillus agilis* strain R16; however, this activity was found with intact cells and has been linked to membrane associated enzyme of this gram positive bacterium (Palop et al., 1995).

The pH optima for most  $\beta$ -O-glucosidases lie in the range 4.0-7.5 but this depends on their cellular location and source (Ketudat Cairns and Esen, 2010). In general the pH optima for most bacterial  $\beta$ -O-glucosidases are reported to be acidic (Coughlan, 1985). Myrosinase-like activity has been reported in bacteria such as *Lactobacillus agilis* strain R16 (Palop et al., 1995), *Bifidobacterium* (*B. pseudocatenulatum*; *B. adolescentis*; *B. longum*) (Cheng et al., 2004), *Bacteroides thetaiotaomicron* (Elfoul et al., 2001), *Bacteroides vulgatus* (Rabot et al., 1993). However, the pH optima of myrosinases of these organisms have not been investigated. Several authors have reported the pH of the buffer that was used for extraction of the crude extract and the same buffer also used for enzyme assay; others have reported the pH of culture medium. The latter is not clear whether it was according to the pH optima for enzyme production or for the organism itself. Ohtsuru et al., (1969) reported the control of the culturing broth to pH (7.0) with sodium hydroxide in order to maximise myrosinase production of the fungus *Aspergillus sydowi* as it was found that decreases in the pH of the growth medium affected myrosinase production (Ohtsuru et al., 1969). Palop et al. (1995) reported the use of buffer of pH 6.5 for extracting *Lactobacillus agilis* crude lysate while Cheng et al., (2004) reported buffer of pH 7.0 for the three species of *Bifidobacterium*, Elfoul et al., (2001) reported pH 7.0 for the medium used for sub-culturing *Bacteroides thetaiotaomicron*. The only bacterial myrosinase characterised is that of *Enterobacter cloacae* no. 506 where the pH optimum is pH 6.8 (Tani et al., 1974). It seems that most if not all species of bacteria that have been reported possessing myrosinase-like activity have an optimal activity between pH 6-7. *Citrobacter* lies in this range and *Citrobacter* is grown in M9 medium at a pH of 6.8. Thus sinigrin will be hydrolysed by myrosinase in these growth conditions. The crude protein extract of these cells has an optimum activity at pH 6. This in agreement with the suggestion that isothiocyanates are produced at neutral pHs (Rask et al., 2000) and was true for *Citrobacter* myrosinase using a cell free extract (Chapter 2 Figure 2.5 & 2.11). This pH (6.0) optimum is very close to that of the

myrosinase of *Aspergillus niger* (pH 6.2) which was also reported as an intracellular enzyme (Ohtsuru et al., 1973) and is comparable with the myrosinase of *Brevicoryne brassicae* (cabbage aphid) (pH 5.5) (Jones et al., 2001). However, it is higher than the pH optima of plant myrosinase of *Brassica napus* (pH 4.5-5.4) but close to another plant myrosinase of *Sinapis alba* (pH 5.8) (Bones and Rossiter, 1996). It is also close to the optimum pH of other bacterial  $\beta$ -glucosidases such as those of *Brevibacterium* sp. strain R312 (pH 6.0) (Legras et al., 1989) and thermophilic anaerobe Wai21W.2 (pH 6.2) (Patchett et al., 1987). Differences in pH optima values between bacterial, fungus, plant and aphid myrosinases may be due to cellular locations as well as structural - mechanistic considerations.

Mesophilic  $\beta$ -glucosidases have temperature optima in the range 30-65 °C and may be inactivated above 55-70 °C (Ketudat Cairns and Esen, 2010). In this study, the temperature versus activity profile of *Citrobacter* crude extract shows a temperature optimum of 25 °C with 50% or greater residual activity from 20 to 35°C. Although *Citrobacter* is a soil isolate the optimum temperature for its myrosinase is similar to that of the bacterial myrosinase *Enterobacter cloacae* (human faeces isolate) that has an optimum activity at 37 °C. It is comparable with optimum temperature of myrosinases of other microbes such as the fungus *Aspergillus niger* 34 °C (Ohtsuru et al., 1973), and also comparable to the assay temperature for three species of *Bifidobacteria* (*B. pseudocatenulatum*; *B. adolescentis*; *B. longum*) (37 °C) (Cheng et al., 2004), and close to cabbage aphid (L.) (30 °C) (Jones et al., 2001). Generally the growth rates for mesophilic organisms (bacterial and fungal) have an optimum temperature below 30 °C and their activity decreases at higher temperatures (Pietikäinen et al., 2005). This applies to *Citrobacter* as it is a soil isolate of a cold climate.

Ascorbic acid at concentrations between 0.7-5.0 mM are known to stimulate the activity of six partially purified plant myrosinases (Wilkinson et al., 1984). The presence of 0.5 mM (Figure 3.5) of L-ascorbic acid in the crude extract pH 7.0 of *Bifidobacterium*



*spp* increased the hydrolysis rate of GLS sinigrin in a time dependent (30-120 min) manner (Cheng et al., 2004). In contrast ascorbate (0.4 mM) inhibited the bacterial myrosinase of *Enterobacter cloacae* no. 506 (Tani et al., 1974). The bacterial myrosinase of *Paracolobacterium aerogenoides* is also inhibited by ascorbate (0.03mM) (Oginsky et al., 1965). The myrosinase of the fungus *Aspergillus sydowi* is neither activated nor inhibited by ascorbate (3-7 mM) (Ohtsuru et al., 1969). Ascorbate (0.1- 20 mM) did not activate aphid myrosinase (Jones et al., 2001); therefore it was of interest to determine whether ascorbate activated *Citrobacter* myrosinase. *Citrobacter* myrosinase, like the cabbage aphid and bacterial myrosinase of *Enterobacter cloacae* and *Paracolobacterium aerogenoides* and fungal *Aspergillus niger* and *Aspergillus sydowi*, do not require ascorbate for activation (Oginsky et al., 1965; Ohtsuru et al., 1969; Tani et al., 1974; Jones et al., 2001). In contrast Cheng et al (2004) reported the activation of *Bifidobacter* myrosinase with ascorbate (0.5 mM), the activation factor for this bacterium was 6 fold which is four times higher than the activation factor for *Citrobacter* myrosinase (1.6). In contrast the plant myrosinase of *B. napus* was activated 40 fold (Björkman and Lönnnerdal, 1973) (Table 3.3).

Organism	Inhibition Ascorbate (mM)	Activation Ascorbate (mM)	Activation factor
<i>Citrobacter sp.</i>	-	0.25	1.6
<i>Bifidobacter adolescentis</i> JCM 7045	-	0.5	6
<i>Enterobacter cloacae</i> no. 506	0.4	-	-
<i>Paracolobacterium aerogenoides</i>	3	-	-
<i>Aspergillus sydowi</i>	-	-	-
Aphid myrosinase	-	-	-
Plant myrosinase ( <i>B. napus</i> )	-	0.7-5.0	40

**Table 3.3** Comparison of ascorbate effect on myrosinase activity of different origin including *Citrobacter*. References of each are mentioned in the text above.

Three enzymes are involved in production of ethanol from cellulose. These are endo-glucanase, exo-glucanase and  $\beta$ -O-glucosidase (Bommarius et al., 2008). First cellulose is cut to a fragment polymer by endo-glucanase followed by an exo-glucanase which hydrolyses these fragments into cellobiose which inhibits the exo-glucanase. Cellobiose is then degraded by  $\beta$ -O-glucosidase into glucose which is known to inhibit the  $\beta$ -glucosidase itself (Philippidis et al., 1993; Wyman, 1999).  $\beta$ -O-Glucosidases play an crucial role in bioethanol production in which high concentrations of glucose are released (> 10 %). Many microbial  $\beta$ -O-glucosidases are sensitive to such concentrations (Riou et al., 1998). For this reason an adequate level of  $\beta$ -glucosidase activity is required in this process (Sternberg et al., 1977). However, we have shown that the *Citrobacter*  $\beta$ -glucosidase does not hydrolyse cellobiose and therefore would not be a candidate enzyme for ethanol production. Despite this we tested the  $\beta$ -O-glucosidase for inhibition by glucose. The enzyme activity is decreased by 50% at 2 mM glucose concentration with a further decrease to about 90% at 10 mM concentration. This inhibitory effect of glucose could be the result of repression of  $\beta$ -glucosidase activity in *Citrobacter*. Concentrations above 10 mM were not investigated. Ideally this should be repeated but with glucosinolate (sinigrin) in order to assay the effect of glucose on the activity of *Citrobacter* myrosinase. *Citrobacter* myrosinase is not inhibited by low concentration of the released glucose (0.04  $\mu$ M) during activity assays used in the current study. Inhibition by 0.1 M glucose was reported for bacterial myrosinase of *Enterobacter cloacae* (Tani et al., 1974). According to the obtained data *Citrobacter*  $\beta$ -glucosidase is not ideal for bioethanol production, a process which requires  $\beta$ -glucosidase that can tolerate a high concentration of glucose as well as being able to hydrolyse cellobiose (10-20 %) (0.55-1.1M) (Riou et al., 1998). There have been no reports on the use of myrosinases as enzymes for producing glucose from disaccharides.

Neither sugars with a  $\alpha$  or  $\beta$  sugar configuration were hydrolysed by a crude protein extract of *Citrobacter* except for trehalose (discussed in induction, Chapter 2) which was slightly hydrolysed (Table 2.2 Chapter 2). Cellobiose is a good substrate for many bacterial  $\beta$ -O-glucosidases such as from *Alcaligenes faecalis* (Han and Srinivasan, 1969) and also fungal  $\beta$ -O-glucosidases such as the rumen fungus *Neocallimastix frontalis* EB 188 (Li and Calza, 1991) but is hardly hydrolysed by  $\beta$ -O-glucosidase of *Acetobacter xylinum* BPR2001 (Tahara et al., 1998). *Citrobacter* myrosinase grows well on M9 medium supplemented with cellobiose suggesting that *Citrobacter* produces a glucosidase that hydrolyses the  $\beta$ -O-glucoside of this sugar (discussed in Chapter 2). The cell protein extract of sinigrin-induced cells was totally inactive against disaccharides (cellobiose and lactose) and hence does not have cellobiase (EC 3.2.1.21) activity. This also means that *Citrobacter* myrosinase has no effect on cellobiose and supports the fact that cellobiose does not induce *Citrobacter* myrosinase (Chapter 2).

It has been found that metal ions have an inhibitory effect on *Citrobacter* myrosinase (Table 3.2). Metallic ions may react with the breakdown products or with GLS precursors, thus modifying the metabolites and related effects. The myrosinase of white mustard has been crystallised and the mechanism of the enzyme reaction elucidated. One key feature of this enzyme is that it is a dimer held together by  $Zn^{2+}$ . It is not clear if the  $Zn^{2+}$  is necessary for activity but it seems likely that it must have a role in the assembly of the active protein.

Based on substrate specificity  $\beta$ -glucosidases are classified into three classes, aryl  $\beta$ -glucoside in class (1), true cellobiase in class (2) and broad substrate specificity enzymes in class (3) (Krisch et al., 2010). Many  $\beta$ -glucosidases from different sources have been reported to have both aryl-  $\beta$ -glucoside activity and cellobiase activity, an

example of which is *Thermotoga neapolitana*. This phenomenon is observed in most of the GH family 1 of  $\beta$ -glucosidases. The Michaelis-Menton constant for *Citrobacter* myrosinase was estimated from a Lineweaver-Burk plot. The enzyme has greatest affinity to aryl- $\beta$ -glucoside pNPG ( $K_m$  0.0183 mM) while showing lower affinity towards salicin (0.8 mM) and sinigrin (0.54 mM). A similar result was reported for *Enterobacter cloacae* myrosinase; however, the latter has a greater affinity to sinigrin ( $K_m$  0.37 mM) than pNPG ( $K_m$  0.7 mM) (Tani et al., 1974). Theoretically since the crude lysate of *Citrobacter* which also contains myrosinase did not hydrolyse cellobiose (Table 3.1) but strongly hydrolysed pNPG it suggests this enzyme belongs to class 1 (aryl  $\beta$ -glucoside). On the other hand the enzyme hydrolyses sinigrin and hence a place in broad substrate specificity of class 3 is also true for this enzyme. *Citrobacter* myrosinase like fungal myrosinase of *Aspergillus sydowi* and *Aspergillus niger* which has a greater affinity to pNPG than to sinigrin. *Citrobacter* myrosinase closely resembles the plant myrosinase in its capability of hydrolysing  $\beta$ -thioglucoside and aryl  $\beta$ -glucoside; however, the affinity to sinigrin ( $K_m$ ) is higher in plant myrosinase than with *Citrobacter* myrosinase but not for pNPG where the latter has a greater affinity. A full peptide or gene sequence of *Citrobacter* myrosinase is necessary for proper classification of this enzyme. The maximum velocity ( $V_{max}$ ) of *Citrobacter* glucosidase for sinigrin ( $3.1 \text{ nmole.L}^{-1}.\text{min}^{-1}$ ) was higher than for pNPG ( $0.6 \text{ nmole.L}^{-1}.\text{min}^{-1}$ ) and salicin ( $0.526 \text{ nmole.L}^{-1}.\text{min}^{-1}$ ). Despite a lower (6 fold)  $V_{max}$  for pNPG than sinigrin the *Citrobacter* glucosidase has a greater affinity to pNPG. A similar result was reported for the aphid myrosinase (*Brevicoryne brassicae*) which has a lower  $V_{max}$  for epiprogoitrin (0.021 U/mg) than sinigrin (1.038 U/mg) but it has a greater affinity ( $K_m$ ) to the former (0.164 mM) than to latter (0.359 mM) (Frédéric et al., 2002).

## Conclusion

The purpose of using two glucosinolate substrates (sinigrin & gluconasturtiin) was to assay the crude extract of *Citrobacter* for  $\beta$ -S-glucosidase (myrosinase) action. Thus the obtained data for the pH, temperature optima, effect of ascorbate and  $K_m$  value of sinigrin experiments are directly linked with *Citrobacter* myrosinase activity. On the other hand the crude extract of *Citrobacter* may contain  $\beta$ -O-glucosidase. In fact this is supported by the  $k_m$  study. The use of the chromogenic substrate ( $\rho$ NPG) shows that this enzyme has a greater affinity towards this substrate than myrosinase has towards sinigrin (as mentioned earlier). It was difficult to differentiate between these two enzymes in terms of whether if they are different or the same enzyme especially when data from other organisms is compared to data obtained here. Myrosinase activity of many microorganisms has been studied against the aryl  $\beta$ -glucoside such as salicin and  $\rho$ NPG, for example, the only pure bacterial myrosinase of *Enterobacter cloacae* no. 506 has specific activity of 0.616  $\mu\text{mol}/\text{min}/\text{mL}$  (concentration of protein not given in this paper) towards this chromogenic substrate which is comparable with that of sinigrin for the same enzyme 1.073  $\mu\text{mol}/\text{min}/\text{mL}$  (Tani et al., 1974). Unfortunately the current  $k_m$  study didn't provide much information about the two enzymes. Additional  $k_m$  studies using other glucosinolate substrates especially those found in rapeseed such as progoitrin would be useful in terms of examining enzyme affinity.

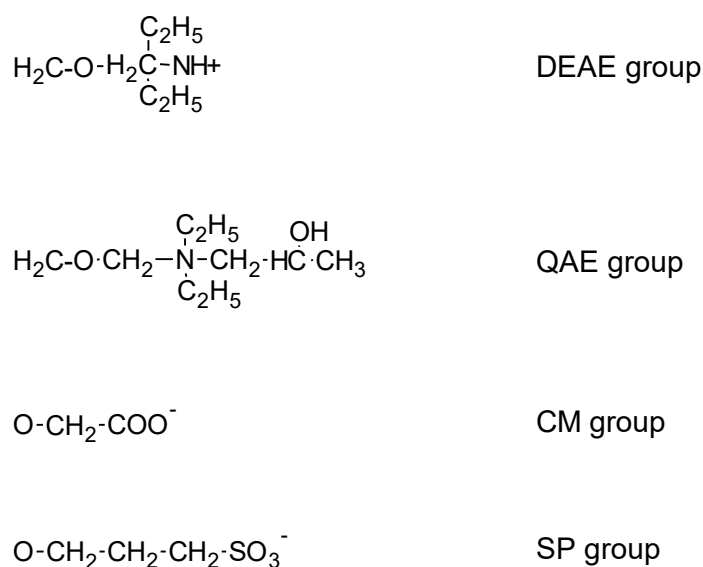
**CHAPTER FOUR**  
**PURIFICATION OF A *CITROBACTER* MYROSINASE**

## Introduction

### 4.1 Protein purification and sequencing strategies for myrosinase

#### 4.1.1 Ion exchange

The separation of proteins by ion exchange chromatography is based on the charges carried by protein molecules. Matrices (stationary phase) of ion exchangers consist of either basic or acidic groups. The acidic ion exchanger containing negative groups is the cation exchanger, whereas the basic ones containing positive groups is the anion exchanger. The matrix can be made from different media; the commonly used are cellulose, agarose and dextran. Ion exchangers are classified into weak or strong exchangers (Figure 4.1) according to the  $pK_a$  values of their charged groups. Strong cation exchangers are the sulfonates (S) and sulfopropyl groups (SP), the weak cation exchanger is carboxymethyl (CM) (Millner, 1999). The strong anion exchangers have quaternary amines such as QAE (quaternary ammonium ethyl). Amines such as diethyl-amino-ethyl group (DEAE) are weak anion exchangers. DEAE is the most common functional group used in the anion exchangers. The pH of the starting and elution buffers (mobile phase) must be higher or lower than the isoelectric point (pI) of the protein and the elution buffer is usually two pH units either side of the pI. The charged protein will be bound to the ion exchange that carries an opposite charge. Proteins in solutions of pH above their pI will be negatively charged and will bind to anion exchanger whereas in solutions of pH below their pI will be positively charged and bind to cation exchanger while unbound protein is washed away. The target protein is eluted by increasing concentration of a competing ion. This is achieved either by increasing (gradient) the ionic strength of the eluting buffer or changing its pH (Scopes, 1994; Millner, 1999).



**Figure 4.1** Ion exchange functional groups; DEAE is a weak anion exchanger; QAE is a strong anion exchanger; CM is a weak cation exchanger; SP is a strong cation exchanger (Millner, 1999).

Plant myrosinase was reported as an abundant protein in some cruciferous seeds and requires a relatively simple purification where the enzyme is precipitated by ammonium sulphate fractionation followed by desalting and ion exchange chromatography (Bones and Rossiter, 2006). The purification of aphid myrosinase from *Brevicoryne brassicae* was achieved by a five step purification: ammonium sulphate fractionation, gel filtration using Sephacryl (S 200) column, Concanavalin and two ion exchange steps using a Resource Q column (Jones et al., 2001). Later this purification protocol was reduced to three steps using ammonium sulphate fractionation, ion exchange using a DEAE 650 Fractogel column followed by gel filtration using Superdex 200 (Frédéric et al., 2002).



### 4.1.2 Gel filtration

This technique separates proteins, peptides, and oligonucleotides on the basis of molecular size. The media in this type of chromatography are usually small beads that contain an open meshwork of cross linked polysaccharides. Molecules move through a bed of porous beads diffusing into the beads to greater or lesser degrees. Small molecules can enter the gel but larger molecules are excluded and leave the column first while the smaller last. This method is also used to estimate the molecular weight. The common gel filtration matrixes are dextran (trade name Sephadex), polyacrylamide (BioGel P), agarose (Sepharose) and combination of these media such as dextran/bisacrylamide (Sephacryl) and agarose/dextran (Superdex). They all can be manufactured with different degrees of porosity and so will fractionate different size ranges of proteins (Lars, 1998).

### 4.1.3 Gel electrophoresis

The gel is usually made of polyacrylamide which creates the gel pores. The higher the concentration of acrylamide the smaller the pore size of the gel. Due to electric field influence proteins molecules with a net charge will migrate towards the electrode of the opposite charge, the greater the net charge the faster the protein will migrate. Polymerization of acrylamide is initiated by the addition of ammonium persulphate and N,N,N',-tetramethylethylenediamine (TEMED). In native polyacrylamide gels the separation of proteins will be according to their net charge, while by including the strong anionic detergent sodium dodecyl sulphate in the buffer for polyacrylamide gel electrophoresis (SDS PAGE) this will denature by disrupting the noncovalent bonds in the proteins and unfolding the polypeptide chains of the proteins and coat with an overall negative charge, thus the separation will be according to the proteins molecular

weight only. In SDS PAGE protein is also treated with a reducing agent such as 2-mercaptoethanol or dithiothreitol to break all disulfide bonds and to ensure complete denaturation. SDS-PAGE is thus a quick and reproducible method for comparing, quantifying and characterising proteins (Scopes, 1994).

#### **4.1.4 Isoelectric focusing (IEF)**

This electrophoretic method separates proteins according to their isoelectric point (pI). Proteins, peptides and enzymes are amphoteric molecules. They carry either negative, positive or zero charge depending on the pH of their local environment. The net charge of proteins is the sum of all positive and negative charges of the amino acids side chain and carboxyl- amino termini. The pI of the protein is the specific pH at which the net charge is zero. Proteins are negatively charged at pH value above their pI while positively charged at pH lower than their pI. When proteins are placed in medium of gradient pH and under the influence of an electric field, they will initially migrate toward the electrode of the opposite charge. During migration proteins either lose or gain protons, and as a result the charge and mobility of the protein will decrease and the protein slow down until it reaches at the point in the pH gradient that is equal to its pI. At this point the protein becomes neutral in terms of charge and it will not move. If diffusion of the protein occurs then electrophoretic forces will push the protein back to its pI. In this way each protein will be focused into a narrow band. The establishment of a pH gradient requires the use of polymeric buffers which have many pI values known as polyampholytes. These buffers are mixtures of molecules containing multiple aliphatic and carboxylic groups. They are a spectrum of low molecular weight (approximately 300-1000 Da) ampholytes with closely related pI and high conductivity. Analytical IEF is carried out in agarose or polyacrylamide gels. The polyacrylamide gel used in this method has large pore sizes (5% acrylamide, 3% bisacrylamide) and

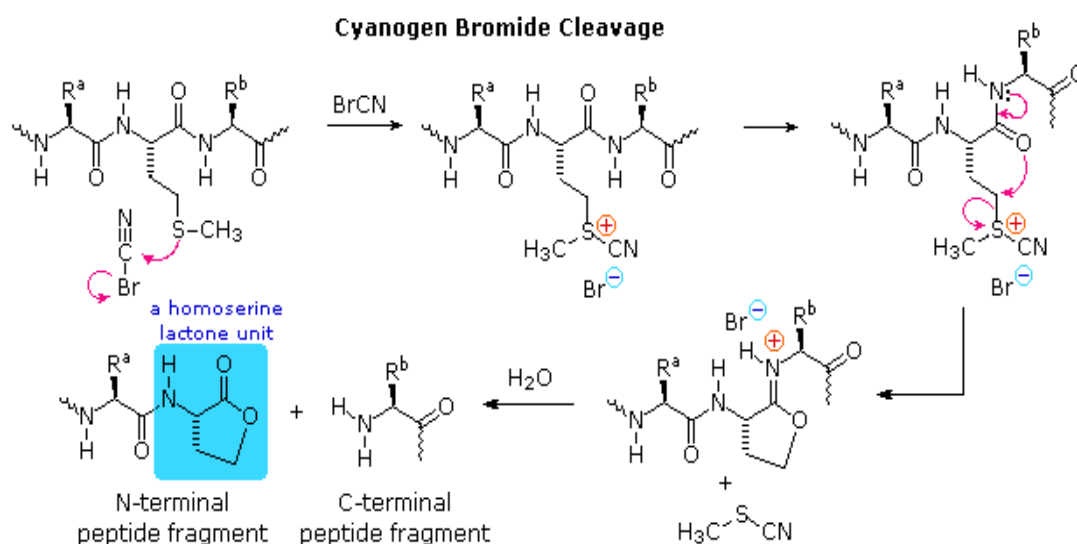
contains ampholyte. Agarose IEF gels usually contain 0.8-1.0 % agarose (Westermeier, 2005).

Under the effect of the electric field the ampholyte migrates and forms a pH gradient in the gel, following which separation of proteins occurs as mentioned earlier. For high resolution separations this technique can be combined with SDS-PAGE in a procedure known as two dimensional (2D) gel electrophoresis. Here the protein is first focused in a narrow strip of gel that contains the polyampholyte and after focusing the strip is applied to the top of polyacrylamide gel and electrophoresed. Spots of separated protein will appear in horizontal direction on the basis of their pI and in vertical direction according to their molecular weight (Scopes, 1994).

## 4.2 Protein sequencing

Proteins are polymers of amino acids and can contain a combination of twenty different amino acids. The typical structure of an amino acid consists of an amine group ( $\text{NH}_2$ ), carboxyl group ( $\text{COOH}$ ) and contain a side chain (R). The amino acids are linked via an amide bond which occurs due to a condensation reaction between the amine and carboxyl groups. Peptides of more than 10 amino acid residue are termed polypeptides. A polypeptide has two ends *N*-terminal and *C*-terminal. The arrangement of the amino acids (sequence) of a protein is known as a primary structure, this sequence is written from *N*-terminal end to *C*-terminal end of the protein because proteins are synthesized in this direction (Hames and Hooper, 2000). The knowledge of the primary structure of a protein is essentially required to understand how this protein works. Protein secondary structure refers to the regular folding of regions of the

polypeptide chains into coiled or pleated structure held together by hydrogen bond,  $\alpha$ -helix and  $\beta$ -pleated sheets are the most common types of secondary structure. Tertiary protein structure refers to the overall interrelationship of the various regions and arrangement of a single polypeptide chain (three dimensional structure). The protein is said to possess quaternary structure if it consist of more than one polypeptide chain linked together by non covalent forces which stabilise protein tertiary structure. Proteins with multiple polypeptide chains are oligomeric. The structure formed by interaction of two monomers in oligomeric protein is known as quaternary structure. The amino acid composition of a protein can be determined by hydrolysing the peptide bonds, this involves splitting of the protein into a number of a smaller peptides using chemical reagents such as cyanogen bromide (which cleaves only after methionine residues) or specific proteolytic enzymes such as trypsin (which cleaves only after lysine or arginine). Cyanogen bromide (Figure 4.2) is the most frequently used chemical for cleavage of the polypeptide chain at the carboxylic side of methionine. Peptides can then be analysed by Edman degradation either manually or in an automated sequencer machine (Kraft, 1997).



**Fig 4.2** Mechanism of peptide cleavage using cyanogen bromide

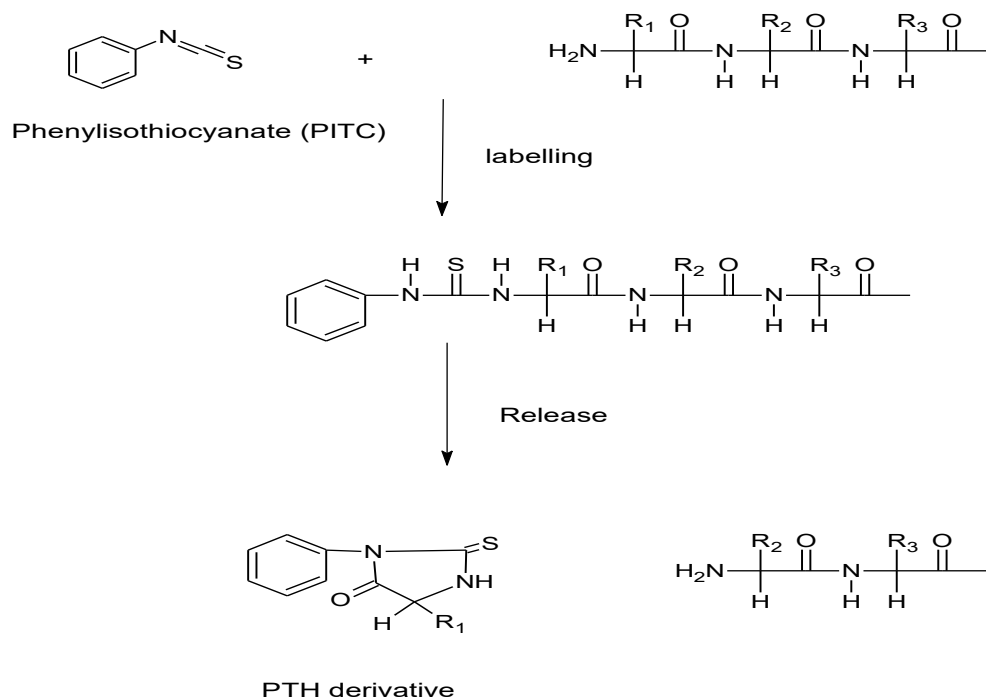
(<http://www2.chemistry.msu.edu/faculty/reusch/virttxtjml/protein2.htm>)

### 4.3 Edman degradation

Edman degradation (Figure 4.3) was developed by Pehr Edman in 1950. This method remains a reliable method for obtaining the *N*-terminal sequences of proteins. The Edman chemistry involves three different reactions:

- i) Coupling of the reagent phenylisothiocyanate (PITC) to the amino group under basic conditions.
- ii) Release of the first residue from the remaining intact polypeptide as 2-anilino-thiazolinone derivative (ATZ amino acid).
- iii) Isomerisation (conversion) of the ATZ amino acid into more stable phenylthiohydantoin (PTH).

The coupling reagent (PITC) is the key reagent in this method; PITC reacts with the free  $\alpha$ 1 *N*-terminal amino acid to form the phenylthiocarboamoyl (PTC) derivative. This reaction is performed for about 15 min at 45-48 °C. Derivatisation of the first amino acid residue is carried out by treatment with strong acid such as trifluoroacetic acid (TFA) to release this residue from the remaining polypeptide chain as ATZ amino acid. The final step is conversion of ATZ amino acid by treatment with aqueous acid resulting in more stable PTH. The released PTH amino acid is identified by high performance liquid chromatography (HPLC) (Wittmann-Liebold, 1997; Hames and Hooper, 2000).



**Fig 4.3** Edman degradation. The *N*-terminal amino acid is labelled with PITC. Under mild acid hydrolysis this residue is released as acyclic PTH derivative (amino acid). The peptide is shortened by one residue available for another round of labelling and release. Adapted from (Hames and Hooper, 2000)

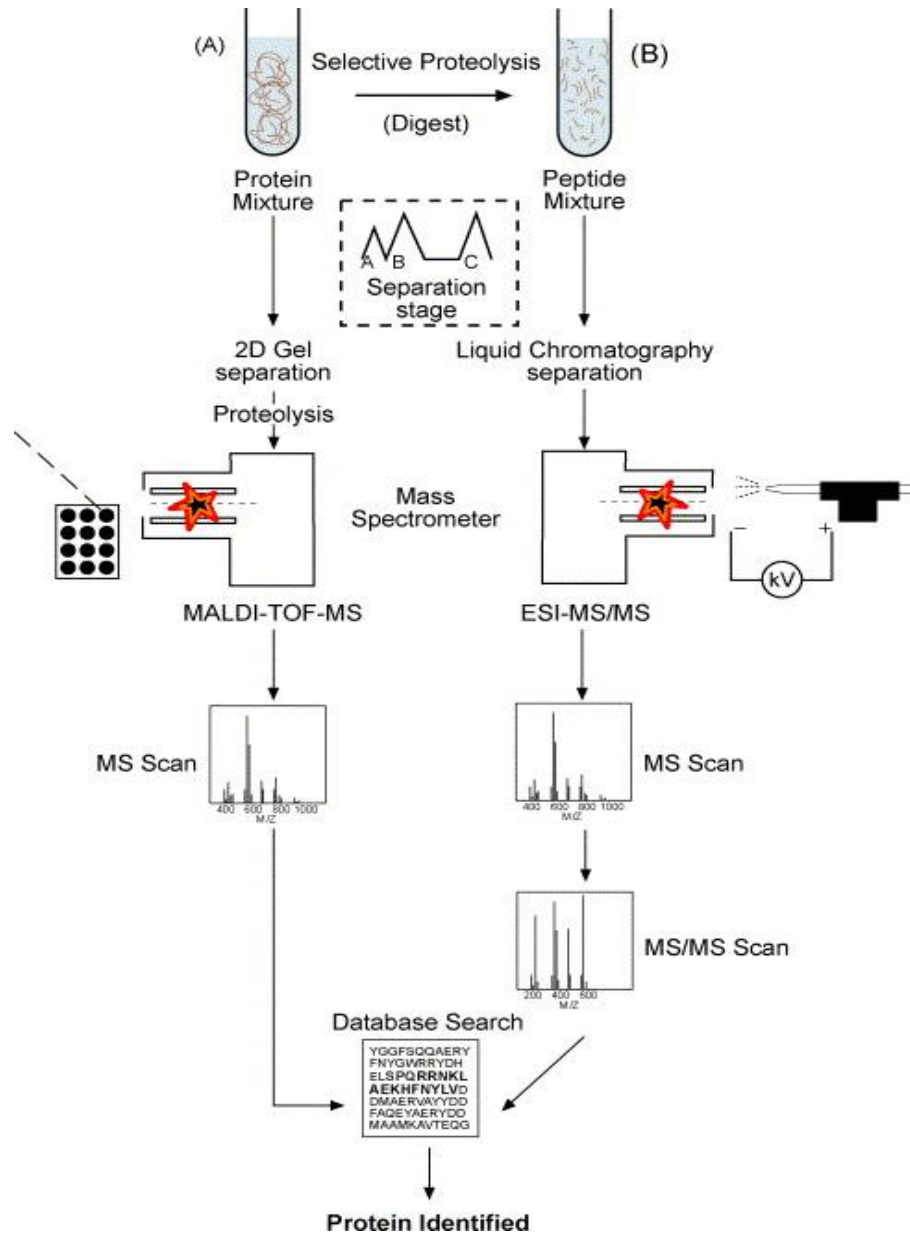
#### 4.4 Peptide sequencing by mass spectrometry

Historically the amino acid sequence of a protein was obtained by the Edman process on purified peptides from tryptic digests. However, advances in mass spectrometry have enabled amino acid sequences of peptides to be obtained and this, coupled with bioinformatics approaches using genome data bases, has enabled the full sequences of proteins to be obtained. Mass spectrometry consists of three principle components, an ionization source to generate characteristic charged ionic fragments; a mass analyser that measures the mass to charge ( $m/z$ ) ratio; and an ion detector which counts the numbers of ions. Molecules of sample are first ionised into gaseous ions before introduced into a mass analyser where they separated according to their  $m/z$

ratio. The signals are registered in the detector and pass the information to a computer for spectrum recording and analysis. Two ionisation methods are most frequently used to study peptide and proteins through mass spectrometry; the Matrix Assisted Laser Desorption Ionisation (MALDI) and electrospray ionization (ESI) (Manz et al., 2004).

#### 4.4.1 Matrix Assisted Laser Desorption Ionisation- Time of Flight Mass Spectrometry (MALDI-TOF/MS)

Molecules are not degraded during the ionisation process using this method and for this reason this method is referred to as soft ionisation. Ionisation is based on the soft desorption of the solid sample molecules into vacuum. The sample is placed on a metallic plate and first co-crystallised with a small organic UV absorbing molecule matrix. The co-crystallised protein is then blasted with a laser beam into an ionised gas. Each molecule of ionised gas carries one or more positive charge. The ionised gas is accelerated in an electric field and then enter the field free tube where they drift along at various speeds according to their mass and charge ratio and at the end of the tube they hit a detector. The more highly charged ions move more quickly and reach the detector first while large mass ions move slowly and reach the detector later. The precise mass is then determined by analysis of the ions with a single charge. The resulting fingerprint or masses are used to search protein sequence database (summarised in figure 4.4).



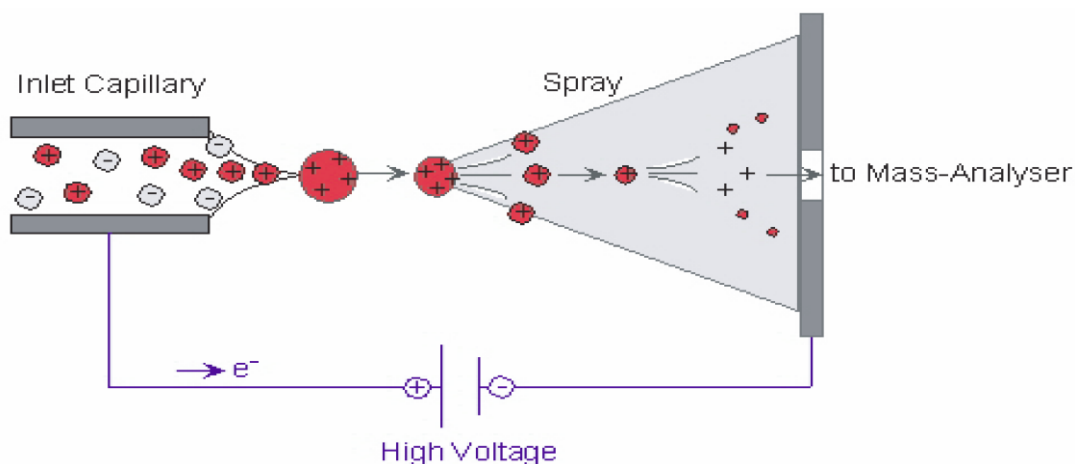
**Fig 4.4** Proteomic analysis. The two common mass spectrometry approaches. (A) peptide mass mapping, (B) peptide sequencing. Adapted from (Kicman et al., 2007).

Serine proteases such as trypsin (EC 3.4.21.4) are commonly used in internal sequencing to breakdown proteins and specifically cleave peptide bonds in which the carboxyl side (C-terminally) comprises the amino acids lysine (Lys) or arginine (Arg).



## 4.4.2 Electrospray ionisation mass spectrometry (ESI-MS)

In this ionisation method (Figure 4.5) an electrospray is generated by dispersing the liquid into fine droplets via a high potential difference. The sample is dissolved in a solvent and pumped through a narrow heated chamber (capillary). A high voltage (3-4 kilo volt) is applied between capillary and the opposing chamber wall. As a result of the strong electric field, the sample is dispersed into highly charged droplets. Due to heat assisted by the flow of warm gas (usually nitrogen), the droplets lose solvent and shrink, this will lead to an increase of the charge density on the surface of the droplets. The sample ions eventually free of solvent are released from the droplets and transferred into the mass analyser (Manz et al., 2004).



**Fig. 4.5** The principle of electrospray ionisation mass spectrometry (ESI-MS). adapted from (Manz et al., 2004)

#### 4.5 Aims

The main goals in this section are:-

- Purify *Citrobacter* myrosinase to homogeneity and estimate its molecular weight.
- Obtain the amino acid sequence of this enzyme and compare with plant myrosinase and other  $\beta$ -glucosidases with a view to cloning the gene.
- Develop analytical methodology for peptide analysis.
- Development of a screening method for bacterial myrosinase.

## 4.6 Methods

### 4.6.1 pI determination

A range of buffers at different pH (Table 4.1) were used to determine the approximate pI of the crude extract of *Citrobacter myrosinase*. Mini anion Q columns (90010 Thermo scientific, USA) were equilibrated with the appropriate buffer (400  $\mu$ L) and centrifuged (Eppendorf 5425 centrifuge, Hamburg Germany) (2000 x g, 4  $^{\circ}$ C, 5 min). The crude protein extract was desalted using desalt spin columns 0.5 mL (89882 Thermo scientific, USA) against the appropriate pH buffer and applied to the mini anion Q column and then centrifuged (2000 x g, 4  $^{\circ}$ C, 5 min). The column was washed twice with the appropriate buffer (400 $\mu$ L), finally the protein was eluted with the buffer that contained NaCl (1 M) then desalted against citrate phosphate buffer (CPB) (20 mM, pH 6.0) before being assayed for activity. All future reference to a crude protein extract refers to a desalted lysate.

Buffer (0.2M)	pH range
N-methyl piperazine	4.5
N-methyl piperazine	5.0
L-Histidine	5.5
L-Histidine	6.0
bis-Tris	6.4
bis-Tris propane	7.0
triethanolamine	7.6
Tris	8.0

**Table 4.1** Buffers used for the isoelectric point determination of *Citrobacter myrosinase*, the ion exchange column (Q column) was equilibrated with relevant pH buffer and spun (2000 x g, 4  $^{\circ}$ C, 5 min) before adding sample (400  $\mu$ L) and centrifuged twice (2000 x g, 4  $^{\circ}$ C, 5 min). The column was washed twice with buffer of the relevant pH (400  $\mu$ L) and eluted with the same buffer (0.2 M) with 1.0 M NaCl then desalted and was kept until assay.

#### 4.6.2 Large scale preparation of myrosinase for purification

An overnight culture of *Citrobacter* was grown on nutrient broth (NB) and inoculated into M9 medium (500 mL) and incubated aerobically overnight (at 30 °C). The bacteria were harvested by centrifugation (Avanti J26 XP, Beckman coulter, USA) (8000 x g, 15 min, 4°C) and washed twice with CPB (20 mM, pH 6.0). The cells (25 mL) were lysed using one shot constant cell disruption system (Constant systems Ltd, UK), cell lysis was achieved by placing the bacterial suspension under high pressure (30 kps) followed by a sudden release of pressure which causes the bacterial cells to burst. The cell debris was pelleted by centrifugation (20000 x g, 20 min, 4°C). The supernatant was desalted using a desalting column (Econo-Pac 10DG Desalting Columns, BIORAD, USA), against CPB (20 mM, pH 6.0) and concentrated (11 mL) using a ultrafiltration membrane tube (molecular weight cut-off 10 KDa, 4 mL UFC801024, Amicon ultra, Millipore, USA). The protein concentration (Chapter 2) and specific activity of the crude extract was determined. In a final step the sample was desalted against TRIS buffer (20 mM, pH 8.0) to obtain the crude lysate.

#### 4.6.3 Purification of *Citrobacter myrosinase*

##### 4.6.3.1 Ion exchange

##### 4.6.3.1.1 First ion exchange run

A Mono Q HR 515 column (50 x 5 mm I.D., Pharmacia, Uppsala, Sweden), an anion exchanger was used in the first and the second steps of myrosinase purification. The column was attached to a WATERS HPLC purification system (650 E, Millipore, USA) that consisted of injector part (Waters 650E, Millipore, USA), system controller part

(Waters 600E). The programme utilised in this purification step is shown in Table (4.2). The protein elution was monitored at  $A_{280\text{nm}}$  on a UV detector (ABI 757 absorbance detector, Applied Biosystems). Both the initial starting buffer (Tris 20 mM, pH 8.0) and elution buffer (Tris 20 mM, plus NaCl 1M pH 8.0) were filtered using a Millipore membrane filter. The column was pre-equilibrated with starting buffer. The sample (40 mg) was desalted against initial starting buffer (Tris, 20 mM, pH 8.0) using Econo-Pac 10DG desalting columns then filtered through a 0.2 $\mu\text{m}$  mini-Sart filter prior to injection. Fractions (1.5 mL) were collected every 2 min at flow rate of 0.75 mL/min. The bound protein was eluted by using a linear buffer concentration gradient from 0-1M NaCl.

Time	Flow (mL)	% A	% B
0	0.75	100	0
15	0.75	100	10
45	0.75	90	25
55	0.75	75	50
65	0.75	50	100
75	0.75	0	100
85	0.75	0	0
90	0.75	100	0
120	0.75	100	0
121	0.75	100	0

**Table 4.2** The composition of the gradient system to separate myrosinase on a Mono Q column. For equilibration; the column was prewashed with the start buffer (Buffer A, TRIS, 20 mM, pH 8.0) prior to loading the sample. The sample was eluted with the same buffer but with NaCl (Buffer B, 1M), the collected fractions were dialysed using a multiwell dialysis system against CPB (20mM, pH 6.0). Flow rate of 0.75 ml/min.

The collected fractions were dialysed using a multiwell dialysis system against CPB (20 mM, pH 6.0) then assayed for myrosinase activity using GOD Perid method (Chapter 2.2.9).

#### 4.6.3.1.2 Second ion exchange run

For the second purification step, fractions having *Citrobacter myrosinase* activity (GOD Perid, Chapter 2.2.9) were pooled, combined and desalted against initial starting buffer (Tris 20 mM, pH 8.0) using Econo-Pac 10DG desalting columns and concentrated using a ultrafiltration membrane tube. The sample (0.77 mg) was then applied to an ion exchange column (Mono Q column) equilibrated with same buffer. The separation was carried out exactly as previously outlined above (Table 4.2). However, fractions (0.75mL) were collected every 1 min with flow rate 0.75mL/min. The bound protein was eluted as mentioned for first ion exchange run.

#### 4.6.3.2 Gel filtration

The active fractions following the ion exchange purification second step were combined, desalted against CPB (50 mM, pH 6.0) using Econo-Pac 10DG desalting columns and concentrated using a spun column ultrafiltration tube. The protein concentration was measured by the Bradford method (Chapter 2.2.7). The sample (0.05 mg) was then loaded onto a Superdex 75 gel filtration column (Superdex 75, GE health care, Sweden) equilibrated with CPB buffer (50 mM, pH 6.0) containing NaCl (150 mM) and sodium azide (0.02%). Fractions (0.35 mL) were collected every 1 min at flow rate of 0.35mL/min then assayed for myrosinase activity using the GOD Perid method (Chapter 2.2.9).

#### 4.6.4 Estimation of molecular weight of *Citrobacter myrosinase*

##### 4.6.4.1 Gel filtration

At room temperature the chromatographic column (80 x 2.5 cm) Superdex 75 was equilibrated with the buffer (CPB, 20 mM pH 6.0) containing sodium azide (0.02 %). Molecular weight markers 12 - 79 kDa were used to calibrate the column, cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), albumin (45 kDa), albumin bovine serum (66 kDa) and transferrin (79 kDa). Fractions (45 of 0.35 mL) volume each were collected and assayed for the enzyme activity. The molecular weight of the subunit was extrapolated from a plot of log (molecular weight) versus elution time.

##### 4.6.4.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

###### Resolving gel (13%)

Proteins are separated by size according to the method of Laemmli (1970). The resolving gel (13%) was prepared by mixing Milli Q H<sub>2</sub>O (3.2 mL), acrylamide/bis (161-0158 BIORAD, USA) (4.3 mL), Tris-HCl buffer (2.5 mL of 1.5M pH8.8) and 0.1 mL of 10% SDS (B2008 Melford Ltd, UK) were mixed and prior to pouring the gel ammonium persulfate APS (50µL of 10%) (BIORAD, USA) and TEMED (T7024 Sigma, UK) (5µL) were added to the resolving gel then gently swirled and poured into Mini-protein Tetra cell (BIORAD, USA). After pouring the gel was layered with Milli Q H<sub>2</sub>O and left to polymerise.

### Stacking gel (5%)

The stacking gel was made by mixing Milli Q H<sub>2</sub>O (2.3 mL), acrylamide/bis (0.67 mL of 30%), Tris-HCl buffer (1.0 mL, of 0.5 M pH 6.8) and 10% SDS (0.1 mL). APS (30 μL of 10%) and TEMED (5 μL) were added prior to pouring, mixed gently and then poured on the top of resolving gel, and the comb placed into the gel. The running buffer was (tris HCl 25 mM, glycine 192 mM, SDS 1g)/L<sup>-1</sup>. The gel was run at room temperature (200 V / 50 min).

### 4.6.5 Two dimension gel electrophoresis (2D gel)

#### 4.6.5.1 First dimension

##### 4.6.5.1.1 Cell lysis

The cells were washed twice with Milli Q H<sub>2</sub>O then resuspended in lysis buffer (2 mL, 7 M urea, 2 M thiourea, 4% CHAPS, 2% Immobilized pH Gradient (IPG) Buffer, 40 mM DTT) and mixed with protease inhibitor cocktail (Melford Labs Ltd, UK) (25 μL) before sonicated (Sonics Vibracell VCX 130, Sonics & materials inc., USA) with 40% power (2 seconds on and 2 seconds off) over a period of 10 min in total. The crude lysate was transferred into Eppendorf tubes and centrifuged (Eppendorf 5415D, Hamburg, Germany) (16100 x g, 4 °C, 10 min). The supernatant was carefully transferred into a fresh Eppendorf tube. The sample was concentrated to approximately 0.5 mL using desalt spin columns. The protein concentration was measured using Bradford reagent (Chapter 2.2.7).



#### 4.6.5.1.2 Sample clean up

The purpose of this step was to remove contaminating substances such as salts, lipids, detergents and nucleic acids which interfere with 2-D electrophoresis. For this reason a 2D clean up kit (1632130, BIORAD, USA) was used and the protein sample (~100 µg) was transferred to an Eppendorf tube and precipitating agent 1 (300 µL) added and mixed by vortexing and then incubated on ice (15 min). A precipitating agent 2 (300 µL) was added to the mixture and mixed well prior to centrifugation (12000 x g, 5 min). The supernatant was removed and wash reagent 1 (40 µL) was added on the top of the pellet and centrifuged (12,000 x g, 5 min). The wash was discarded and Milli Q H<sub>2</sub>O (25 µL) was added to the pellet and mixed (10-20 sec) and a prechilled (- 20 °C) wash reagent 2 (1 mL) added together with wash 2 additive (5 µL) and mixed (1 min) and incubated (- 20 °C) for 30 min and mixed by vortexing (30 sec) every 10 min during the incubation period. The supernatant was removed and the protein precipitate was air dried (3-5 min) and stored at - 80 °C.

#### 4.6.5.1.3 Preparation of rehydration buffer (sample buffer)

The rehydration buffer contains urea (7 M, U 6504, sigma, USA), thiourea (2 M, RPN 301, GE Healthcare, UK), CHAPS (2% w/v, C9426, Sigma, UK), IPG buffer (0.5% v/v, 17600440, GE Healthcare, Sweden), bromophenol blue (B 6896, Sigma, USA) 0.002% of 1% stock solution that contains bromophenol blue (1%) and Tris-base (50 mM, T1503 sigma, UK), were dissolved in Milli Q H<sub>2</sub>O to final volume of 25mL then decanted (1.25 mL) into Eppendorf tube and stored at -20 until use, Dithiothreitol (DTT) (3.5 mg, MB 1015 Melford, UK) was added just prior to use.

#### 4.6.5.1.4 Isoelectric focusing (IEF)

Dry polyacrylamide gel strips with an immobilized nonlinear pH gradient (pH 3-11 nonlinear (NL), 11 cm, GE Healthcare, Sweden) were rehydrated overnight in the rehydration tray contained the denaturing rehydration buffer (200  $\mu$ l) and covered with mineral oil to prevent dehydration. The strips were transferred into isoelectric focusing unit (Ettan IPGphor 3, GE Healthcare, Sweden) for IEF. The cleaned up sample was re-suspended in sample buffer (100  $\mu$ L) and loaded into the cup loading of each strip. Finally the strips were overlaid with mineral oil to prevent dehydration. The IEF process was carried out using three sequential voltages: 500 V (60 min), gradient 1000 V (60 min) and gradient 8000 V (7 h). The temperature was kept at 20 °C.

#### 4.6.5.2 Second dimension

##### 4.6.5.2.1 Preparation of SDS equilibration buffer solution

The following ingredients urea (6 M), glycerol (29.3% v/v, 17132501, GE Healthcare, Sweden), SDS (2% W/V, B2008 Melford, UK) and bromophenol blue (0.002% w/v) were dissolved in Milli Q water , the total volume 200 mL was dispensed in falcon tubes (50 mL) and stored at -20.

##### 4.6.5.2.2 Electrophoresis

After completion of IEF, the IPG strips were equilibrated into (10 mL) SDS equilibration buffer (4.7.5.2.1) containing DTT (1%) with gentle shaking for 15 min after which the

equilibration buffer was replaced with the same solution except that DTT was exchanged with iodoacetamide (2.5%) and equilibrated for 15 min. The strips were then placed on the gel (Criterion precast gel 4-20% Tris-HCl, 3450104 BIORAD, USA) and sealed with agarose solution (0.5% agarose, 1% bromophenol blue in a Laemmli SDS electrophoresis buffer) together with high range (12-225 kDa) rainbow molecular weight marker (RPN 756E, GE Healthcare, Sweden). These were loaded before the addition of the running buffer 2-(N-morpholino) ethanesulfonic acid (MES), (1610789, BIORAD, USA). The gel then was subjected to electrophoresis at 40 V (10 min) followed by 150 V (50 min). Staining was performed using a Coomassie instant blue (ISB 1L, Expedeon, UK), for 1h after which the stain was replaced with Milli Q water. Gel images were analysed by Progenesis SameSpots software (Nonlinear Dynamics Ltd, USA).

#### 4.6.6 Detection of myrosinase activity in the culture medium supernatant of *Citrobacter*

The *Citrobacter* was grown over night (30 °C) on M9 media containing sinigrin. From the original large scale purification (4.7.3) the supernatant (500mL) was stored (at 4 °C). Ammonium sulphate (90%, 3.93 M, pH 6.8) was added to the supernatant in order to precipitate protein and left overnight at 4°C with gentle stirring using magnetic stirrer. The precipitated protein was collected by centrifugation (20000 x g, 20 min, 4°C), the supernatant was discarded, and the pellet reconstituted with 3 mL of CPB (20 mM /pH 6), filtered using a 0.2 µm filter to remove any remaining bacteria. The sample was then desalted against the same buffer using an Econo-Pac 10DG desalting column before the addition of sodium azide, and concentrated to 200µL using a Millipore ultrafiltration spun column (4 mL). The activity of the myrosinase was assayed by the GOD Perid method (Chapter 2.2.9). The sample was run on SDS PAGE in order to confirm the

molecular weight and purity of the obtained myrosinase. The SDS Page was prepared as mentioned earlier. Sample (25  $\mu$ L) was mixed with 5 x sample buffer and boiled (3 min) prior to loading into the gel wells before the addition of running buffer (tris 0.025M, glycine 0.192M, SDS 0.1%)/L<sup>-1</sup>. The gel was run under constant voltage (180 V / 45 min). After completing electrophoresis the gel was stained with Coomassie instant blue (1h) after which the stain was replaced with Milli Q water and left destaining over night with gentle agitating.

#### 4.6.7 Elution of *Citrobacter myrosinase* from native polyacrylamide gel

A native polyacrylamide gel was used for this experiment. The gel was prepared as for SDS gel (4.7.4.2) but SDS was omitted. The protein crude extract of induced cells and the control (non induced) were mixed with sample buffer (tris HCl 0.312M, glycerol 50% and bromophenol blue 0.05%) and loaded into gel wells. Electrophoresis buffer (tris HCl 25 mM, glycine 192 mM)/L<sup>-1</sup> was poured into gel tank. The gel was run (140 V / 90 min) but with the gel rig in an ice bath. Following electrophoresis the gel was washed twice with CPB (20 mM, pH 6.0) and incubated (25 °C) either with freshly prepared esculin reagent (1-2 mL) or with *p*NPG (1-2 mL) of 10 mM stock. This was carried out to confirm  $\beta$ -O-glucosidase activity only. The active bands (dark brown) were excised from the gels. One selected active band from the gel was incubated (25 °C, 1h) with sinigrin (0.2  $\mu$ M) and CPB (20 mM, pH 6.0) in order to confirm myrosinase activity. For protein elution the remaining active bands were minced into small gel pieces and incubated in CPB (4mL, 20 mM, pH 6.0) and left overnight at 4°C and then briefly centrifuged in order to pellet the gel pieces. The supernatant was carefully pipetted into a fresh Falcon tube and desalted against the same buffer. An aliquot (20  $\mu$ L) of the

eluted protein sample was assayed for myrosinase activity using the substrate sinigrin (0.2  $\mu$ M). The sample was subjected to SDS PAGE in order to confirm the molecular weight and purity of sample. Prior to electrophoresis the sample (4 mL) was concentrated to approximately 1 mL. The SDS PAGE gel was prepared as mentioned earlier, the sample (22  $\mu$ L) was combined with 4X sample buffer (7.5  $\mu$ L) and boiled (3 min). Sigma low molecular weight markers were used (14-66 kDa, SDS7, Sigma USA); the voltage was set at 200 V (45 min). Following electrophoresis the gel was placed in Milli Q water (10 min) and then stained with Coomassie instant blue for one hour after which the stain was replaced with Milli Q water and left destaining over night with gentle agitating.

#### 4.6.8 Detection of *Citrobacter* myrosinase activity in SDS PAGE gels

SDS PAGE gel was prepared as mentioned earlier (section 4.7.4.2). The boiling step of sample was omitted. The removal of SDS from the gel was done according to the procedure described by Blank et al., (1982). After electrophoresis the gel was washed (3 Xs, 30 min) with CPB (20 mM, pH 6.0) containing isopropanol (25% v/v) (Blank et al., 1982). After the final wash the gel was incubated (25 °C, 60 min) with either esculin reagent or  $\rho$ NPG.

#### 4.6.9 Initial purification of *Citrobacter* myrosinase

Resource- Q-resin 1 mL column (GE Healthcare Bio-Sciences AB, Sweden) an anion exchanger was used under the same conditions mentioned in section 4.7.3.1.1 with the following modification the collected fractions were 3 mL. The collected fractions were

dialysed using a multiwell dialysis system against CPB (20 mM, pH 6.0) then assayed for myrosinase activity using GOD Perid method (Chapter 2.2.9).

#### 4.6.10 Preparation of protein bands or spots of putative myrosinase for peptide sequencing

##### 4.6.10.1 Destaining

The band (1D gel) or spot (2D gel) of interest was excised from the gel and transferred into a siliconized tube (T3406-250EA, Sigma-Aldrich) and diced into small pieces and vortexed (10 min) with 100  $\mu$ L of  $\text{NH}_4\text{HCO}_3$  (25 mM) in 50% acetonitrile (ACN) and centrifuged (16100 x g, 10 min) this step was repeated twice and the supernatant was removed after each step. The gel was completely dried using speed vacuum concentrator (Heto vacuum centrifuge VR-1 Heto Lab, Denmark) (15 min).

##### 4.6.10.2 Reduction and alkylation

25  $\mu$ L of freshly prepared dithiothreitol (DTT) (10 mM in 25 mM  $\text{NH}_4\text{HCO}_3$ ) was added to the dried gel, vortexed, centrifuged (16100 x g, 1 min) and incubated in a heat block (56  $^{\circ}\text{C}$ , 1 h). The supernatant was removed. 25  $\mu$ L of iodoacetamide (55 mM in 25 mM  $\text{NH}_4\text{HCO}_3$ ) was added to the gel and mixed by vortex and spun (16100 x g, 1 min) and incubated in darkness (room temperature, 45 min). The supernatant was discarded and the gel washed with 100  $\mu$ L of 25 mM  $\text{NH}_4\text{HCO}_3$  and vortexed (10 min) and spun (16100 x g, 1 min). The supernatant was discarded and the gel rehydrated with 100  $\mu$ L of 25 mM  $\text{NH}_4\text{HCO}_3$  in 50% ACN and vortex (5 min) then spun (16100 x g, 1 min), the

last step was repeated once. The gel pieces were completely dried in a speed vacuum concentrator (20 min).

#### 4.6.10.3 Tryptic digestion

Freshly diluted trypsin (10  $\mu$ L of 12.5 ng/ $\mu$ L) (V511A, Promega, USA) was added to the gel and briefly centrifuged (10 sec) and incubated on ice (10 min) for rehydration. Approximately 50 $\mu$ L of 25 mM  $\text{NH}_4\text{HCO}_3$  was added to rehydrated the gel pieces in order to keep them immersed during digestion then centrifuged briefly (10 sec) and incubated overnight at 37 °C. The digest solution was transferred into a fresh siliconized tube. Formic acid (50  $\mu$ L of 5%) in 50% ACN was added and mixed by vortexing (30 min) then spun (16100 x g, 1 min) and sonicated using a water ultrasonic bath XB2 (Grant instruments Cambridge Ltd, Herts, UK) (5 min) and centrifuged (16100 x g, 1 min), (this step was repeated once). The liquid was transferred into the digest solution tube and the volume reduced to 15-20  $\mu$ L using a speed vacuum concentrator and the sample was stored at -20 °C for analysing by LC/MS.

#### 4.6.10.4 Peptide identification and analysis using LC-MS

##### 4.6.10.4.1 All protein bands & 2D spots except of initial purification step

An Applied Biosystems QTrap MS coupled to an Agilent 1100 LC stack was used to analyse samples. The agilent 1100 LC stack consisted of three parts; a Binary pump, Capillary pump and Well Plate auto-sampler and a column oven with integrated 6-port valve. Samples were loaded into a trap column (Agilent Zorbax SB 5 $\mu$ m x 0.3mm x

35mm), the trap column was washed using the binary pump and then switched into the capillary flow; peptides were separated on a capillary column (Agilent SB 5  $\mu\text{m}$  0.5mm x 150mm column). The LC was interfaced to the MS with a Turbo Ion Spray Source. Milli Q H<sub>2</sub>O containing 0.2% HCOOH, 0.02% TFA at flow rate 150  $\mu\text{L}/\text{min}$  was used as loading/ washing solvent. A gradient system of %B (0-40%) over 60 min with a flow rate of 10  $\mu\text{L}/\text{min}$  (A: 94.9% H<sub>2</sub>O, 5% CH<sub>3</sub>CN, 0.1% HCOOH; B: 94.9% CH<sub>3</sub>CN, 5% H<sub>2</sub>O, 0.1% HCOOH) was used as a resolving solvent. The column oven was heated to 40°C, and the valve was switched to direct the flow from the trap on to the resolving column after a 5 min wash. The MS parameters were normally set to curtain gas (CUR)10psi, nebulizer gas (GS1) 20psi, turbo gas (GS2) 20psi, interface heater on, turbo gas temperature (TEM) 150 °C, declustering potential (DP) 65.

The acquisition method consisted of an Enhanced Mass Spectrum (EMS) survey scan(350-1200m/z) followed by an Enhanced Resolution (ER) scan and then Enhanced Product Ion scans (100-1500 m/z) of selected ions. The Information Dependent Acquisition (IDA) was set for the top 4 most intense peaks after dynamic background subtraction of the survey scan. The criteria include mass-to-charge ratio (m/z) > 325 <1200 with a charge state of 2-3, the ER scan was used to determine charge state, rolling collision energy was used, +1 precursors were excluded for the dependent scans. Former target ions were excluded after two occurrences for 2 min.

#### 4.6.10.4.2 Protein identification and database searching

The data from mass spectrometry was analysed by database search engine ProteinPilot using the Paragon algorithm (4.0.0.0, 459) (Applied Biosystems). Sample parameters was set as follows: trypsin digestion, cysteine alkylation set to iodoacetamide, urea denaturation and acetylation emphasis. The C-terminal cleavage



at lysine and arginine was selected for trypsin specificity. “Biological modification” was set for processing parameters, and a thorough ID search effort was selected. The peptide and fragment mass tolerances were < 10 ppm. During the search by Protein Pilot, an automatic mass recalibration of the data sets based on highly confident peptide spectra was carried out. A first search iteration was specifically performed to select high confidence peptide identifications. These were then used to recalibrate both the MS and MS/MS data, which are automatically re-searched. Tandem MS data were searched against *Citrobacter* database from National Centre for Biotechnology Information (NCBI). All a reported proteins were identified with at least two peptides having a confidence (Conf.) interval of  $\geq 95\%$  ( $p < 0.05$ ) as determined by ProteinPilot™ Unused scores ( $\geq 1.3$ ) with the corresponding false positive discovery rate below 1%.

#### 4.6.10.4.3 Peptide bands of initial purification step

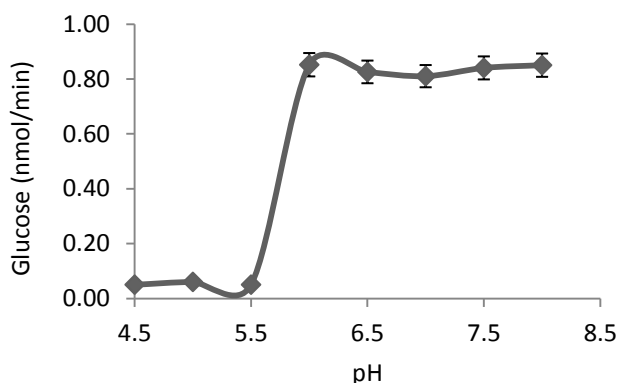
This was carried using LTQ-Orbitrap mass spectrometer (Thermo Scientific) nanoflow-HPLC system (nanoAcquity, Waters Corp.) samples were applied onto a pre-column Symmetry C18 5 $\mu$ m beads, 180  $\mu$ m x 20 mm column, Waters Corp) connected to an analytical column (25 cm) (BEH 130 C18 1.7  $\mu$ m beads, 75  $\mu$ m x 250 mm column, Waters Corp.). A gradient (2 to 30%) of acetonitrile in formic acid (0.1%) was used to elute peptides over 40 min at a flow rate of 250 nL min<sup>-1</sup>. Mass spectrum (MS1) were acquired by Orbitrap with 60,000 resolution while a selection of four most abundant ions in each cycle (up to five data dependent acquisition of MS/MS) were acquired in

the LTQ: MS mass-to-charge ratio (m/z) 300 to 1700, minimum signal 5000, collision energy 35, 1 repeat hit, 60 sec exclusion. Operation of the mass spectrometer was in a positive ion mode with a nano-spray source (Proxeon) and a capillary temperature of 200 °C, no sheath gas was employed. The source voltage and focusing voltages were optimised for the transmission of the peptide MRFA. The files were extracted using extract\_msn (ThermoScientific). Peptide data were subsequently submitted to the MASCOT database for protein identification. A BLAST analysis was then carried out using <http://blast.ncbi.nlm.nih.gov>.

## 4.7 Results

### 4.7.1 Determination of the pI of *Citrobacter myrosinase*

The following data (Figure 4.6) shows that pH 6.0 was the optimum for *Citrobacter myrosinase* enzyme to bind to ion exchange column. Proteins are positively charged at pH below their pI while negatively charged above their pI. For anion exchange the pH of the buffer should be about one unit above the pI of the sample (Millner, 1999). Thus pH 8 was selected as the optimum ion exchange binding pH for the Mono Q column (see later).



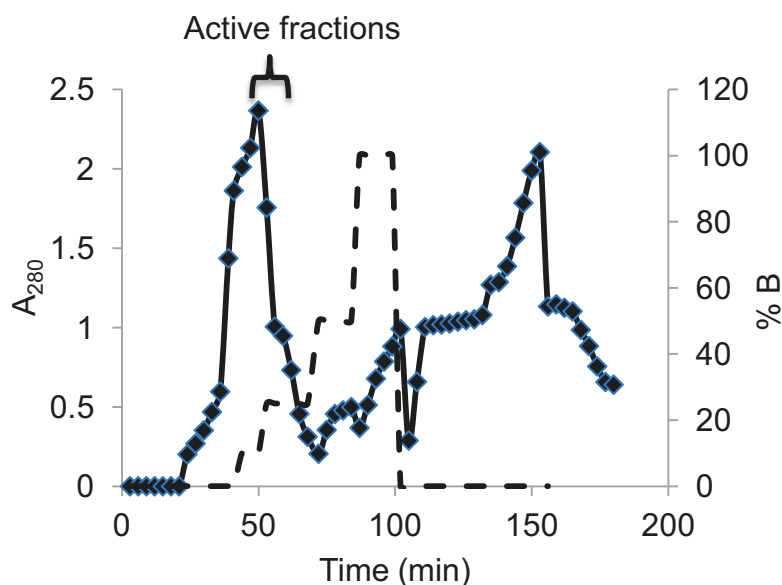
**Fig 4.6** Preliminary pI Determination. The crude protein extract (2.5  $\mu\text{g}$ ) was desalted against appropriate buffer and applied into Mini Q columns pre-equilibrated with the same buffer. The sample was eluted with appropriate buffer that contained NaCl (1 M). The sample was desalted against CPB (20 mM, pH 6.0) and mixed with sinigrin (0.2  $\mu\text{M}$ ) and incubated (25  $^{\circ}\text{C}$ , 1h). The reaction was stopped by boiling (5 min). The liberated glucose was measured by GOD Perid (1 mL). The highest activity was recorded at pH 6.0.

4.7.2 Purification of *Citrobacter myrosinase*

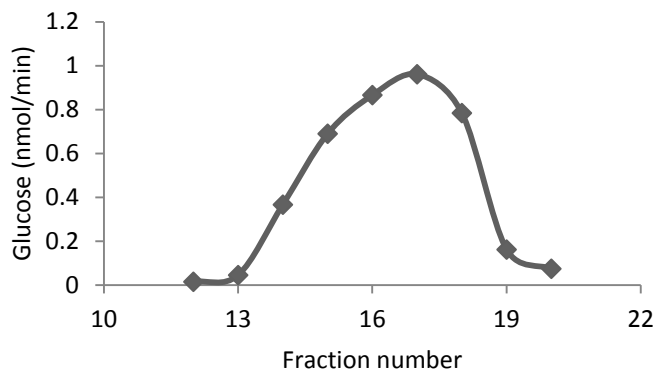
## 4.7.2.1 Ion exchange

## 4.7.2.1.1 First purification step

The ion exchange purification of the *Citrobacter myrosinase* (Figure 4.7) was carried out using a salt gradient as described in the methods. Each fraction was assayed after being desalted against CPB (20 mM, pH 6.0). Most myrosinase activity was obtained in fractions 15-18 of the first run (Figure 4.8) with less activity in other fractions.



**Fig 4.7** Ion exchange (1<sup>st</sup> run) chromatogram of collected fractions. An anion Mono Q column was used with flow rate 1.5mL / min for 2 min with a starting buffer containing Tris-HCl buffer (20 mM, pH 8) and eluted with same buffer but with a sodium chloride (1 M). - - - - salt gradient.

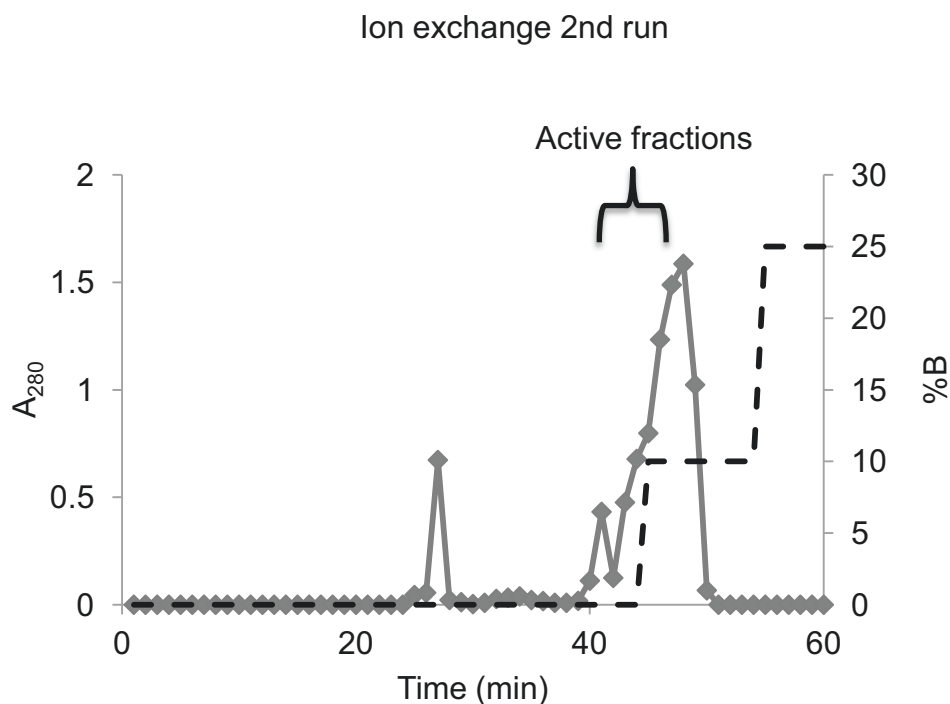


**Fig 4.8** Corresponding myrosinase activity from ion exchange purification on a Mono Q column. The collected fractions were desalted against CPB (20 mM, pH 6) then assayed for myrosinase activity by mixing enzyme with sinigrin and CPB (20mM, pH 6) then incubated at 25 °C, 2h, the released glucose was measured by GOD Perid reagent. The protein concentration was measured using the Bradford method. Fractions 15-18 have the highest activity.

The active fractions (15-18) were combined and concentrated (2.5 mL) using a ultrafiltration membrane tube before being desalted against Tris buffer (20 mM, pH 8.0) using Econo-Pac 10DG desalting columns in order to be ready for the second ion exchange run.

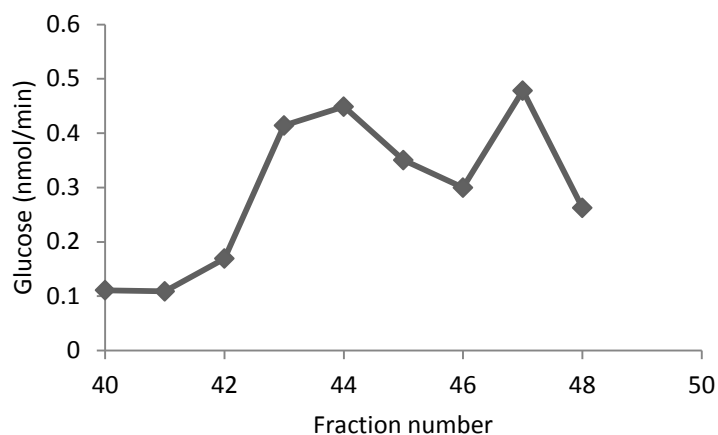
#### 4.7.2.1.2 Second purification step

The combined fractions (2.5 mL) from the first ion exchange purification step with a total of 0.77 mg myrosinase active protein were loaded onto an ion exchange column as used in the first step (Mono Q) for a second round of purification using exactly the same chromatography conditions. After completion of the second run the active fractions (Figure 4.9) were pooled and desalted against CPB (20 mM, pH 6.0) and the myrosinase activity was assayed using GOD Perid method (Chapter 2.2.9).



**Fig 4.9** Ion exchange second run of *Citrobacter myrosinase*. An anion Mono Q column was used at flow rate 0.75 mL / min for one minute with a starting buffer containing Tris HCl buffer (20 mM, pH 8) and eluted with the same buffer but with sodium chloride (1 M). Active fractions (41-46). - - - salt gradient.

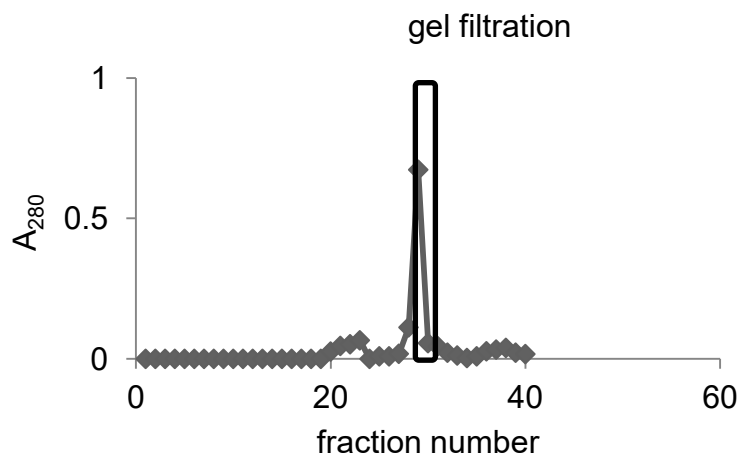
Significant myrosinase activity was found in fractions 43-47 and there is a suggestion that there may be two myrosinase isoforms based on the shape of the activity profile (Figure 4.10). However, this is based on just a single ion exchange chromatogram and was not investigated further. It was decided to combine fractions 43 and 44 for further purification on the basis that combining all active fractions would result in more contamination with other polypeptides. The two fractions (43 and 44) were combined to give 0.05 mg of protein and a specific activity of 593 nmole/mg protein/min.



**Fig 4.10** Corresponding myrosinase activity from ion exchange purification (2) on a Mono Q column. The collected fractions were first desalted against CPB (20 mM, pH 6) then assayed for myrosinase activity by mixing enzyme with sinigrin and buffer CPB (20 mM pH 6) then incubated (25 °C , 2h), the released glucose was measured by GOD Perid reagent

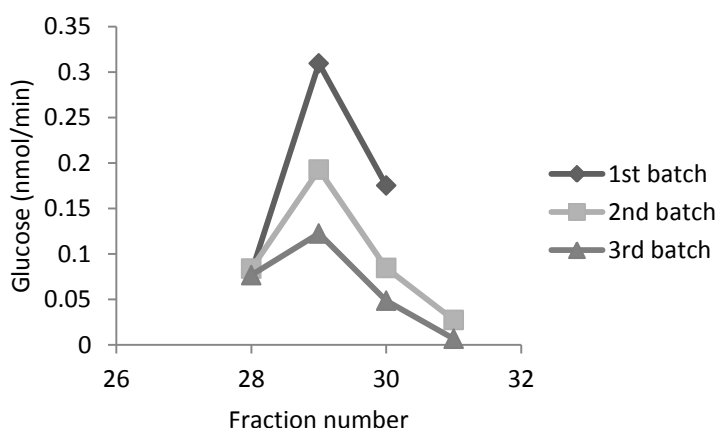
#### 4.7.2.2 Gel filtration

The active fractions from the second purification step (43 and 44) were desalted against CPB (50 mM, pH 6.0) using Econo-Pac 10DG desalting columns and concentrated using a ultra filtration membrane tube and loaded onto a Superdex 75 high resolution (GE Healthcare, Sweden) gel filtration column which has an optimal resolution between 3000 and 70000 Dalton's. An assay of the fractions obtained by gel filtration (Figure 4.11) showed that fractions 26-32 were active for myrosinase.



**Fig 4.11** Gel filtration chromatogram of collected fractions. A Superdex 75 column was used with flow rate 0.35 mL/ min. the column was calibrated against CPB buffer (50 mM pH 6.0) plus 0.15 NaCl and 0.02% sodium azide. The highlighted peak corresponds to the active fractions 28-31.

The highest activity was detected in fraction 29 (Figure 4.12). The estimated protein in this fraction was 0.0045 mg with specific activity 2.69  $\mu\text{mole/mg protein/min}$ . This fraction was selected to run on SDS PAGE to check the myrosinase purity and to obtain an *N*-terminal peptide sequence.



**Fig 4.12** Gel filtration fraction (28, 29, 30 & 31) myrosinase assay. The enzyme was incubated (25 °C, 1h) with sinigrin (0.2  $\mu\text{M}$ ) and CPB (20 mM, pH 6.0). The reaction was stopped by boiling (5 min) and the released glucose was measured by GOD Perid (1 mL). The highest activity was found in fraction 29.

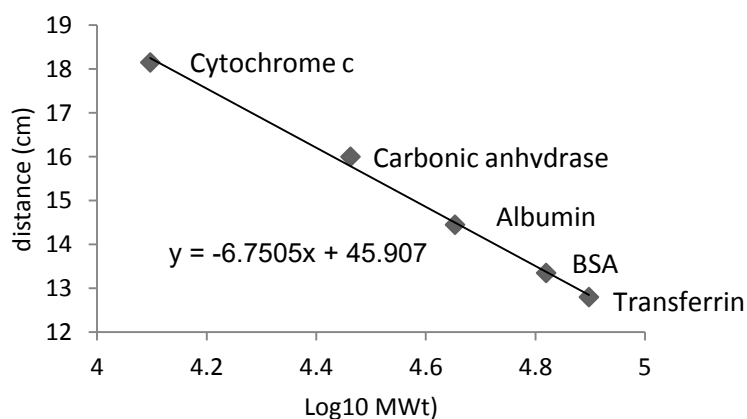


4.7.3 Estimation of the molecular weight of *Citrobacter myrosinase*.

A Superdex 75 column was calibrated with protein markers (Table 4.3) and Figure (4.13).

MW marker	MW (Da)	Distance (cm)	log MW
Transferrin	79000	12.8	4.89
BSA	66,000	13.35	4.81
Albumin	45000	14.45	4.65
Carbonic anhydrase	29,000	16	4.46
Cytochrome C	12,400	18.15	4.09

**Table 4.3** Calibration of Superdex 75 column.



**Fig 4.13** Calibration curve of gel filtration column (Superdex 75) obtained by plotting the logarithm of the molecular weight (log<sub>10</sub> molecular weight) V distance (cm).

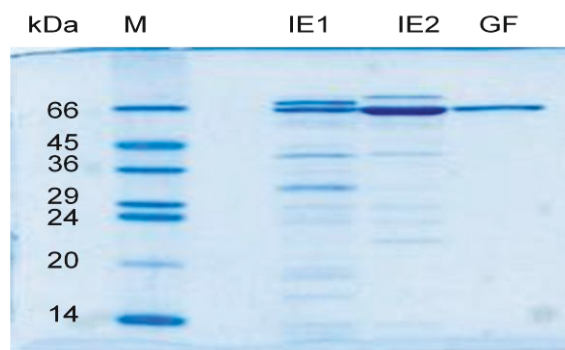
Crude lysate of *Citrobacter* containing myrosinase was applied to the column and fractions collected and assayed for activity. Myrosinase activity was found in fractions 21 to 31; however, the highest activity was found in fraction 29 which correspond to a molecular mass of approximately 66 kDa.

The purification of *Citrobacter* myrosinase activity is summarised in Table (4.4). Complete purification of *Citrobacter* myrosinase to homogeneity was achieved using both ion exchange (two steps using an anion Mono Q column) and gel filtration chromatography (Superdex 75 column).

Purification step	Protein (mg)	Total activity*	Specific activity*	Purification fold	Recovery (%)
Crude extract	40	24444.4	611	1.00	100
Ion exchange 1st (Mono Q column )	0.77	861.1	1111	1.82	3.52
Ion exchange 2nd (Mono Q column )	0.05	29.6	593	0.97	0.12
Gel filtration (Superdex 75 column)	0.0045	12.1	2686	4.40	0.05

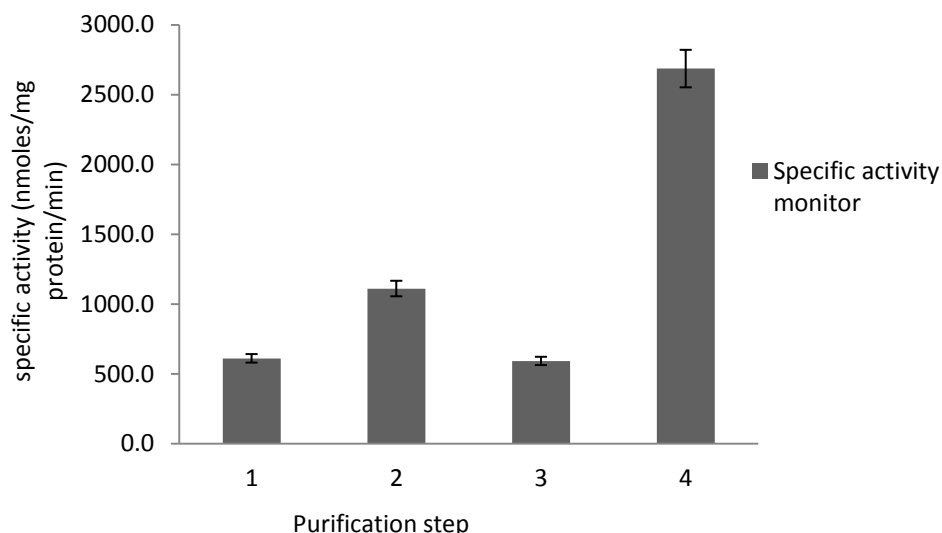
**Table 4.4** Summary of purification of *Citrobacter* myrosinase. At all steps assays were conducted with sinigrin (10 mM) as substrate at 25 °C at pH 6.0 (CPB 20 mM). Activity is based on glucose release measured using the GOD-Perid assay. \*Total activity expressed as nmole while the specific activity expressed as nmole/mg protein/min

On the basis of SDS gel electrophoresis (Figure 4.14) a single protein band was obtained which migrated according to a relative molecular weight of 66 kDa which is in agreement with the data obtained from the gel filtration of native protein.



**Fig. 4.14** SDS PAGE of purification steps of *Citrobacter myrosinase*; M= marker; IE1= Ion exchange first run; IE2= Ion exchange second run; GF= gel filtration fraction (29) pure myrosinase at 66 kDa. After electrophoresis the gel was washed three times with Milli Q H<sub>2</sub>O (100 mL) and stained with SimplyBlue SafeStain (Invitrogen LC6060) for an hour and destained in Milli Q H<sub>2</sub>O (100 mL).

Prior to ion exchange the measured protein concentration in the crude extract was 40 mg. This sharply decreased to 0.77 mg & 0.05 mg after the first and second ion exchange steps respectively while in the last purification step of gel filtration the measured protein was 4.5 $\mu$ g. There was a significant drop in recovered protein and the overall recovery of the pure myrosinase was 0.05 % indicating that it is present in low concentrations. On the other hand an increase of specific activity (Figure 4.15) was achieved after both ion exchange steps compared with the activity of crude extract. The second ion exchange step gives a specific activity of 592.6 nmole/min/mg protein while in the final purification step by gel filtration there is a sharp increase in the specific activity to 2686.2 nmole/min/mg protein. This was detected in fraction 29 which contained the pure protein. The use of two steps of purification using ion exchange had a significant effect on the purification process, and many contaminant proteins were removed. This was clear on the SDS PAGE gel (Figure 4.14) which showed fewer bands than the previous ion exchange step. Purification of *Citrobacter*  $\beta$ -S-thioglucosidase to homogeneity was finally accomplished by gel filtration chromatography.

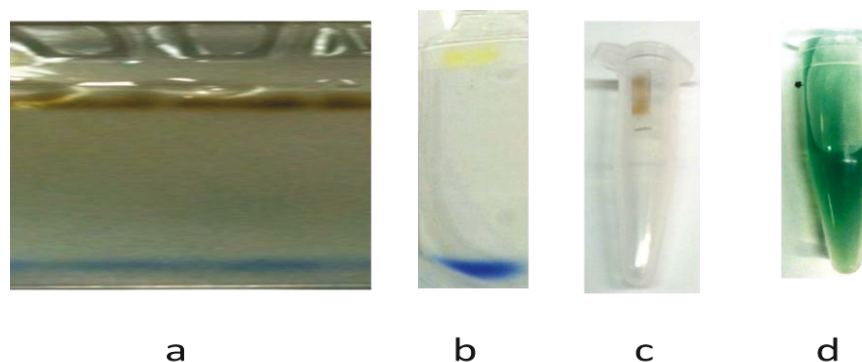


**Fig 4.15** Summary of the purification step versus specific activity; the enzyme (was incubated with substrate (sinigrin) and buffer; 1= crude extract; 2= first Ion exchange; 3 = second ion exchange; 4= gel filtration step.

#### 4.7.4 Elution of *Citrobacter myrosinase* from native polyacrylamide gel

The aim of this experiment was to develop a screening method for bacterial myrosinase of *Citrobacter* by using native polyacrylamide gel and esculin reagent for activity assay. Esculin is a glycoside composed of glucose and a dihydroxycoumarin compound, it is a product of a coumarin derivative extracted from the bark of flowering ash (*Fraxinus ornus*). Esculin is used as a differential agent to facilitate the identification of many organisms such as *Enterobacteriaceae*, *Enterococci* and anaerobes. Hydrolysis of esculin yields esculetin and glucose. In the presence of an iron salt, esculetin forms a brown-black complex that diffuses into the surrounding medium. The  $\beta$ -glucosidase activity of the crude extract of *Citrobacter* was confirmed by incubating the gel with esculin reagent or with *p*NPG (10 mM in CPB). A dark brown colour was developed within 3 min of incubation with esculin reagent (Figure 4.16 a), while a yellow colour was formed with *p*NPG (Figure 4.16 b) after 10-20 min of

incubation. The myrosinase like activity was confirmed by incubating one band of esculin gel (Figure 4.16 c) with sinigrin and buffer (CPB, 20 Mm, pH 6) (Figure 4.16 d).



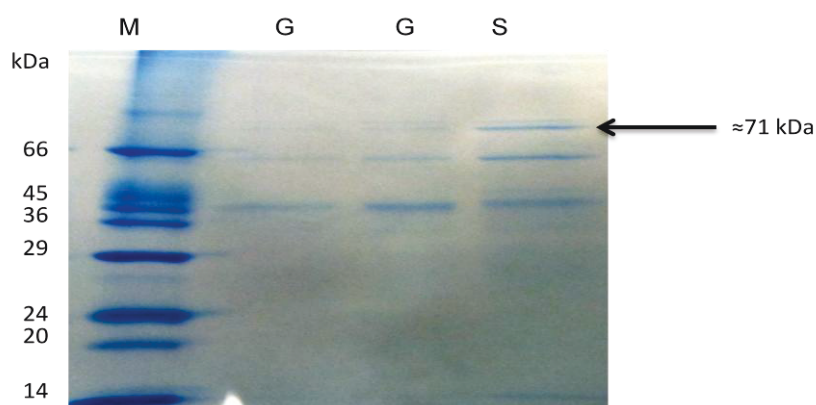
**Fig 4.16** Native gel with esculin reagent from left; (a) the top dark brown colour is indication of esculetin due to  $\beta$ -glucosidase; (b) the yellow stain is caused by reaction with pNPG; (c) the first microfuge tube from left contained the cut band of the esculin reaction which was then assayed for  $\beta$ -glucosidase activity by using sinigrin; a positive reaction was observed (second green tube) (d).

A negative reaction was recorded for non induced cells (control) which was grown on M9 containing glucose as the only carbon source lane 1 (Figure 4.17). Thus it is evident that the *Citrobacter*  $\beta$ -glucosidase is an inducible enzyme.



**Fig 4.17** Native Page assay with esculin reagent: after electrophoresis the gel was incubated with esculin reagent; 1= control (cells was grown on glucose); 2= induced cells, dark band indicated activity of *Citrobacter*  $\beta$ -glucosidase. This experiment was repeated three times.

The eluted proteins were subjected to SDS PAGE, only five to six bands migrated indicating the semi purity of protein, interestingly two of these bands were above 66 kDa and appeared only in the sinigrin extract cells (induced cells) but not in the control. The highlighted band was sequenced (Figure 4.18). The estimated molecular mass of this band is  $\approx 71$  kDa.

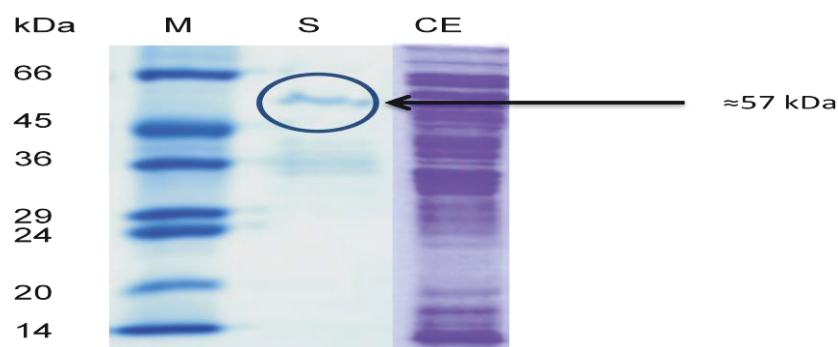


**Fig 4.18** SDS PAGE of semi-purified  $\beta$ -glucosidase of *Citrobacter* eluted from native gel and run on SDS gel. M= marker; G= glucose extract (non induced cells); S= sinigrin extract (induced cells).The highlighted band ( $\approx 71$  kDa) was sequenced.

The activity of *Citrobacter* myrosinase could not be recovered after removal of SDS from the gel indicating that *Citrobacter* glucosidase was fully denatured.

#### 4.7.5 Detection of myrosinase activity in the culture medium supernatant of *Citrobacter*

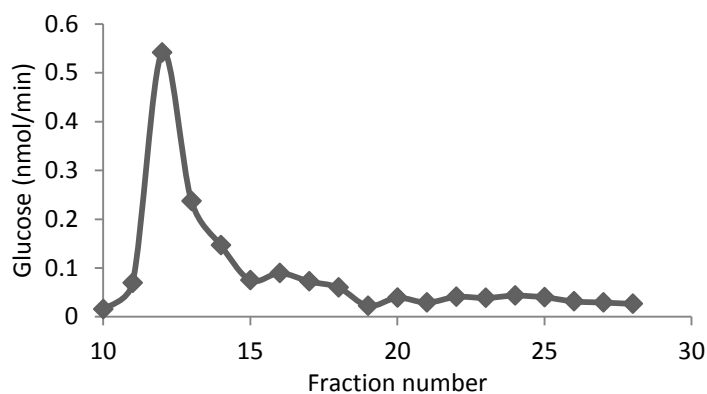
The myrosinase like activity was confirmed by incubating the enzyme with sinigrin. The released glucose was measured using the GOD Perid method (Chapter 2.2.9). Analysis of the sample on SDS PAGE (Figure 4.19) shows a few bands, of which the highest molecular weight was found to be  $\approx 57$  kDa. On one hand this suggests that this band (highlighted) is not the same band while it also suggests that *Citrobacter* may produce different  $\beta$ -glucosidases, or it could be a dimeric protein or active degraded protein. Given also that the protein is present in very small quantities it may well be that the enzyme although active may not be detectable at this level by SDS-PAGE analysis.



**Fig 4.19** SDS PAGE of partially purified beta glucosidase from M9 culture supernatant of *Citrobacter* spp. M= protein marker; S= sample; CE= crude extract of cells.

4.7.6 Initial purification of *Citrobacter myrosinase*

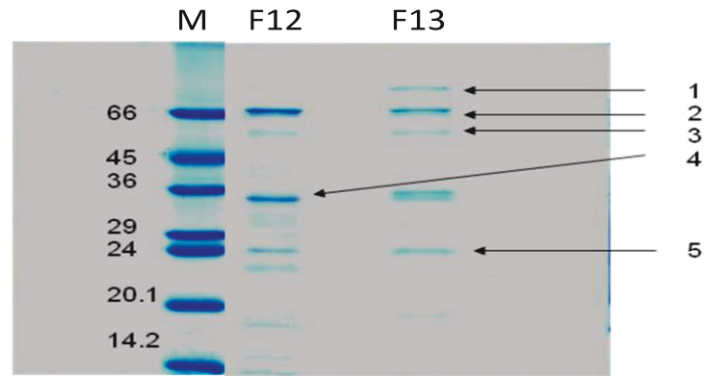
The aim of the experiment was to obtain partially purified peptide sequences of *Citrobacter myrosinase*. The purification of the myrosinase was carried out using a salt gradient as described in the methods. Each fraction was assayed after desalting against CPB (20 mM, pH 6.0). Figure 4.20 shows that the maximum activity was obtained in fractions 12 & 13. Less activity was obtained in the other fractions.



**Fig. 4.20** Corresponding myrosinase activity from ion exchange purification on a Resource Q column. The collected fractions were first desalted against CPB (20 mM, pH 6) then assayed for myrosinase activity by mixing enzyme with sinigrin and CPB (20 mM, pH 6) then incubated at 37 °C / 2h, the released glucose was measured by GOD Perid reagent.

Fractions 12 & 13 were concentrated and run on an SDS gel. Total protein in the crude extract (from 100 ml of culture) was 14.4 mg of protein with an activity of 0.29 nmole/min/mg protein. After ion exchange the measured protein in fraction 12 was 0.31 mg with activity of 4.13 nmole/min /mg protein while in fraction 13 the measured protein was 0.19 mg with activity of 2.82 nmole/min /mg protein. The highlighted bands were selected, excised and subjected to peptide sequencing (Figure 4.21).

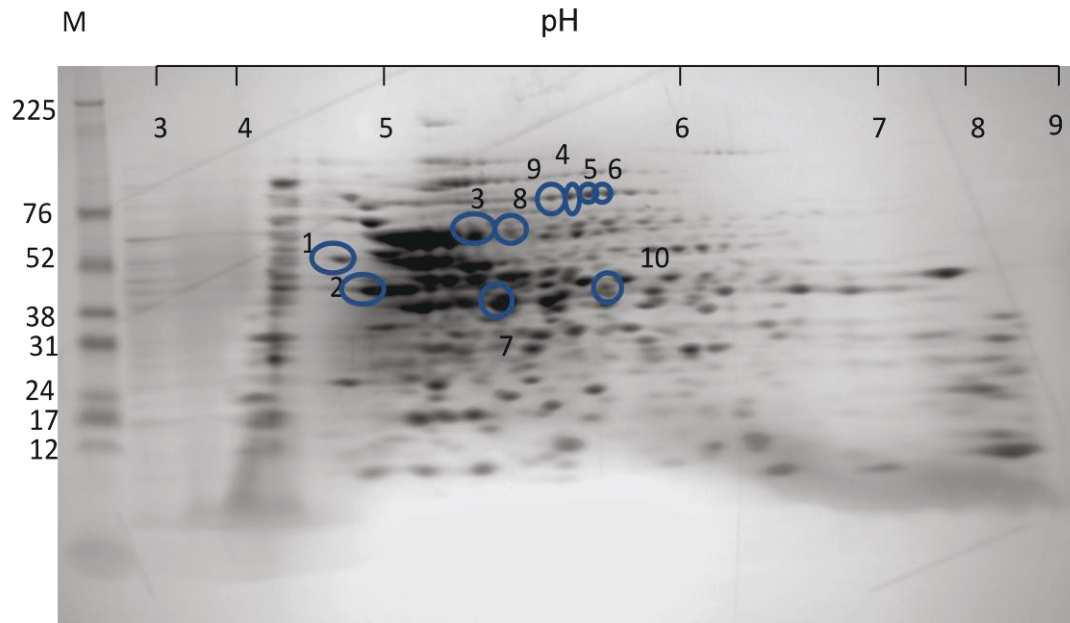




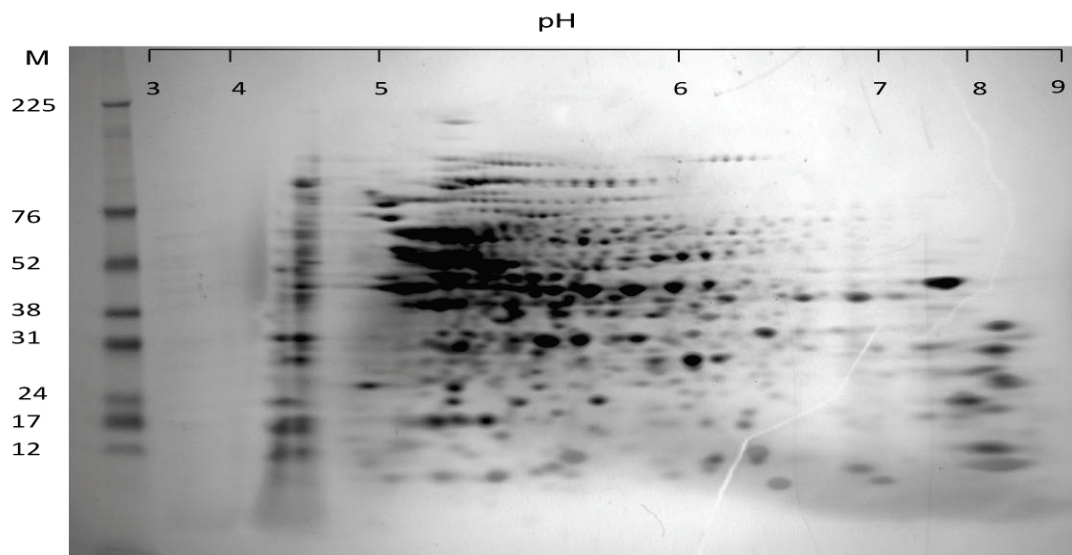
**Fig. 4.21** SDS PAGE of initial purification of *Citrobacter myrosinase*. The myrosinase was partially purified by ion exchange on a Resource Q column using the gradient listed in section 4.7.3.1.1. Two fractions (F12 & F13) were selected for SDS-PAGE analysis. The numbers indicate the bands that were cut out and sent for sequencing. M = marker

#### 4.7.7 Two dimensional gel electrophoresis (2D)

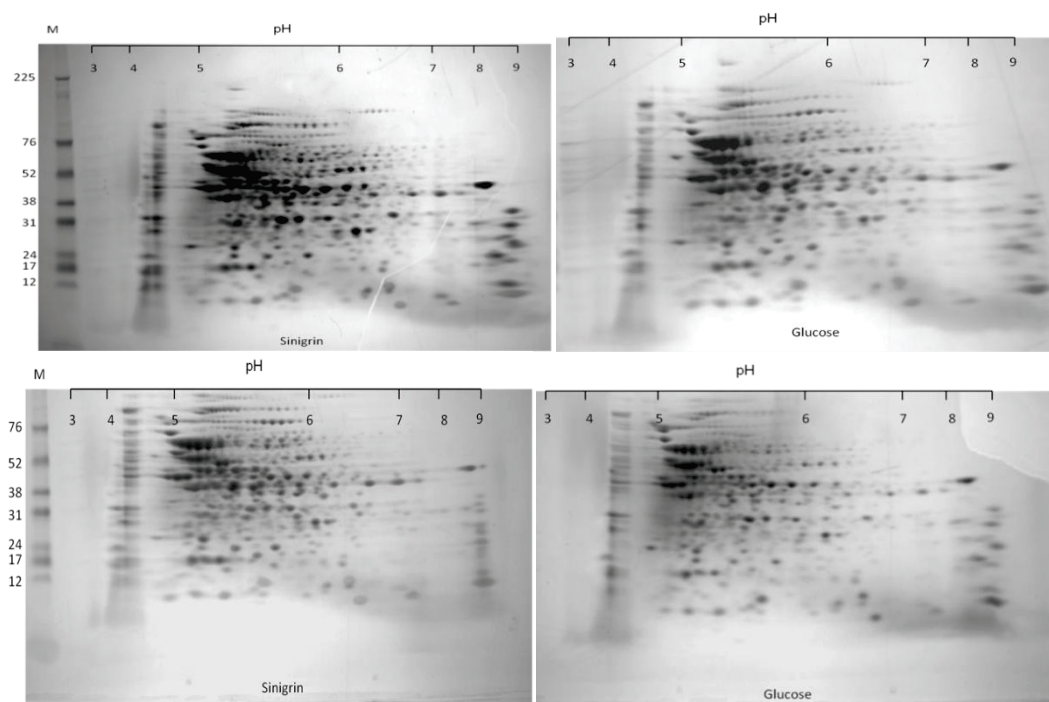
The second dimensional procedure was performed under denaturing conditions. This will give the highest resolution and cleanest results; however, myrosinase activity cannot be assayed due to denaturing conditions. The protein concentration is usually measured after lysis or after precipitation (clean up) step but the use of many reagents used to solubilise sample, including reductants, carrier ampholytes, chaotropes and detergents are incompatible with common protein assays (Rabilloud et al., 2007). The main objective of 2D gel is to compare sinigrin inducible proteins against proteins from glucose-grown *Citrobacter* (myrosinase absent). Figures (4.22) and (4.23) are 2D images and replicates are shown in figure 4.24. The highlighted spots are examples of differences between the two samples and were selected for peptide sequencing.



**Fig 4.22** Potential spots of *Citrobacter myrosinase* on a 2D gel. The *Citrobacter* was grown on M9 containing the myrosinase inducer (sinigrin); protein (300 $\mu$ g) was applied into immobiline dry strip (11 cm, pH 3-11) and focused (9.5 h) using IpG phore then transferred into a precast gel and run 10 min/ 40 V then 45 min / 140 V. The gel is stained with instant blue. The highlighted bands were selected for peptide sequencing. M = molecular weight marker.



**Fig 4.23** Control, the bacteria was grown on M9 containing the non inducer (glucose), Protein (300  $\mu$ g) was applied into immobiline dry strip (11 cm, pH 3-11) and focused (9.5 h), using IpG phore then transferred into precast gel and run 10 min/ 40 V then 45 min / 140 V. The gel is stained with instant blue. M = molecular weight marker.



**Fig 4.24** Replication of 2D gels.

#### 4.7.8 Peptide sequence analysis of putative myrosinase

##### 4.7.8.1 *N*-terminal sequence

The purified *Citrobacter myrosinase* (fraction 29) was submitted to the Biochemistry Department, Cambridge UK for *N*-terminal sequence determination. The following 16 amino acids letters are present in the *N*-terminal sequence of *Citrobacter myrosinase*.

***MSIQSAQQPELGYDTV***

BLAST search shows that the obtained *N*-terminal sequences of *Citrobacter myrosinase* did not show any significant similarity to any known myrosinase such as aphid and plant myrosinase; however, as it can be seen from Table 4.5 it has some

homology with glycosyl hydrolase family 20 of *Paenibacillus* sp. HGF5. It has not been reported that the latter has myrosinase like activity.

<i>Citrobacter</i> N-terminal sequence		MSIQSAQQPELGYDTV		Blasted against
glycosyl hydrolase family 20 [ <i>Paenibacillus</i> sp. HGF5]	601	SAQQ+ELGYD	611	blind search
Glycosyl hydrolase family 3 N terminal [ <i>Vibrio</i> <i>parahaemolyticus</i> 16]	31	IQSAQQ	36	beta glucosidase
<i>E. cloacae</i> subsp. <i>cloacae</i> ATCC 13047	322	A+++PELDYD	330	<i>Enterobacter cloacae</i>
<i>Citrobacter</i> sp. 30_2	26	AQNIPQPELG	35	<i>Citrobacter</i>
<i>Citrobacter</i> sp. 30_2	438	QSAQ	441	<i>Citrobacter</i>
<i>Arabidopsis thaliana</i> beta-glucosidase 26	100	YD	101	myrosinase
[ <i>Arabidopsis thaliana</i> ] beta-thioglucoside	191	GYDT	194	thioglucosidase
glucohydrolase [ <i>Carica papaya</i> ]	304	QRAQDFKLG	312	thioglucosidase
beta-thioglucoside glucohydrolase [ <i>Carica papaya</i> ]	1	MAIQ	4	thioglucosidase

**Table 4.5** Alignment of *Citrobacter* myrosinase N-terminal sequence with different  $\beta$ -glucosidases. The blast was carried out using <http://blast.ncbi.nlm.nih.gov>.

4.7.8.2 Peptide sequence of the band of  $\approx 71$  kDa

The blast search was aligned to search for peptide sequence similarities to the  $\beta$ -glucosidase of *Citrobacter* 30\_2. The result has shown that 5 peptides with score of 99% were similar to  $\beta$ -glucosidase of *Citrobacter* sp. 30\_2 (Table 4.6).

Peptide sequence	Similarity (%)
AVGITEALSPQADIATEPR	99
ISGTFGEDPELAR	99
LPFALPASMDAVLK	99
NSYQALAGISNPAGK	99
PAVLTGVTDK	99
ISGTFGEDPELARK	30.7
NILPLKPGTR	25.7

**Table 4.6** Alignment of peptide sequence of the band  $\approx 71$  kDa with *Citrobacter* sp. 30\_2  $\beta$ -glucosidase. 5 peptides hit 99 % similarity to *Citrobacter* sp. 30\_2  $\beta$ -glucosidase sequence. Two peptides show lower similarity.

## 4.7.8.3 Amino acid sequence of peptides obtained from protein present in the bacterial cell supernatant

The peptide sequence of secreted enzyme protein present in the bacterial cell supernatant Figure 4.17 has shown some similarity with Glycoside hydrolase, family 3 of *Ktedonobacter racemifer* DSM 44963. However, it doesn't have any similarity with any known plant or aphid myrosinases (Table 4.7).

	Database	Accession	Score	Mass	Number of matches	Description
1	NCBIInr	gi 136429	85	24394	2	RecName: Full=Trypsin; Flags: Precursor RecName: Full=Glyceraldehyde-3-phosphate dehydrogenase, cytosolic
2	NCBIInr	gi 120666	76	36662	1	
3	NCBIInr	gi 7706848	68	49027	2	ATP synthase beta subunit [ <i>Amaranthus hypochondriacus</i> ]
4	NCBIInr	gi 28317	60	59492	1	unnamed protein product [Homo sapiens] oligopeptide transporter with chaperone properties [ <i>Citrobacter sp. 30_2</i> ]
5	NCBIInr	gi 237731743	59	64386	6	
6	NCBIInr	gi 315466374	58	65806	1	ascorbate oxidase [ <i>Cucumis sativus</i> ] hypothetical protein HMPREF0994_02503 [ <i>Lachnospiraceae bacterium 3_1_57FAA_CT1</i> ]
7	NCBIInr	gi 336426487	55	81488	1	
8	NCBIInr	gi 108743972	53	51944	1	8S globulin alpha isoform precursor [ <i>Vigna radiata</i> ] glycoside hydrolase family 3 domain protein [ <i>Ktedonobacter racemifer</i> DSM 44963]
9	NCBIInr	gi 298244743	52	66706	1	
10	NCBIInr	gi 449438161	52	25208	1	predicted: protein phloem protein 2-like A1-like [ <i>Cucumis sativus</i> ] electron transport complex, RnfABCDGE type, B subunit
11	NCBIInr	gi 255280016	47	27051	1	[ <i>Bryantella formatexigens</i> DSM 14469] AMP-dependent synthetase and ligase [ <i>Desulfovibrio fructosovorans JJ</i> ]
12	NCBIInr	gi 303249386	41	54663	1	
13	NCBIInr	gi 187736658	37	21959	1	beta actin [ <i>Medicago sativa</i> ] predicted: low quality protein: ATP-binding cassette sub-family A member 10 [ <i>Callithrix jacchus</i> ]
	NCBIInr	gi 390463752	37	108783	1	

**Table 4.7** Peptide sequence of secreted myrosinase

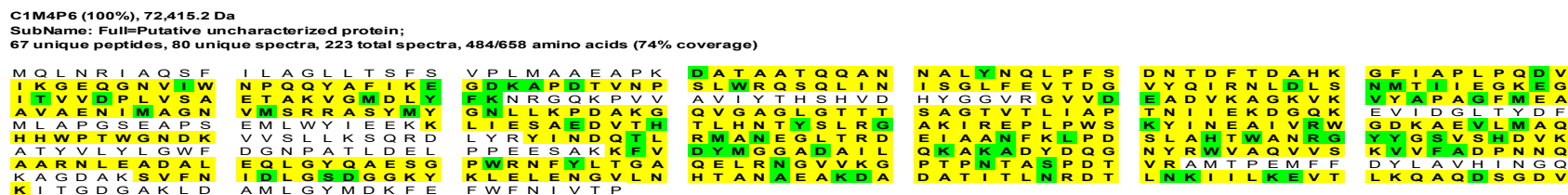
#### 4.7.8.4 Amino acid sequence of peptides obtained from gel slices of partially purified *Citrobacter myrosinase* (initial purification)

Gel slices were taken from the gel (Figure 4.5) and treated with trypsin and subjected to LC-MS analysis (Thermo Fisher Scientific LTQ Orbitrap) for peptide identification. All data of the amino acids sequences were imported into Scaffold <http://www.proteomesoftware.com>, a data software package that interprets peptide sequences based on the masses of daughter ions derived from the parent ion. The sequence is shown in Figure 4.25. An example of a spectrum of a peptide is shown in Figure 4.26. Other proteins identified (150) have little homology with beta glucosidases and are not discussed. A BLAST analysis (Table 4.8) was then carried out for each set of peptides and with the *Citrobacter sp 30\_2* proteome (Alex Jones, Sainsbury Laboratory). However, for band 2 peptide matches were found for an uncharacterised protein (C1M4P6) which blasts to alkylsulphur transferases and putative hydrolases and has a molecular mass of 72 kDa. Other proteins identified (150) have little homology with beta glucosidases and are not discussed.

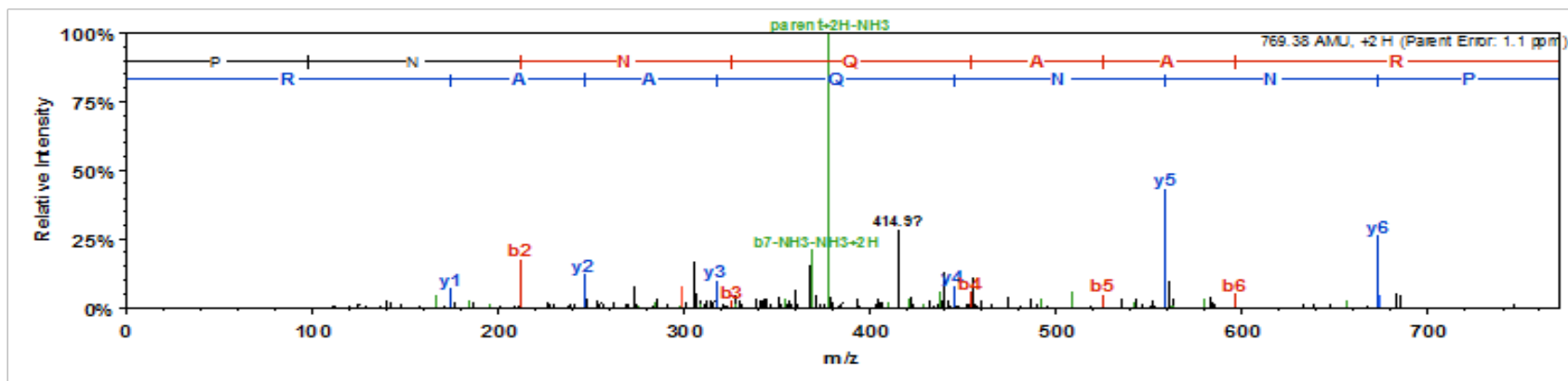
Band No	Accession No.	Description	MWt (Da)	Peptide No.	Coverage (%)
1	B5MU02	Alkyl/aryl sulfatase	72698	2	53
2	C1M4P6	Putative uncharacterised protein	72415	67	74
3	B2NXQ9	Beta glucosidase periplasmic	83489	1	2
4	A9MR82	Transaldolase	35149	33	78
5	Q5PHM1	Putative hydrolase	72585	1	4
5	A9MH26	Putative hydrolase	26925	2	7

**Table 4.8** Examples of the obtained peptides sequence of partially purified *Citrobacter myrosinase* using single ion exchange step.





**Fig 4.25** The sequence of C1M4P6 derived from a BLAST search (*Citrobacter* sp 30\_2, proteome) from band 2 digested proteins. The colour green represents a probability of >95% and yellow > 80-95%.



**Fig**

**4.26** Mass Spectrum of peptide sequence (D) PNNQAAR (N) matching C1M4

## 4.7.8.5 Amino acid sequence of peptides obtained from spots of 2D gel

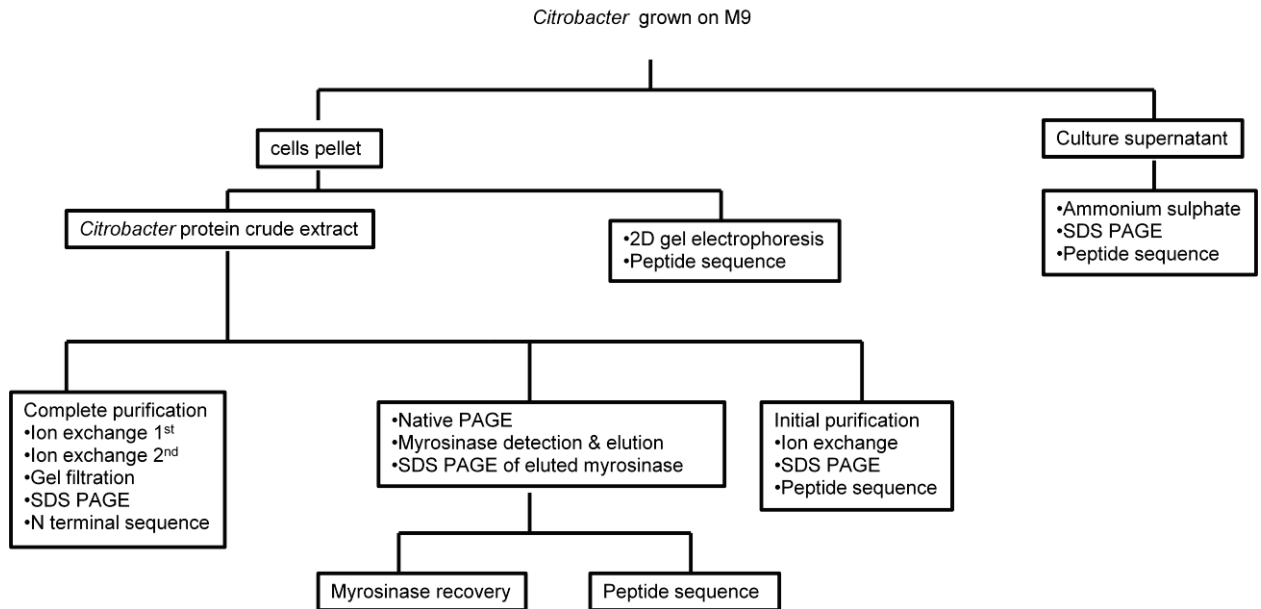
Although many spots were present in the sinigrin extract but not found in the 2D gel of glucose-grown protein extract the peptide sequence of these spots did not result in any similarity with the target protein (myrosinase) Table 4.9 shows an example of potential spots that have been identified by 2D software on 2D gel of crude lysate of induced cells. However, these spots don't represent *Citrobacter myrosinase*.

Band No.	Accession No.	Peptide	MWt(Da)	Description	Coverage (%)
1	A6TG38	SVDAMIPIGR	55,169	ATP SYNTHASE SUBUNIT ALPHA	4
2	C1MDJ9	TDTDILISQR	55,484	Putative uncharacterised protein	5
3	B6FG85	AFEYLMTR	56,454	Putative uncharacterised protein	4
4	A2X22W9	IQNAGTEVVEAK	42,221	protein	3
5	A2IBY3	NMSTYVDYK	40,564	OmpE36	13
6	A1A9U6	FSIDDVAK	58,649	Glucans biosynthesis protein	11
7	B1LHK1	GLVTKQLSGK	59,617	Phosphoenolpyruvate carboxkinase [ATP]	16
8	A8ADW9	ALAGAWR	61,246	SEPHCHC synthase	3
9	B6FD9	CSEVVVYFK	60,333	Glucose-6-phosphate 1-dehydrogenase	6
10	C2B636	HFLQSESEPK	94,800	Putative uncharacterised protein	5

**Table 4.9** Peptide sequences and putative identity of spots present on 2D gels.

## 4.8 Discussion

The summary of this chapter is illustrated in the following figure (Figure 4.27).



**Figure 4.27** Summary of work conducted in Chapter 4

Many fungi and bacteria isolated from different sources such as human & insect gut and soil and have been reported to have myrosinase activity. In contrast the only bacterial myrosinase to be purified to homogeneity is that of *Enterobacter cloacae* no. 506. This was purified by precipitation with ammonium sulphate and chromatography on CM-Sephadex followed by two steps of gel filtration using Sephadex G-200 & G-100 respectively (Tani et al., 1974). A soil isolate belonging to the genus *Citrobacter* possessed myrosinase activity with the enzyme present in extracts in soluble form (Chapter 3), and therefore the enzyme was chosen for purification and characterisation. This is the first time that  $\beta$ -glucosidase with myrosinase-like activity

has been isolated from this organism. It is the second bacterial myrosinase that has been successfully purified to homogeneity.

The use of large scale culture in M9 medium (500 mL) and a three step chromatography procedure allowed the complete purification to homogeneity (Figure 4.27). The estimated pI was found at the ~pH 6 at which the protein bound to the ion exchange mini Q column. This was investigated in order to plan a large scale ion exchange purification step. Since proteins are negatively charged at pH above their pI, tris buffer (pH 8.0) was used to equilibrate the anion exchanger. A sharp decrease in the total protein was observed after the first ion exchange run (0.77 mg) compared to the start point of 40 mg (crude extract) indicating that the target protein is not abundant. The first ion exchange step yielded approximately two fold purification while the second ion exchange step yielded approximately one fold. A further purification (four fold) was achieved by gel filtration (Figure 4.27). This is much less than with the myrosinase of *Enterobacter cloacae* no. 506 which was purified approximately 1000 fold. The bacterial myrosinase of *Enterobacter cloacae* no. 506 was much easier to purify than *Citrobacter* myrosinase. Although the start culture medium for this bacterium is not known the amounts of protein in the crude extract (41000 mg) and in the pure fraction (15 mg) (Tani et al., 1974) gives a yield of 0.0366% compared to 0.011% for *Citrobacter*. Thus in both cases the myrosinase is not an abundant protein. Plant myrosinase of some seeds are relatively abundant and can be easily purified by fractionation with ammonium sulphate followed by ion exchange chromatography (Bones and Rossiter, 2006). The *Citrobacter* myrosinase is highly purified with efficient yields in term of specific activity and it is similar to that reported for *Enterobacter cloacae* enzyme ( $k_m$  values are discussed in Chapter 3). Although the specific activity (Table 4.4) of *Citrobacter* myrosinase increased after the first ion exchange step but declined after the second ion exchange step then increased after the gel filtration step. Overall, the specific activity increased but total activity declined. A similar result was

reported for the bacterial myrosinase of *Enterobacter cloacae* no. 506 (Tani et al., 1974) and for aphid myrosinase (Jones et al., 2001). Thus it is possible that although a single band (Figure 4.14) was obtained the protein it may well be that some of the protein is inactive. This is not unusual as it is quite common to stabilise pure proteins with other proteins such as BSA.

Many  $\beta$ -glucosidases purified from bacteria or archaea such as *Streptomyces reticuli* and *Clostridium thermocellum* were reported as monomeric proteins; however, some have also been described as dimeric, tetrameric and hexameric in structure (Gueguen et al., 1997). The range of molecular masses for  $\beta$ -glucosidase monomers is 55-62 kDa (Esen, 1993). The estimated molecular mass of the purified *Citrobacter myrosinase* using SDS-PAGE (denaturing) and gel filtration (native) analysis was 66 kDa indicating that the enzyme is monomeric as only a single band migrated on SDS PAGE, and is very close to if not within the monomeric upper range of  $\beta$ -glucosidase. The molecular mass is much closer to that of the *Enterobacter cloacae* enzyme (61 kDa) than to aphid myrosinase of *Brevicoryne brassicae* (L.) (53 kDa) (Jones et al., 2001); however, it is not comparable with the molecular weight of the dimeric plant myrosinase that are in the range of 125 to over 150 kDa (Bones and Rossiter, 1996).

The *Citrobacter* spp possess the ability to secrete extracellular  $\beta$ -glucosidase with myrosinase activity when switched to M9 culture medium containing sinigrin as the only carbon source. If this protein resulted from broken cells during cultivation of the *Citrobacter* then one would expect to see more proteins as in crude extract. Interestingly fewer contaminant proteins were observed on SDS PAGE (Figure 4.17) and thus the obtained myrosinase was considered a semi purified protein. This can be purified further by using a single step of ion exchange or gel filtration step or by use of different concentrations of ammonium sulphate precipitation. This method saved time compared with the traditional purification methods that include ion exchange and gel

filtration as these processes require time and more chemicals such as buffers, desalting and concentrating sample several times before and after many steps. A potential origin of the activity from dead cells cannot be ruled out. The molecular weight of the semi purified enzyme is about 55 kDa, this is lower than the molecular weight of intercellular myrosinase which was fully purified in this study of the same organism (66 kDa). The protein of 55 kDa could be an isoenzyme which has myrosinase activity against sinigrin but with a molecular mass lower than that of the purified myrosinase in this study (66 kDa) or may just be a proteolytic degradation product from dead bacterial cells. In the first ion exchange step two myrosinase active peaks were observed, one major and one minor. This might indicate that the minor peak contains the potential 55 kDa active myrosinase. The potential of myrosinase as a secreted protein requires further investigation. Due to insufficient material this experiment was carried out only once and requires further work. However, the data obtained looks promising especially if the plan is to obtain peptide sequence.

Detection of enzyme activity using esculin is the modified version of the method described by Kwon et al. (1994) who reported this method for  $\beta$ -glucosidases but not myrosinase (Kwon et al., 1994). Myrosinase activity of plant origin was detected by incubating isoelectric focusing (IEF) gel with barium sulphate assay which is described by Bones and Slupphaug (1989) but the myrosinase was not eluted from the gel. Zymogram technique of a number of enzymes including  $\beta$ -glucosidase such of maize was developed after SDS PAGE (Esen, 1993). In the present study myrosinase like activity of *Citrobacter* was detected in the induced cells but not in the control (Figure 4.17). This method is simple and fast in terms of time and reagents. Unlike the method described by Bones and Slupphaug (1989) that required sinigrin (0.5 mL) of 12 mM or barium sulphate (0.5 mL) of 18 mM and longer incubation time (30 min or more). Here only two  $\beta$ -glycoside substrates are used esculin & pNPG, the result can be seen within 3 min of incubating the native gel with esculin reagent or about 10 min with pNPG. The

purpose use of these two substrates was first to confirm  $\beta$ -glucosidase activity and secondly to check if this glucosidase possess S-glucosidase activity. This was confirmed by incubating one of the active bands with sinigrin (Figure 4.18d). This is a strong evidence that the *Citrobacter*  $\beta$ -glucosidase has both  $\beta$ -O-glucosidase and  $\beta$ -S-thioglucohydrolase activity. SDS PAGE analysis of the eluted bands (Figure 4.18) showed fewer protein bands, indicating that this method can be employed as a purification step and hence allows a shortcut approach towards protein sequencing. This is comparable with the traditional purification methods such as ion exchange and gel filtration. The number of protein bands obtained by this method (Figure 4.18) is much less than that obtained by ion exchange second run (Figure 4.14). One of the major advantages of using this method is the reduction of culture volume from 500 to 20 mL and subsequently a reduction in sinigrin use. Ion exchange & gel filtration methods are generally used to purify proteins from large scale culture medium and hence a large amount of substrate is required. Where the substrate is cheap it does not pose a limitation in terms of cost, however, for sinigrin this is not the case. The use of the current method in a single step represented a promising tool for obtaining the amino acid sequence thus allowing gene cloning strategies to be devised and ultimately protein over expression systems. Furthermore this method can be used to scan more than one organism at the same time for myrosinase like activity. The method can be improved by repeating the same steps once more. This may result in huge purification step for the target protein. A single gel filtration step can be employed after this method after which one would expect to see a pure *Citrobacter* myrosinase. It was not possible to recover *Citrobacter* myrosinase activity after removal of SDS of the SDS PAGE. This suggests that this enzyme doesn't possess rigid or compact structure similar to  $\beta$ -glucosidase of maize and thermophilic bacteria. To confirm this a future study should extend the study to test the *Citrobacter* myrosinase activity in presence of different concentration of SDS.

The peptide and N-terminal sequences of *Citrobacter myrosinase* have shown little similarity with glucosidases of the same genus especially *Citrobacter sp. 30\_2*. The latter is known to have a  $\beta$ -glucosidase at approximately 55 kDa. However, this bacterium failed to show myrosinase activity when tested in our lab (data not shown). Induction and detection of myrosinase activity of *Citrobacter sp. 30\_2* was done exactly as for our new isolate. Investigation of induction of  $\beta$ -glucosidases other than myrosinase from this organism was not carried out. As no bacterial myrosinase has been sequenced it is not possible to rule out potential activity from the proteins identified. The data obtained from LC-MS confirm the enzyme identity as  $\beta$ -glucosidase; however, no significant sequence homology existed. In the 2D gel, many spots appeared in sinigrin-induced extract but not in the negative control; however, the peptide sequences of these spots did not yield any homology with the target protein (myrosinase). Interestingly sinigrin seems to induce other proteins in *Citrobacter* but these proteins were not further investigated as the aim of sequence to obtain peptide sequence of the myrosinase. The use of crude extract in such conditions is more likely to complicate the experiment and as a result there are many protein spots that appeared but not related to the target. The 2D experiment was done at early stage of this project and before the molecular weight and pI (pH 6) of *Citrobacter myrosinase* (66 kDa) was known and hence for future study one should only select spots with these two criteria. The 2D procedure used here was conducted under denaturing condition and thus the myrosinase activity cannot be assayed under such condition.

The sequence data of the band with molecular weight of  $\approx 71$  kDa (Figure 4.18) shows a partial match with the  $\beta$ -glucosidase of *Citrobacter 30\_2*. This could be either myrosinase isozymes or another  $\beta$ -glucosidase that is induced in the presence of sinigrin. The molecular weight of this protein possibly similar of that partially purified protein data (72 kDa) (Figure 4.21). However, the latter came up with uncharacterised protein (C1M4P6) which blasts to alkylsulphur transferases and putative hydrolases.



While the genome for *Citrobacter* sp. 30\_2 is known it is possible that the *Citrobacter* isolate used in this work is a variant. This may explain why only partial homologies were obtained from the peptides resulting from the tryptic digests. Ideally the genome of the *Citrobacter* isolate should be obtained to enable a cloning strategy based on the peptide sequences of the putative myrosinase. The draft genome of the *Citrobacter* isolate is currently being undertaken.

### Conclusion

Since all of these attempts to obtain peptide sequence of *Citrobacter* myrosinase were only partially successful and ideally the whole genome sequence for the *Citrobacter* spp. is required. This may enable the identification of the myrosinase at the gene level.

**CHAPTER FIVE**  
**GENERAL DISCUSSION**

## General discussion

An intracellular *Citrobacter* myrosinase, possibly located in the periplasmic space between the cell wall and cell membrane, was isolated and purified to homogeneity.  $\beta$ -O-Glucosidases have been reported in several *Citrobacter* species, such as *Citrobacter freundii*, *C. diversus* and *C. amalonaticus* (Kämpfer et al., 1991), *Citrobacter* sp. 30\_2 NCBI accession ZP\_04559043, *C. youngae* ATCC 29220 NCBI accession EFE10211, *C. rodentium* ICC168 NCBI accession CBG88999 and *C. koseri* ATCC BAA-895 NCBI accession ABV11816 [www.pubmed.com](http://www.pubmed.com); however, none of these species have been reported to possess myrosinase-like activity. The data presented here is the first evidence to suggest myrosinase-like activity in a *Citrobacter* species. This new species of *Citrobacter* was isolated from field grown oilseed rape (*Brassica napus* L.) (Wye, UK) through an enrichment process where soil was incubated with 2-propenyl glucosinolate followed by continuous subculturing (Dr John Rossiter, unpublished data).

The time required for maximum induction of  $\beta$ -glucosidase production varies among microbial species. For example, *Thermoascus aurianticus* produces a maximum  $\beta$ -O-glucosidase activity after 48 h (Parry et al., 2001) whilst *Melanocarpus* sp requires 72 h (Kaur et al., 2007). For *Candida peltata*, maximum production was reported at 96 h (Saha and Bothast, 1996). In general the optimal production of the  $\beta$ -glucosidases is dependent on several conditions, such as the microbial strain, its genetic makeup, and the culture and environment conditions (Sharma and Bajaj, 2005; Sudan and Bajaj, 2007). The bacterial myrosinase of *Enterobacter cloacae* 506 is an inducible enzyme (Tani et al., 1974); however, due to the high commercial cost of the

substrate 2-propenyl glucosinolate (sinigrin) and the time required to carry out extraction and purification of myrosinase, alternative sources, such as the crude extract of brown mustard seed (*Brassica juncea*) alone (Palop et al., 1995) or with 0.01% sinigrin (Tani et al., 1974) has been used. In the presence of the substrate sinigrin, *Citrobacter* produces myrosinase. Production of this enzyme in *Citrobacter* is not constitutive as myrosinase activity is not induced by glucose or in media such nutrient broth (Figure 2.1). As cells of *Citrobacter* were found to completely metabolise sinigrin (10 mM) within 12-24 h of cultivation under aerobic conditions (Figure 2.12), overnight cultures were used for induction and preparation of protein cell free extracts. A similar induction time for *Lactobacillus agilis* R16 has also been observed (Palop et al., 1995).

Sugars such as cellobiose, lactose, and maltose were easily metabolised by *Citrobacter*. Some of these sugars are known to induce bacterial and fungal  $\beta$ -O-glucosidases. In soil enrichment cultures, cellobiose was reported to be the active inducer in synthesis of  $\beta$ -O-glucosidases by specific proliferation of cultures of both *Pseudomonas* and *Aspergillus* (Busto et al., 1995). However, the myrosinase activity of the soil isolates (*Citrobacter*) is not induced by these sugars and could not be detected in cell free extracts (Section 2.3.1). Cellobiose contains a  $\beta$ -O-linkage which requires the presence of a  $\beta$ -O-glycosidase that is able to hydrolyse this substrate. A mixture of cellobiose and sinigrin was used to investigate the hydrolysis of cellobiose by *Citrobacter* to see if this was due to myrosinase activity or other enzymatic activity. The increase in both cell density (OD) and protein concentration of cell free extracts was not correlated with an increase in *Citrobacter* myrosinase, suggesting that the hydrolysis of cellobiose is independent of myrosinase. The same finding also applies to lactose and maltose. The hydrolysis of these  $\beta$ -O configurations may due to the action of a membrane bound  $\beta$ -O-glucosidase such as that isolated from *Physarum polycephalum* (Hayase et al., 2008). One interesting observation was the induction of myrosinase by trehalose; however, it was considered a residual activity of a different

enzyme that may be induced in the presence of sinigrin and was not comparable with the specific activity of *Citrobacter* myrosinase obtained from cultures grown in sinigrin. *p*-Nitrophenyl  $\beta$ -D-glucopyranoside (*p*NPG) was among the tested substrates for myrosinase inducibility; despite the enzyme possessing a greater affinity for the substrate. *Citrobacter* growth with *p*NPG was limited in M9 medium containing *p*NPG (10 mM) (Figure 2.1) and may be due to the accumulation of inhibitory levels of the product *p*-nitrophenol. The yellow colouration of the culture which occurs after several hours of incubation with *Citrobacter* indicates metabolism and production of *p*-nitrophenol.

Poor growth of *Citrobacter* was observed for several  $\alpha$ -glucoside substrates such as methyl- $\alpha$ -D-glucoside and 4-nitrophenyl- $\alpha$ -D-glucoside, while growth on  $\beta$ -glucosides such as methyl- $\beta$ -D-glucoside and esculin was variable. Surprisingly, *Citrobacter* did not grow on octyl- $\beta$ -1-thioglucopyranoside, which resembles sinigrin in having an *S*-linkage to the sugar group. Since myrosinase is a thioglucosidase, it was expected that *Citrobacter* would be able to metabolise this substrate, but this was not the case. This could be due to the presence of the octyl functionality which is long and bulky or the lack of a sulphonated oxime. None of the tested  $\alpha$ -glucosides were hydrolysed by *Citrobacter* myrosinase thus confirming that this enzyme is a  $\beta$ -*S*-glucosidase with  $\beta$ -*O*-glucosidase activity (Figure 2.1). Cell free extracts with all tested substrates did not induce myrosinase activity, suggesting the inducer sinigrin could not be replaced by these substrates (Figure 2.5). This is in agreement with Duerksen & Halvorson (1959) who reported that *Saccharomyces cerevisiae*  $\beta$ -*O*-glucosidase was poorly induced by aryl  $\beta$ -glucoside but strongly induced by alkyl glucosides.

Cells of *Citrobacter* were found to assimilate sinigrin preferentially over glucotropaeolin and gluconasturtiin (Figure 2.7 & 2.9). *Citrobacter* grown in the presence of sinigrin metabolised this substrate within 8 h of incubation. Both gluconasturtiin and glucotropaeolin were metabolised in the presence of sinigrin to

varying degrees. A decrease in gluconasturtiin was observed during 10 h of cultivation and at this time point sinigrin levels in the culture medium decreased sharply. Levels of glucotropaeolin metabolised by *Citrobacter* cells was much less than that of gluconasturtiin. At 10 h, over 90% of sinigrin, 40% of gluconasturtiin (Figure 2.7) and 30% of glucotropaeolin (Figure 2.9) had been metabolised, suggesting the side chain functionality impacts on the rate of metabolism.

Although the starting pH of the culture medium (M9 plus sinigrin) was neutral (pH 7) at which isothiocyanates (ITCs) are reported to be produced (Bones and Rossiter, 2006), no ITCs could be detected even after the complete metabolism of sinigrin. This finding is in contrast to many bacterial species (intact cells) that have been reported to metabolise sinigrin and produce ITCs such as *Lactobacillus agilis* (Palop et al., 1995). ITCs were only detected in cell free extract of *Citrobacter* (myrosinase) in contrast to *L. agilis*. This suggests that if *Citrobacter* is found in the human gut, it may not produce ITCs. The metabolism of sinigrin by *Citrobacter* both *in vivo* and *in vitro* have been studied; the product ITC was clearly produced by cell free extracts but hardly produced by the resting cell and it is likely that the ITC is being further metabolised. Using NMR to establish the metabolism of sinigrin *in vivo* for both *Citrobacter* and *L. agilis*, Chu (2009) demonstrated metabolism of sinigrin by these two bacterial to be different. Whereas *L. agilis* produces ITCs, *Citrobacter* produced an unknown metabolite. This unknown product was not comparable to allylcyanide (nitrile), allylamine or isothiocyanate standards (Chu, 2009).

Characterisation of myrosinase activity was carried out using crude protein extract and not the purified enzyme. *Citrobacter* myrosinase showed maximum activity at pH 6.0. However, significant activity was also observed over the pH range of 5.6 to

7.0. The optimum pH is close to the previously described fungal myrosinase of *Aspergillus niger* (pH 6.2) (Ohtsuru et al., 1973) and to the myrosinase of *Enterobacter cloacae* (pH 6.8) (Tani et al., 1974), and is within the range of various plant myrosinases (pH 5.0-7.0) (Botti et al., 1995) but slightly higher than the cabbage aphid myrosinase (pH 5.5) (Jones et al., 2001). In general the bacterial  $\beta$ -glycosidases have an acidic pH optimum (Coughlan, 1985), but for many  $\beta$ -glycosidases, optimum pH ranges between 4.0-7.5. This optimum is dependent on the source and cellular location of the  $\beta$ -glycosidases (Ketudat Cairns and Esen, 2010).

The optimum temperature of *Citrobacter* myrosinase is 25 °C; however, significant activity is over the range of 10-35 °C. *Citrobacter* myrosinase is not a thermo stable enzyme, as activity was not detected at temperatures over 50-75 °C. Organisms that live in cold environments produce enzymes characterised by high hydrolytic efficiency at low temperature and low stability (Collins et al., 2003). This applies to *Citrobacter* as it is an isolate from a cold environment (Wye, UK). The optimum temperature of *Citrobacter* myrosinase is close to that of the cabbage aphid myrosinase of 30 °C (Frédéric et al., 2002) but lower than of both *Aspergillus niger* (34 °C) (Ohtsuru et al., 1973) and of bacterial myrosinase *E. cloacae* (37 °C) (Tani et al., 1974).

*Citrobacter* myrosinase assays showed that the enzyme is slightly activated in the presence 0.25 mM of ascorbate, although not significantly. This suggests that *Citrobacter* myrosinase, like the aphid myrosinase is not activated by ascorbate and behaves in terms of mechanism as a classical  $\beta$ -O-glucosidase. Both myrosinases of the bacteria *E. cloacae* (Tani et al., 1974) and aphid (Jones et al., 2001) are not activated by ascorbate.

Glucose at a concentration of 10 mM inhibited *Citrobacter*  $\beta$ -O-glucosidase activity to approximately 90 % suggesting that this enzyme cannot be employed for

bioethanol production, a process at which highly glucose (0.55-1.1M) tolerant  $\beta$ -glucosidase is required. In addition to this, the selected  $\beta$ -glucosidase should function at low pH values (pH 2.5-3.8) (Riou et al., 1998). Generally most microbial  $\beta$ -glucosidases are sensitive to glucose (Riou et al., 1998) as it competes with the substrate for the active site of the enzyme (Krisch et al., 2010). The metal ion chelator EDTA inhibits  $\beta$ -O-glucosidase activity with a loss of 70% while  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $Fe^{3+}$  and  $Mg^{2+}$  inhibit the enzyme to different degrees with  $Fe^{3+}$  causing the largest loss of 76% (3.7.6). These experiments were carried out to establish if any of the tested ions promoted enzyme activity. This is important as metal ions can act as co-factors but this is clearly not the case with *Citrobacter* myrosinase where the opposite occurred i.e. inhibition. *Sinapis alba* myrosinase (Burmeister et al., 1997) possesses a zinc atom which binds the dimeric protein although it is not clear if this is a requirement for activity. In contrast the aphid myrosinase is a monomeric protein and does not contain a zinc atom (Husebye et al., 2005) and it seems likely that this is also true for the *Citrobacter* myrosinase which is also a monomeric protein.

The *Citrobacter* myrosinase could not hydrolyse any sugars with  $\alpha$  and  $\beta$  configurations. The exception was for trehalose {glu ( $\alpha$ 1- $\alpha$ 1) glu} which is slightly hydrolyzed. However, the rate of reaction did not match that of sinigrin and was considered as residual activity or might be due to the action of different enzymes. Maltose {glu ( $\alpha$ 1-4) glu}, sucrose {glu ( $\alpha$ 1- $\beta$ 2) fru}, lactose {gal ( $\beta$ 1-4) glu}, cellobiose {glu ( $\beta$ 1-4) glu} and galactose were not hydrolyzed. Cellobiose induces  $\beta$ -glucosidases of many microbes such as the rumen fungus *Neocallimastix frontalis* EB 188 (Li and Calza, 1991); however, none of the microbial myrosinases is reported to hydrolyse cellobiose and this includes *Aspergillus niger* (Ohtsuru et al., 1973) and *Enterobacter cloacae* (Tani et al., 1974). This suggests that bacterial myrosinase does not have cellobiase activity. Based on substrate specificity  $\beta$ -glucosidases are classified into three classes, aryl  $\beta$ -glucoside in class (1), true cellobiase in class (2) and broad substrates



specificity enzymes in class (3) (Krisch et al., 2010). As previously discussed *Citrobacter* grows on cellobiose, but does not produce myrosinase.

Studies on Michaelis Menton constant ( $K_m$ ) for *Citrobacter* myrosinase show that this enzyme has a greater affinity for the  $\beta$ -O-glucoside substrate (*p*NPG) with a  $K_m$  value of 0.0183 mM; however, the enzyme has also a significant affinity to the inducer sinigrin ( $K_m$  0.54 mM). Salicin is also hydrolysed, but with a very high  $K_m$  value (0.8 mM) compared to *p*NPG and sinigrin. This indicates that *Citrobacter* myrosinase is a broad substrate enzyme according to substrate specificity classification. Similar findings were reported for the myrosinase of the fungus *Aspergillus niger* which also has a greater affinity for *p*NPG (1.5 mM) over sinigrin (3.3 mM) (Ohtsuru et al., 1973). However, it is unlike the bacterial myrosinase of *Enterobacter cloacae* which has a greater affinity towards sinigrin (0.37 mM) over *p*NPG (0.7 mM) (Tani et al., 1974). Some plant myrosinases have a similar  $K_m$  value towards sinigrin, for cress (*Lepidium sativum*) myrosinase (0.3 mM) but a higher  $k_m$  for *p*NPG (2 mM) (Durham and Poulton, 1990).

Purification of *Citrobacter* myrosinase to homogeneity was accomplished using two ion exchange steps and one gel filtration chromatography step. An earlier attempt to purify this enzyme from a small scale culture (100 mL, 14 mg protein) and using two chromatography steps Ion exchange and gel filtration, respectively, was unsuccessful (data are not shown). The protein concentration obtained after the final gel filtration step was too low to be considered for further purification steps. The complete purification required the use of a larger culture (500 mL, 40 mg protein) and the use of additional step of ion exchange. The yielded 0.011% of purified protein (myrosinase) which is a low amount indicating that this protein is not abundant. This is comparable with the bacterial myrosinase of *Enterobacter cloacae* (0.0366%). However, based on assays and measurement of protein the latter was purified 1000 fold while the *Citrobacter* myrosinase was purified 4 fold. It seems likely that the *Citrobacter*

myrosinase is not as stable as that of *Enterobacter cloacae* and loss of activity rather than protein explains the difference in the magnitude of purification. This is evident from SDS-PAGE analysis of purified fractions where it is clear that the *Citrobacter* myrosinase has been purified to a much greater extent than assays alone represent. The myrosinase of *Enterobacter cloacae* is also not an abundant protein which required three purification steps (ammonium precipitation, ion exchange and gel filtration). This is different to the situation with the myrosinases of many cruciferous seeds where myrosinase is an abundant protein that can be easily purified by using ammonium sulphate fractionation followed by ion exchange (Bones and Rossiter, 2006).

The purity of the *Citrobacter* enzyme was examined and confirmed by SDS PAGE (denature protein), which showed a single band migrating with a molecular mass of approximately 66 kDa (Figure 4.14), in agreement with the estimated molecular weight that obtained from native protein by using gel filtration (Superdex 75 column). This is evidence that *Citrobacter* myrosinase is monomeric and close to the range of  $\beta$ -glucosidase monomers (55-62 kDa) reported by Esen (1993). The estimated molecular mass is very close to other bacterial myrosinase reported by Tani et al. (1974) for *Enterobacter cloacae* (61 kDa), but lower than *Aspergillus niger* (90 kDa). The molecular weight of these two microbial myrosinase was only estimated by gel filtration. The molecular mass of *Citrobacter* myrosinase is also close to the aphid myrosinase (*Brevicoryne brassicae*) (60 kDa) (Jones et al., 2001; Frédéric et al., 2002).

Using native PAGE and esculin reagent to detect putative myrosinase activity allowed a partial purification of the *Citrobacter* myrosinase. After electrophoresis,  $\beta$ -O-glucosidase activity was observed (dark brown band) but only from a glucosinolate induced culture and activity could not be detected in the control (glucose grown bacteria) on the same gel even after long periods of incubation with esculin reagent. Incubation of this band (positive for the esculin reaction) with sinigrin confirmed the

myrosinase identity. This supports the previous suggestion that *Citrobacter* myrosinase possess broad activity ( $K_m$  study) and has both  $\beta$ -S-glucosidase and  $\beta$ -O-glucosidase activities. Interestingly the yielded bands are less than those obtained after the ion exchange second run and hence the obtained myrosinase is considered as a semi purified protein. This is a very simple method to investigate and screen myrosinase activity. Thus it could be used as purification step which would minimise the number of protein bands for peptide sequencing. In comparison to traditional methods such as ion exchange and gel filtration chromatography this method is less labour intensive. The possibility of protein losses is also reduced compared to traditional methods. Additionally, the amount of inducer which may limit the use of large or small scale purification is greatly reduced. A partial purification combined with 2D gel electrophoresis may be more informative in terms of less spots to analyse which would enable an easier comparison with a control.

The amino acid sequence of the only known purified bacterial myrosinase from *Enterobacter cloacae* is not yet available. All other bacteria which have been reported to metabolise sinigrin or other glucosinolates (in *vitro* or in *vivo*) which may be myrosinases have not been purified or sequenced. Most work has focused on the conversion of glucosinolates into isothiocyanates or nitriles. The *N*-terminal sequence has limited homology with other bacterial  $\beta$ -glycosidases (4.7.8.1). It is not known if these  $\beta$ -O-glycosidase possess myrosinase activity and these bacteria are not related to the family *Enterobacteriaceae* in which the genus *Citrobacter* is placed. Thus similarity is based on some peptide homology but not on gene identity. Although the *N*-terminal sequence hits the  $\beta$ -O-glucosidase of closely related species *Citrobacter* 30\_2, myrosinase activity was not detected in the latter (4.7.8.1). This indicates that the gene responsible for myrosinase induction in our isolates does not exist in *Citrobacter* sp. 30\_2 although it's likely to have a similar sequence to the  $\beta$ -O-glucosidase indicated in Table 4.8. Peptide sequencing of the band of  $\approx 71$  kDa (from native gel-SDS-PAGE)

shows some similarity with the  $\beta$ -glucosidase of *Citrobacter* sp. 30\_2 (Section 4.7.8.2) while the peptide sequence of secreted myrosinase of  $\approx 57$  kDa (Section 4.7.8.3) has shown similarity with Glycoside hydrolase, family 3 of *Ktedonobacter racemifer* DSM 44963. Peptide sequence of band number two of partially purified myrosinase has homology with alkylsulphur transferases and putative hydrolases and has a molecular mass of 72 kDa (Section 4.7.8.4). It seems likely that 71 and 72 kDa proteins are the same protein but arrived at using two different purification techniques. In conclusion a full genomic sequence of the new isolate of *Citrobacter* is required in order to establish a cloning strategy based on the peptides obtained in this work. This will enable a better understanding of bacterial myrosinase especially that of *Enterobacter cloacae* is not available in the protein banks.

## **APPENDICES**

Appendices

Appendix A: Bradford reagent calibration curve (Section 2.2.7)

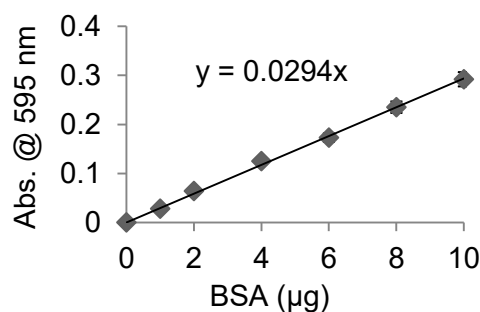
BSA conc. ( $\mu\text{g/ml}$ )	Milli Q H <sub>2</sub> O ( $\mu\text{l}$ )	Bradford reagent ( $\mu\text{l}$ )
0	500	500
10	490	500
20	480	500
40	460	500
60	440	500
80	420	500
100	400	500

Calibration of Bradford reagent against BSA. (0-100  $\mu\text{g/mL}$ ) of BSA was mixed with Milli Q H<sub>2</sub>O (400-500  $\mu\text{L}$ ) then Bradford reagent (500  $\mu\text{L}$ ) was added and incubated at room temperature (10 min). The colour intensity was measured by spectrophotometer at 595 nm.

BSA ( $\mu\text{g}$ )	Abs. @595 nm				
	1st	2nd	3rd	mean	SD
10	0.291	0.276	0.311	0.292	0.0176
8	0.236	0.204	0.26	0.235	0.0281
6	0.178	0.183	0.16	0.173	0.0121
4	0.116	0.131	0.13	0.125	0.0084
2	0.06	0.062	0.07	0.064	0.0053
1	0.012	0.052	0.022	0.028	0.0208
0	0	0	0	0	0

Calibration of Bradford reagent against BSA, the average reading was used to create the Bradford standard curve (see below).

## Appendices



Bradford reagent standard curve, BSA = Bovine Serum Albumin, the obtained equation  $x = y / 0.0294$  will be used in the estimation of the *Citrobacter* protein,  $x$  = protein concentration (µg);  $y$  = absorbance at 595 nm.

### Appendix A: Glucose assay standard curve (Section 2.2.9.2)

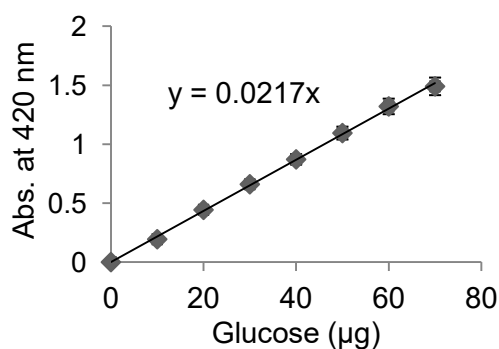
Glucose (µg)	Stock (1/10) Glucose (µL)	Deionised H <sub>2</sub> O (µL)	Perid Reagent (µL)	Total assay (µL)
0	0	300	1000	1300
1	10	290	1000	1300
2	20	280	1000	1300
3	30	270	1000	1300
4	40	260	1000	1300
5	50	250	1000	1300
6	60	240	1000	1300
7	70	230	1000	1300

Glucose assay (0-7 µg glucose) using GOD Perid reagent, in triplicate glucose (0-70 µL) of stock solution was mixed with Milli Q H<sub>2</sub>O (230-300 µL) before adding GOD Perid (1 mL) then incubated (37 °C, 15 min), the colour intensity was measured at 420 nm using spectrophotometer.

## Appendices

glucose ( $\mu\text{g}$ )	Abs. At 420 nm				
	1 <sup>st</sup>	2nd	3rd	mean	SD
0	0	0	0	0	0
10	0.198	0.19	0.195	0.194	0.00404
20	0.448	0.432	0.451	0.443	0.01021
30	0.666	0.671	0.643	0.66	0.01493
40	0.868	0.89	0.855	0.871	0.01769
50	1.087	1.094	1.101	1.094	0.007
60	1.324	1.324	1.311	1.32	0.00751
70	1.486	1.497	1.482	1.49	0.00777

The measurement of released glucose amount using GOD Perid reagent, the obtained data of colour intensity will be used to generate glucose standard curve.



Glucose standard curve,  $x$  = amount of glucose ( $\mu\text{g}$ );  $y$  = absorbance at 420 nm, the obtained equation  $x = y / 0.0217$  will be used in the estimation of released glucose by the action *Citrobacter* myrosinase.

### Appendix A: *p*-Nitrophenol standard 2.2.9.4)

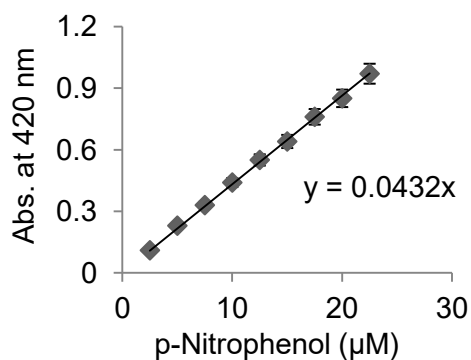
*p*-Nitrophenol (Sigma 1048) (1 mM/ 10 mL) was prepared by dissolving 1.4 mg in 10 mL  $\text{H}_2\text{O}$ . Different concentrations were mixed with CPB (20 mM, pH 6.0) to volume of 300 $\mu\text{L}$  before the addition of  $\text{Na}_2\text{CO}_3$  (BDH 102404H) (1 mL). The colour intensity was measured at 420 nm using spectrophotometer.



Appendices

<i>p</i> -Nitrophenol (μM)	CPB	Absorbance at 420 nm			
	pH 6				
	(μl)	1st	2nd	3rd	mean
2.5	275	0.112	0.113	0.117	0.11
5	250	0.233	0.225	0.225	0.23
7.5	225	0.335	0.332	0.333	0.33
10	200	0.446	0.433	0.433	0.44
12.5	175	0.555	0.538	0.552	0.55
15	150	0.647	0.643	0.642	0.64
17.5	125	0.76	0.76	0.76	0.76
20	100	0.847	0.86	0.854	0.85
22.5	75	0.978	0.973	0.963	0.97

Construction of *p*-nitrophenol standard; (n = 3) total volume of 300 μL of *p*-nitrophenol (2.5 to 22.5 μM) was mixed with the buffer (CPB pH 6.0) then Na<sub>2</sub>CO<sub>3</sub> (1000 μL) was added. The colour intensity was measured on a spectrophotometer at 420 nm.

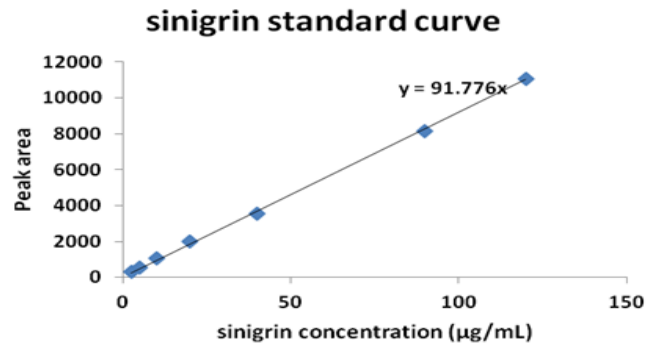


Construction of a *p*-nitrophenol calibration graph; different concentration of the *p*-nitrophenol were mixed with CPB (20 mM, pH 6.0) and Na<sub>2</sub>CO<sub>3</sub> (1000 μL of 0.1 M) added. The yellow-orange colour was measured with a spectrophotometer at 420 nm. The obtained equation ( $y = 0.0432X$ ) was used to estimate the amount of *p*-nitrophenol released.

Appendices

Appendix A: HPLC sinigrin standard (Section 2.2.11.2)

Sinigrin degradation was calculated from standard



HPLC sinigrin standard; this will be used to calculate sinigrin decrease in culture medium of *Citrobacter*. ( $y=91.776X$ ).

## Appendices

### Appendix B: The $K_m$ value (Section 3.6)

#### Sinigrin & salicin (3.6.1)

Substrate (mM)	Substrate stock (10 mM) ( $\mu$ L)	Myrosinase ( $\mu$ L)	Buffer (20mM, pH 6.0.)
0	0	5	295
0.1	3	5	292
0.2	6	5	289
0.3	9	5	286
0.4	12	5	283
0.5	15	5	280
0.6	18	5	277
0.7	21	5	274
0.8	24	5	271
0.9	27	5	268
1	30	5	265
1.5	45	5	250
2	60	5	235
3	90	5	205
4	120	5	175
5	150	5	145

Determination of  $K_m$  value of *Citrobacter* myrosinase. Two substrates were used sinigrin and salicin, the substrates were mixed with the buffer (CPB, 20 mM, pH 6.0). The crude lysate was added to the substrate and buffer and incubated (37 °C, 30 min). The reaction was stopped by boiling (100 °C, 5 min). GOD Perid reagent (1 mL) was added to the tubes and incubated (37 °C, 15 min). The colour intensity was measured at 420 nm

Appendices

*p*-Nitrophenyl- $\beta$ -D-glucopyranoside (*p*NPG) (Section 3.6.2)

<i>p</i> NPG (mM)	Abs.(420 nm)*
0.015	0.095
0.03	0.114
0.045	0.136
0.06	0.159
0.075	0.164
0.09	0.168
0.105	0.195
0.12	0.176

Determination of  $K_m$  value of *p*NPG. Different concentrations of *p*NPG were mixed with CPB (20 mM, pH 6.0) and crude extract (1.11  $\mu$ g) then incubated (25 °C, 1h). The reaction was terminated by  $\text{Na}_2\text{CO}_3$  (1 mL of 0.1 M). The formed colour was measured by a spectrophotometer (420 nm). \* n=3.

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