STUDIES ON THE METABOLISM OF LACTOSE, MELIBIOSE, SUCROSE AND RAFFINOSE BY *ERWINIA CHRYSANTHEMI*

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DECLARATION

All the work reported in this thesis was the author's own work.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

BSA	Bovine serum albumin
CVP	Crystal violet-pectate
DOC	Deoxycholate
EMB	Eosin-methylene blue
EMS	Ethyl methane sulfonate
Glu-DH	Glucose dehydrogenase
HPR	Histidine-containing protein
IPTG	Isopropyl-β-D -galactopyranoside
NG	Nitrosoguanidine
ONPG	*o*-Nitrophenyl-D-galactopyranoside
PEP	Phospho-enol pyruvate
PEP:PTS	Phospho-enol:sugar phosphotransferase
SDS	Sodium dodecyl sulphate
TCZ	Tetrazolium
TLC	Thin layer chromatography
TMG	Thiomethyl-β-D-galactopyranoside
X-gal	5-Bromo-4-chloro-3-indolyl-D-galactopyranoside
YS	Yeast-salts

CHAPTER 1

INTRODUCTION

One of the challenging problems for a bacterial cell is to detect chemical changes that constantly occur in its environment, and to regulate and integrate its metabolism in response to such changes. The primary function of many cellular activities in these organisms is directed towards this task. These activities may include transport processes, cellular locomotion and chemotaxis, and the secretion of digestive enzymes (Saier, 1977; Dills et al., 1980; Postma and Lengeler, 1985; Mitchell, 1985).

The transport of nutrients from the external environment through the cell envelope into the cytoplasm constitutes the first step of the [illegible]quisition process. Bacterial cells utilize a variety of mechanisms in the transport of carbohydrates and their subsequent metabolic degradation. Effective regulation of this transport process by the organism is necessary for the prevention of futile energy expenditure and the maintenance of a fine balance between energy-consuming biosynthetic pathways and energy-producing catabolic reactions (Dills et al., 1980; Postma and Lengeler, 1985; Mitchell, 1985; Reizer et al., 1985). The past twenty years has seen some progress being made in identifying and defining the mechanisms involved in bacterial carbohydrate metabolism, and characterizing proteins involved in catalyzing these reactions (Saier and Reizer, 1992; Tao et al., 1993; Bruckner et al., 1993).

Nutrients that cross the envelopes of Gram-negative bacteria have to traverse a complex structure consisting of a pair of membranes (periplasmic and cytoplasmic) interspersed by the periplasmic space or periplasm. The two membranes have points of contact (zones of adhesion) with one another, suggesting a more complex structure (Hirst and Welch, 1988). The surface of the cell wall has proteins (porins) incorporated into it that function as hydrophilic pores of various diameters through which water-soluble solutes can diffuse. The periplasm contains about 5% of the total cellular protein (Saier, 1987).

Among proteins found in the periplasm is a special class of digestive enzymes that cleave cytoplasmic membrane-impermeable compounds into molecules whose translocation across the cytoplasmic membrane is catalyzed by the presence of specific transport systems or permeases. These transport systems are localized primarily in the cytoplasmic membrane and they exhibit specificities for different nutrients such as sugars, vitamins, amino acids, nucleotides and many other compounds that are of nutritional value to the organism (Saier, 1977, 1987; Postma and Lengeler, 1985; Hirst and Welch, 1988). There are two distinct major transport mechanisms by which carbohydrates enter the cell, namely, group translocation and active transport.

Group translocation, normally referred to as the phospho-enol:sugar phosphotransferase (PEP:PTS) system, is a multicomponent system consisting of a number of constitutively expressed, cytoplasmic (soluble), substrate non-specific proteins and membrane-bound (inducible), sugar specific proteins (Kundig and Roseman, 1971; Postma and Roseman, 1976; Saier, 1977; Postma and Lengeler, 1985; Mitchell, 1985; Saier, 1987). The PEP:PTS transport system is widely distributed in bacteria (Table 1). It involves the participation of at least four separate proteins that function within the cell as phosphocarriers of the high-energy phosphate group from phosphoenolpyruvate to the incoming sugar (Fig. 1), and the overall reaction requires Mg^{2+} (Dills et al., 1980; Ingraham et al., 1983; Saier and Reizer, 1992).

The last member of the chain, Enzyme II (EII), which forms an integral part of the membrane, also serves as the carrier protein that brings the sugar across the membrane. The phosphocarrier enzyme III (EIII), is a peripheral membrane protein which plays a critical role in the regulation of carbohydrate transport (Mitchell, 1985). The first two members of the system, Enzyme I (EI) and the histidine-containing protein (HPR) are cytoplasmic, constitutively expressed and sugar non-specific in their action. These properties are responsible for the common function of these proteins in all PEP:PTS transport systems in the cell. The other proteins, EII and EIII are both inducible, substrate-specific and membrane-bound proteins (Dills et al., 1980; Postma and Lengeler, 1985; Mitchell, 1985; Ebner and Lengeler, 1988). Collectively, these proteins catalyze a single step translocation and phosphorylation of solute molecules across the cytoplasmic membrane (Anderson et al., 1971; Saier, 1977; Saier et al., 1983; Dills et al., 1980; Erni et al., 1982; Postma and Lengeler, 1985; Mitchell, 1985; Saier, 1987).

Table 1. Distribution of the PEP:PTS system in prokaryotes

Genus	Bergey group	Gram stain reaction	Substrate(s)
Rhodopseudomonas	1	-	Fructose
Rhodospirillum	1	-	Fructose
Thiocapsa	1	-	Fructose
Thiocystis	1	-	Fructose
Ancalomicrobium	4	-	General
Spirochacta	5	-	Mannitol
Pseudomonas	7	-	Fructose
Alcaligenes	7	-	Fructose
Beneckea	8	-	General
Escherichia	8	-	General
Klebsiella	8	-	General
Photobacterium	8	-	General
Salmonella	8	-	General
Serratia	8	-	General
Fusobacterium	9	-	Fructose
Megasphaera	11	-	Glucose, fructose
Staphylococcus	14	+	General
Streptococcus	14	+	General
Bacillus	15	+	General
Clostridium	15	+	Fructose, mannitol
Brochotrix	16	+	General
Lactobacillus	16	+	General
Arthrobacter	17	+	Fructose, rhamnose
Mycoplasma	19	+	General

(Adapted from Dills et al., 1980)

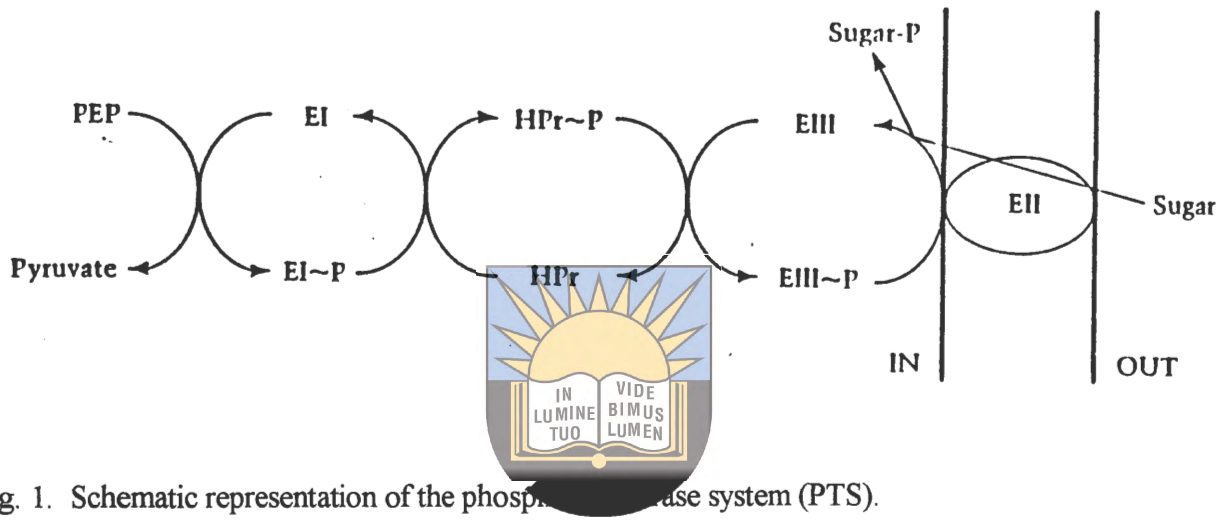


Fig. 1. Schematic representation of the phosp[illegible]ase system (PTS).
(A high-energy phosphate bond from phosphoenolpyruvate (PEP) is transferred through a chain of carriers-Enzyme I (EI), a small histidine containing protein (HPR), Enzyme II (EII), and Enzyme III (EIII) to the incoming sugar. Enzyme II serves as the carrier protein of this transport system).

(after Ingraham et al., 1983).

Active transport, on the other hand, is driven by a mechanism which involves energy coupling. Translocation of the sugar is tightly coupled to ion (Na^+ and H^+) translocation such that the electro-chemical flux across the membrane drives the accumulation of the sugar against a concentration gradient (Saier, 1977., Dills et al., 1980; Thomas and Crow, 1984; Poolman, 1990; King and Wilson, 1990).

The fundamental difference between the two transport systems is in the nature of the end product released into the cytoplasm. In group translocation the corresponding carbohydrate phosphates accumulate in the cytoplasm whereas in active transport the molecule that appears in the cytoplasm is the same chemical species that was removed from the extracellular medium (Saier, 1977; Dills et al., 1980; Erni et al., 1982; Postma and Lengeler, 1985; Mitchell, 1985; Saier, 1987; Poolman, 1990). Despite recent advances in understanding these transmembrane transport processes, relatively little is known at the molecular level about the mechanisms of these translocation phenomena due to difficulties involved in solubilizing transport permeases from membranes, and the lack of convenient *in vitro* assays (Jacobs [illegible] 83; King and Wilson, 1990).

Among the many disaccharides of natural origin that occur in the free state, sucrose and lactose are the most abundant (Hawker, 1985). The trisaccharide, raffinose, is the first member of a homologous series of oligosaccharides, generally known as the raffinose family (Fig. 2), in which successive D-galactosyl groups are joined to each other by *O*-α-D-galactosyl-(1→6)-linkages. The raffinose family is widely distributed in the plant kingdom (Table 2), and is next to sucrose in its abundance in green plants (Duffus and Duffus, 1984; Dey, 1985). The oligisaccharides of the raffinose family have been recognized as important transport carbohydrates in a large number of plants (Bohinski, 1979; Dey, 1985) and the disaccharide, melibiose, is thought to arise by the hydrolysis of raffinose. Sucrose constitutes the major transportable form of carbohydrate synthesized in the leaves during photosynthesis and is found in all organs of some plant species. Being readily soluble, sucrose can be relatively easily extracted from plant crops and purified by crystallization. The two major sources of commercial sucrose, sugar cane stalks and sugar beet, store up to 20% of their fresh weight as this sugar (Duffus and Duffus, 1984; Hawker, 1985).

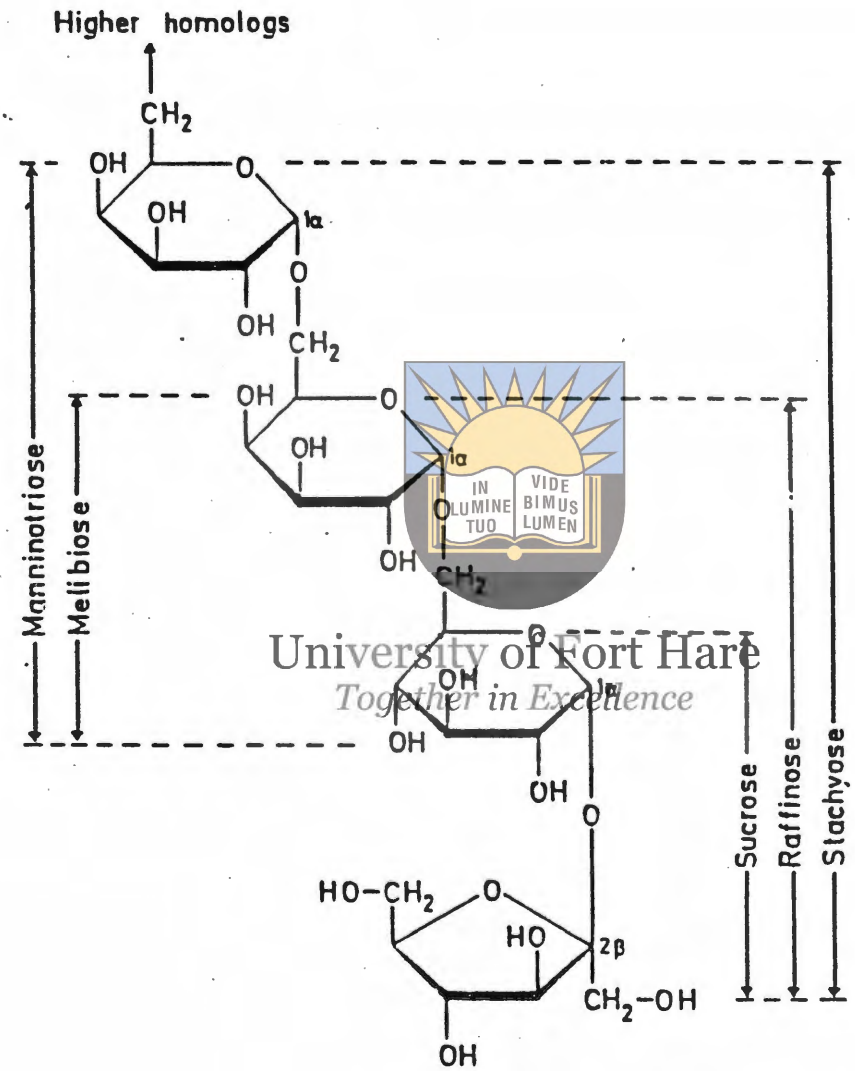


Fig. 2. The raffinose series of oligosaccharides. Raffinose: *O*-α-D-galatopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside; melibiose: *O*-α-D-galactopyranosyl-(1→6)-D-glucopyranose; stachyose: *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside; manninotriose: *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-D-glucopyranose.

(After Kandler and Hopf, 1980)

Table 2. Distribution of sucrose, and the raffinose family of oligosaccharides in various organs of some plant species [a].

Plant species	Sucrose	Raffinose	Stachyose
Andromeda japonica			
Leaf	2.26	0.55	1.16
Stem	2.69	0.64	2.76
Buddleia davidii			
Leaf	3.68	0.22	0.16
Catalpa bignonioides			
Leaf, young	2.58	0.29	0.55
Leaf, old	5.67	0.86	3.61
Lamium maculatum			
Leaf	1.15	0.66	0.68
Root and rhizome	2.07	0.89	9.62
Lycopus europaeus			
Leaf	3.43	2.49	2.12
Root	3.60	3.27	7.70
Marrubium vulgare			
Leaf	2.00	0.65	1.34
Root and hypocotyl	2.65	0.33	1.84
Oenothera pumila			
Leaf	4.75	0.42	0.87
Stem	7.32	0.23	0.15
Origanum vulgare			
Leaf	8.28	0.78	1.40
Root and rhizome	4.86	0.87	1.67
Prunella grandiflora			
Leaf	2.47	0.52	1.88
Root and rhizome	3.20	0.93	2.78

[a] The quantities are expressed in mg/ml fresh weight of tissue (Adapted from Dey, 1985).

Bacterial sucrose metabolism has been reported in *Bacillus subtilis* (Robeson et al., 1983; Steinmetz et al., 1989), and studied extensively in *Streptococcus mutans* (St. Martin and Wittenberger, 1979; Slee and Tanzer, 1979; Thompson and Chassy, 1981; Robeson et al., 1983; Keevil et al., 1984; Russell et al., 1985; Lunsford and Macrina, 1986; Tao et al., 1993). In both these Gram-positive strains, a sucrose specific transport protein or Enz II (EII^{Scr}) of the PEP:PTS and a sucrose-6-phosphate hydrolase (invertase) were found to be involved in sucrose transport and metabolism and were both inducible by the addition of sucrose, fructose or raffinose to the growth medium (Schmid et al., 1988). Steinmetz et al. (1989) have reported the induction by sucrose in *B. subtilis* of both the intracellular sucrase, coded for by the *scr* A gene, and the extracellular levansucrase, coded for by the *scr* B gene. Although these findings suggest the existence of two different sucrose metabolic pathways in *B. subtilis*, the requirement for the different systems and the role of levan, the specific product of levansucrase, is not yet understood (Steinmetz et al., 1989). Studies have shown that sucrose-hydrolyzing enzymes from a wide variety of organisms share common ancestry (Norman et al., 1995). In *S. mutans*, regarded as the principal agent of human dental caries, sucrose is tra[illegible] the PEP:PTS system and subsequently cleaved by invertase yielding glucose-6-phosphate and fructose as intracellular products (St. Martin and Wittenberger, 1979; Lunsford and Macrina, 1986).

While *Escherichia coli* is normally unable to utilize sucrose (Palchaudhuri et al., 1977; Alaeddinoglu and Charles, 1979), some isolates exhibit a sucrose-positive (Scr^+) phenotype (Johnson et al., 1976; Palchaudhuri *et al.*, 1977; Alaeddinoglu and Charles, 1979; Bartlett and Trust, 1980). The genes that code for these variable metabolic processes seem to be located on transmissible plasmids whose origin is not yet fully understood (Wohlhieter et al., 1975; Palchaudhuri et al., 1977; Lengeler et al., 1982; Norman et al., 1995). Thus, strains of *E. coli* K12 and *Salmonella typhimurium* (LT2), which are plasmid-free, are unable to take up and ferment sucrose or to mutate to a sucrose-positive phenotype (Smith and Parsell, 1975; Bartlett and Trust, 1980; Lengeler et al., 1982; Schmid et al., 1982; Garcia, 1985; Hardesty et al., 1987; Schmid et al., 1988).

The sodium-solute co-transport system has been reported in both *S. typhimurium* LT2 (Dills et al., 1980) and *E. coli* (Skulachev, 1987; Pourcher et al., 1995). The uptake of melibiose in *E. coli* and *S. typhimurium* LT2 is stimulated by sodium ions and inhibited by lithium ions in a process in which

the *mel* permease couples substrate uptake to Na^+ uptake in a 1:1 stoichiometry (Dills et al., 1980; Mitchell, 1985). There are at least two mechanisms that control the utilization of melibiose by *E. coli*, a melibiose transport system (permease) and a hydrolyzing enzyme (α-galactosidase) coded for by the melibiose operon genes B and A respectively (Schmitt, 1968; Tanaka et al., 1980; Gray et al., 1986). Substrate specificity of the melibiose permeases among the various members of the enterobacteriaceae differ. In *E. coli*, melibiose permease can transport melibiose, galactose and thiomethyl-β-D-galactopyranoside (TMG) but not isopropyl-β-D-galactopyranoside (IPTG) or lactose (Schmitt, 1968; Dills et al., 1980; Tanaka et al., 1980; Tamura and Matsushita, 1992). Although the *E. chrysanthemi* melibiose permease resembles that of *E. coli* in failing to transport IPTG, it also differs in being able to transport lactose and not TMG (Gray et al., 1986).

The lactose permease in *E. coli* has the distinction of being the first bacterial uptake system to have been described and is known to be coupled to the proton-symport translocation system (Dills et al., 1980). It is coded for by a specific gene within the lactose operon, the *lac Y* gene, which is distinct from the *lac Z* gene coding for β-galactosidase. The *lac A* gene codes for thiogalactoside transacetylase (Zabin and Fowler, 1980; Dills et al., 1980; Mitchell, 1985).

The first two proteins are responsible for the transport and hydrolysis of β-galactosides, but the physiological function of the third enzyme is not known. The coupling of lactose to proton movement is obligatory and transmembrane translocation of one substrate without the other does not occur (Dills et al., 1980; Saier, 1985; Mitchell, 1985). Because of the tight coupling of lactose and proton transport, the flux of protons down their electrochemical gradient can drive the active accumulation of lactose in the cytoplasm against a 1000-fold concentration gradient (Saier, 1987). This unidirectional lactose translocation process involves the passage of the solute and a proton, through the hydrophobic barrier of the cytoplasmic membrane, complexed with the moiety of the carrier protein in a carrier-type mechanism (Dills et al., 1980; Saier, 1985; 1987; Futai and Tsuchiya, 1987). Poolman (1990) has reported the existence of a lactose:galactose antiport transport system in *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. During transport, lactose uptake is coupled to galactose excretion such that no metabolic energy is required for this process.

Common features between the H^+-lactose and Na^+-melibiose transport systems have been observed:

(i) Both the *lac* permease and the *mel* permease function by cation co-transport, probably involving a similar carrier-mediated mechanism (Dills et al., 1980; Tamura and Matsushita, 1992).

(ii) While lactose permease functions exclusively by sugar-H^+ co-transport, the melibiose permease couples sugar uptake to the transport of H^+, Li^+ or Na^+, depending on the bacterial strain and the sugar substrate under investigation (Dills et al., 1980; Tanaka et al., 1980; Pourcher et al., 1995).

(iii) Furthermore, both systems exhibit broad and overlapping sugar substrate specificities (Dills et al., 1980; Tamura and Matsushita, 1992).

Inspite of these similarities, some differences have been noted between the two systems:

(i) While the *lac* permease exhibits stringent cation specificity, that of *mel* permease is relatively broad (Saier, 1985).

(ii) While lactose and H^+ binding to the carrier protein appear to occur independently of each other (Dills et al., 1980), Na^+ and melibiose binding to the *mel* carrier seems to be cooperative (Saier, 1985).

(iii) Treatment of the *lac* carrier with the histidine-specific reagent, diethylpyrocarbonate, abolishes active lactose uptake and counterflow while stimulating facilitated diffusion of the sugar. The melibiose permease, on the other hand, responded to the diethylpyrocarbonate with a decrease in the V_{max} for energy-driven sugar uptake without affecting the K_m of the process (Saier, 1985).

Since the H^+ cycle is widespread among most organisms, it is assumed that the H^+ cycle rather than the Na^+ cycle was the evolutionary initial coupling ion, and that both the lactose and the melibiose permeases are evolutionarily related (Saier, 1985; Skulachev, 1987).

Raffinose occurs in leaves at low concentrations and it accumulates in the storage organs during the process of plant development. Sucrose serves as a precursor for the synthesis of raffinose which in turn can be utilized by plants as a precursor for the synthesis of stachyose (Fig. 2) or degraded by invertase into melibiose and fructose (Duffus and Duffus, 1984; Dey, 1985). The primary role of raffinose and its family of oligosaccharides in the leaves, vegetative organs and seeds is to serve as short- or long-term storage carbohydrates.

Dey (1985) reported that raffinose and its higher homologues serve among the factors responsible for frost resistance in winter-resistant plants. Raffinose and sucrose have also been linked to the viability of seeds as Dey (1985) reported a low level of the two sugars in corn seeds that had lost viability. It is considered that soluble oligosaccharides are the carbohydrates which are mobilized first during seed germination, prior to the utilization of polysaccharides such as starch (Dey, 1985). Members of the raffinose family of oligosaccharides are not digested by humans mainly due to the lack of α-galactosidase in the intestinal mucosa (Duffus and Duffus, 1984; Dey, 1985).

The trisaccharide raffinose (*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-fructofuranoside) cannot be catabolized by *E. coli* K12 since it is unable to induce specific transport or hydrolase activities (Schmid and Schmitt, 1976). Evidence suggests that the raffinose genes may be plasmid borne in certain pathogenic strains (Schmid and Schmitt, 1976; Schmid et al., 1979; Hardesty et al., 1987; Titgemeyer et al., 1994; Muiznieks and Schmitt, 1994). The raffinose operon code genes for the synthesis of three enzymes which are necessary for the metabolism of raffinose. These include a transport system (*raf* permease) coded for by *raf* B, α-galactosidase coded for by *raf* A, and an invertase coded for by *raf* D (Konishi et al., 1985; Kosugi et al., 1986; Muiznieks and Schmitt, 1994). This system is normally referred to as the *raf* system. The three proteins catalyze the uptake and hydrolysis of raffinose into monosaccharides. The *raf* permease catalyzes the uptake of raffinose from the extracellular medium into the cytoplasm. The intracellular raffinose is hydrolysed by α-galactosidase into galactose and sucrose and invertase further cleaves sucrose into glucose and fructose. The active presence of the *raf* system in *E. coli* mutants, which are defective in the melibiose uptake system, suggests different uptake mechanisms for melibiose and raffinose (Schmid and Schmitt, 1976).

The *Erwinia* soft-rot species of plant pathogens are enterobacteria that cause diseases, typified by the maceration of host tissues on a wide range of plants. This group of pathogens is split into two main species, *E. chrysanthemi* and *E. carotovora*. Two other minor pathogens with restricted host range are included in the soft-rot group, namely, *E. cypripedii* and *E. rhapontici* (Perombelon and Kelman, 1980; Kotoujansky et al., 1982; Lelliot and Dickey, 1984; Gray et al., 1984; Reverchon et al., 1985; Kotoujansky, 1987). Besides soft-rot itself, other symptoms are often observed. For instance, *E. chrysanthemi* induces a wilt on carnation and both *E. chrysanthemi* and *E. carotovora* can cause blackleg on potato, which has leaf wilting as a symptom (Perombelon and Kelman, 1980; Kotoujansky, 1987). Infection of chrysanthemum by *E. chrysanthemi* generally induces a canker on the stem starting from the crown (Burkholder et al., 1953). In plants that reproduce vegetatively, the infection can be systemic or, when conditions for development of the disease are not met, latent. The soil is known to be an important source of infection and infection may occur during seedling emergence, by splashing rain droplets or by wind-borne 'dust particles'. Host specificity seems to occur in *E. chrysanthemi* strains (Mildenhall, 1974; Kotoujansky, 1987).

Unlike many fungal pathogens, the soft-rot bacteria are unable to penetrate the cuticle of plants directly and they must therefore gain entry through wounds or natural openings. The natural points of entry of bacteria into plants include stomata and hydathodes in leaves (Lyon, 1989). In maize the first symptom is a wilting of the tips of the uppermost leaves. At this stage a soft-rot develops in the stalk at the base of the whorl. The decay then spreads rapidly downward through the stalk and soon the tops of affected plants droop. When the decay spreads to the nodes close to the ground level, the plants collapse and fall over. On all severely diseased plants, the top cluster of leaves can be pulled out easily showing a soft-rot condition at the breaking point at the base of the whorl (Hoppe and Kelman, 1969; Hartman and Kelman, 1973).

Soft-rot *Erwinias* produce several extracellular enzymes including pectinases, cellulases, and proteases that can attack components of the plant cell wall. The action of the pectic enzymes is responsible for the maceration of the plant tissue by soft-rot *Erwinias* but some interesting features of the disease are still not understood.

For an example, the mode of action involved in the systemic spreading of *Erwinia* within the plant is not known nor are the functions controlling host specificity (Chatterjee and Starr, 1980; Kotoujansky, 1987).

Gray et al. (1984) isolated a lactose non-utilizing *E. chrysanthemi* (EC-C) strain which was found to posses two independently regulated permeases; one associated with the *mel* operon, the other coded for by the *raf* operon, both of which were capable of lactose transport. Both α-galactosidase and lactose permease were induced by melibiose, whereas in cells where raffinose was the carbon source, higher levels of lactose permease were obtained relative to α-galactosidase activity (Gray et al., 1986).

Sucrose and members of its homologous series, melibiose and raffinose, are known to constitute the major carbohydrate source in plants, and may provide a significant nutritional resource for *E. chrysanthemi* during infection. It is therefore apparent that for EC-C to survive in the host plant it would require the existence of specific degrad[illegible]ies and various transport systems.

A better understanding of phytopathogens and the diseases they cause can emerge only from concerted genetic, biochemical, physiological, and pathobiological studies. Because of the nature and inherent complexity of associations between bacteria and plants, elucidation of interaction mechanisms necessitates an interdisciplinary approach. For example, a better insight into the physiological basis of pathogenicity might be obtained from comparative physiological and genetic studies on avirulent mutants and their virulent parents. Knowledge concerning the host and the pathogen must evolve simultaneously; one without the other would defeat the purpose (Starr and Chatterjee, 1972; Chatterjee and Starr, 1980).

Successful infection of a host by a pathogen requires two things. Firstly, it must evade the host defence systems, and secondly, it must grow and replicate in the host. This requires that the pathogen obtains all its nutritional requirements from the host. The pathogen accomplishes this by using the readily assimilable compounds from the host fluids, and by degrading host tissue. The latter process usually results in the gross symptoms of the disease.

It is therefore clear that certain aspects of the metabolism of the pathogen play a fundamental role in determining its ability to colonize a host and cause disease. In general, the uptake and metabolism of carbohydrates by bacteria is a complex system involving a multiplicity of transport and energy-coupling mechanisms which often have overlapping sugar specificities (Schmitt, 1968; Dills et al., 1980; Gray et al., 1984).

The studies reported here investigate aspects of the interrelationship between the transport and utilization of lactose, sucrose, melibiose and raffinose by *E. chrysanthemi*. The use of mutants defective in the metabolism of one or more of these sugars offers an ideal approach to the problem.

This thesis is divided into two sections. The first section (Chapter 2) covers the determination of suitable growth conditions for *E. chrysanthemi* and the production and classification of mutants unable to grow on one of the four sugars mentioned above. The second section (Chapter 3), describes the production of α-galactosidase, β-galactosidase, and invertase, as well as sucrose uptake studies. It also reports on studies unde establish the localization of these enzymes in the bacterial cell.

CHAPTER 2

GROWTH AND MUTAGENESIS OF *ERWINIA CHRYSANTHEMI*

Mutation is a very important biological phenomenon because it is the ultimate source of all biological variation in life. By comparing the properties of mutant strains to those of the parent strain, the function of the affected gene product can be deduced (Ingraham et al., 1983). Mutations may be of two general types, spontaneous and induced. Spontaneous mutations occur in the absence of human intervention whereas induced mutations are alterations caused by specific chemical or physical agents (Boyd, 1988; Prescott et al., 1993).

The most powerful known chemical mutagens are the alkylating agents which add methyl or ethyl groups to the heterocyclic nitrogen atoms of the DNA bases. These mutagens cause a variety of mutations including transitions, [illegible]s and -1 frame-shifts (Ingraham et al., 1983; Prescott et al., 1993). Ethylating agents such as ethyl methane sulfonate (EMS) are favoured mutagens over methylating agents such as nitrosoguanidine (NG) in that with NG-generated mutations one cannot be assured that all the altered phenotypic properties of a mutant strain are a result of the selected mutation (Ingraham et al., 1983).

Intercalating agents are planar molecules that can insert between the stacked pairs of bases in the centre of the DNA molecule. Such incorporation distorts the backbone of the double helix in such a way that frame-shift mutations can occur when the distorted helix is replicated. One or more base pairs can be added or deleted from the molecule. The most useful of the intercalating agents for mutating bacteria is the one synthesized at the Institute for Cancer Research (ICR) in Fox Chase, Pennsylvania, and designated ICR 191 (Ingraham et al., 1983).

The elucidation of the multiple functions of the constituents of the various bacterial transport systems has benefited from the isolation of mutants defective in one or more of these transport components. Mutants unable to utilize all PTS carbohydrates have been isolated and were invariably found to be mutated in the general PTS proteins, EI or HPr or both (Postma and Lengeler, 1985).

In this chapter the methods used in generating and isolating mutants of *E. chrysanthemi* and an investigation of the effects of various carbon sources on the growth of *E. chrysanthemi*, are reported. The isolated mutants are further characterized on the basis of their phenotypic expressions on different carbon sources.

2.1. MATERIALS AND METHODS

2.1.1. Organism.

A locally isolated *E. chrysanthemi* strain (Mildenhall, 1974) was used for these studies. This strain and its mutants were stored as water suspensions prepared as follows: One millilitre of an overnight shake-culture of *E. chrysanthemi*, grown at 30°C in Luria Broth (see below for medium formulation), was centrifuged in an Eppendorf microcentrifuge at room temperature for 5 min, washed once in sterile double distilled water and resuspended in 10 ml of sterile double distilled water. A 100-fold dilution [illegible]spension was made into sterile double distilled water and the strains kept in screw-capped vials at room temperature in the dark. The strains remained viable for at least 12 months.

2.1.2. Chemicals.

Raffinose and melibiose were obtained from BDH Chemicals Ltd. (Poole, England). ICR 191 was obtained from the Department of Microbiology, University of Iowa (U.S.A). EMS was purchased from E. Merck (Federal Republic of Germany). All other chemicals were obtained either from E. Merck or Biolab and were of analytical grade unless otherwise stated.

2.1.3. Media.

The following media, prepared according to Miller (1972), were used in the studies reported in this chapter. All media were sterilized by autoclaving at 101 kPa (121°C) for 20 min. To prepare agar plates, the media were solidified by the addition of 15 g/l of either Difco or Biolab agar before autoclaving. The sugars were made up separately in H_2O and filter-sterilized through a 0.22 µm-pore-size Millex filter (Millipore Corp., Bedford, Mass.).

2.1.3.1. Luria Broth.

Bacto tryptone	10.0 g
Bacto yeast extract (YE)	5.0 g
NaCl	0.5 g
Distilled H_2O	1 l

The pH was adjusted to 7.0 with 1 M NaOH before making up to volume with water. No glucose was added.

2.1.3.2. LB medium.

Bacto tryptone	10.0 g
Bacto yeast extract (YE)	5.0 g
NaCl	10.0 g
Distilled H_2O	1 l

2.1.3.3. Minimal A medium.

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	1.0 g
Sodium citrate $2H_2O$	0.5 g
Distilled H_2O	1 l

2.1.3.4. Minimal M63 medium.

KH_2PO_4	13.6 g
$(NH_4)_2SO_4$	2.0 g
$FeSO_4.7H_2O$	0.5 mg
Distilled H_2O	1 l
pH	7

The following sterile supplements were added per litre, aseptically:

1 M $MgSO_4$	1.0 ml
Various sugars as carbon source (20% w/v)	20.0 ml

2.1.3.5. Eosin-Methylene Blue (EMB) agar.

EMB was prepared as reported by Miller (1972) and contained:

Difco Bacto tryptone	10.0 g
Difco Yeast extract (YE)	1.0 g
NaCl	5.0 g
KH_2PO_4	2.0 g
Difco Agar	15.0 g
Distilled H_2O	930 ml

After autoclaving the following supplements were added aseptically:

Eosin yellow (4% w/v)	10.0 ml
Methylene blue (0.65% w/v)	10.0 ml

The carbon source was added to a final concentration of 0.5% (w/v) prior to dispensing into Petri dishes.

2.1.3.6. MacConkey agar.

MacConkey agar base (Difco) was rehydrated according to the manufacturer's instructions by dissolving 40 g of the dry medium in 1 litre of H_2O. After cooling at room temperature to about 50°C, the carbon source was added to a final concentration of 0.5%, (w/v), mixed, and the medium dispensed into Petri dishes (about 15 ml per dish) at least 24 h before use to ensure a dry surface (Difco Manual, Difco Laboratories, 9th ed., 1972). The plates were stored in plastic bags to prevent dehydration of the medium.

2.1.3.7. Tetrazolium (TCZ) agar.

TCZ agar was prepared according to Miller (1972) as follows:

Nutrient agar (Difco)	23.0 g
NaCl	1.0 g
Distilled H_2O	950 ml

The agar was melted by heating in a microwave oven, and 50 mg of 2,3,5-triphenyltetrazolium chloride (Sigma, St Lous, MO) was added. The TCZ was dissolved by further heating in the microwave oven, after which the medium was autoclaved at 101 kPa (121°C) for 20 min. After cooling to approximately 50°C, the carbon source was added to a final concentration of 0.5% before dispensing into Petri dishes. The plates were stored in plastic bags in the dark.

2.1.3.8. Yeast-salts (YS) agar.

The YS medium used was adapted from that reported by Gray (1985) and had the following ingredients:

$Na_2HPO_4.2H_2O$	1.77 g
KH_2PO_4	0.27 g
NH_4NO_3	0.4 g
Distilled H_2O	964 ml
Agar	15.0 g
pH	7.3

After autoclaving the medium, the following supplements were added, aseptically, to each 250 ml aliquot.

1 M $MgSO_4.7H_2O$	0. 25 ml
YE (9% w/v)	2.5 ml
Sugar or Glycerol (20% w/v)	6.25 ml

2.1.3.9. Crystal Violet-Pectate (CVP) medium.

CVP was prepared as described by Gray (1985). A blender (MSE atomix) was preheated with hot water, and 500 ml of boiling water added. The blender was started at low speed and the following added:

Crystal violet (0.075% w/v)	1.0 ml
1 M NaOH	4.5 ml
$CaCl_2.2H_2O$ (10% w/v)	3.0 ml
$NaNO_3$	1.0 g
Agar	2.0 g

The mixture was blended at high speed for 15 s and 9 g of sodium polypectate (NaPP, Ral Tech Scientific Services, Inc., 3301 Kingman Blvd., Madison, Wisconsin) was added while blending to prevent clumping of NaPP. With the blender still running, 0.5 ml of a 10% (w/v) solution of sodium dodecyl sulphate (SDS) was added and the medium immediately transferred to a 2 l Erlenmeyer flask capped with aluminum foil.

After sterilizing at 101 kPa (121°C) for 20 min, the medium was dispensed into Petri dishes as soon as possible, and left at room temperature for at least 2 days to dry.

2.1.4. Buffers.

The following buffers were prepared according to Miller (1972).

2.1.4.1. Na-citrate buffer (0.1 M, pH 5.5).

0.1 M Citric acid	4.7 volumes
0.1 M Na_3 citrate	15.4 volumes

2.1.4.2. Na-phosphate buffer (0.1 M, pH 7.0).

0.1 M Na_2HPO_4	61.0 ml
0.1 M NaH_2PO_4	39.0 ml

2.1.4.3. Z-buffer.

Na_2HPO_4	16.1 g
NaH_2PO_4	5.5 g
KCl	0.75 g
$MgSO_4.7H_2O$	0.246 g
2-Mercaptoethanol	2.7 ml
Distilled H_2O	900 ml

The pH was adjusted to 7.0 with 5 M NaOH before making up to volume with water.

2.1.4.4. Tris-HCl buffer (0.1 M, pH 7.5).

Tris (Trizma base, Sigma)	6.056 g
Distilled H_2O	450 ml

The pH was adjusted to 7.5 with 2 M HCl. After the volume was made up to 500 ml with water, the pH was measured and readjusted if necessary.

2.1.4.5. Sodium carbonate solution (1 M).

Na_2CO_3	132 g

This was made up to 1 l with distilled water.

2.1.5. Growth of cells

2.1.5.1. Growth in complex and minimal media.

An overnight culture of EC-C or EC-S grown in Luria Broth (30°C, 200 rpm), was used to inoculate either Luria Broth or minimal M63 medium supplemented with 0.4% of the carbon source in a 250 ml side-arm Erlenmeyer flask. The zero time absorbance reading was immediately determined at OD_{600} in a Bausch and Lomb spectronic 20 spectrophotometer and thereafter growth was followed by taking absorbance readings at various time intervals. Either plain Luria Broth or minimal M63 medium was used as a blank.

2.1.5.2. Viable colony counts.

Viable colony counts were determined in parallel with growth determination by making serial dilutions (10^{-1} to 10^{-8}) of cell suspension in Luria Broth. After mixing thoroughly by vortexing, 100 µl of each dilution was spread in triplicate on LB agar plates. The plates were incubated at 30°C for 48 h before viable colony counts were recorded.

2.1.5.3. Reactions of EC-C and EC-S on Indicator plates.

An overnight culture of EC-C or EC-S grown in Luria Broth was used to prepare a 10^{-7} dilution in Luria Broth. The diluted cell suspension (100 µl) was spread in triplicate, on the carbohydrate supplemented indicator agar plates. The plates were incubated at 30°C for 48 h prior to recording the reactions in different media.

2.1.6. Ethyl methane sulfonate (EMS) and ICR 191 mutagenesis of EC-C.

The complex and minimal media used were Luria Broth (Miller, 1972), minimal A medium (Miller, 1972) and modified minimal M63 medium (Gray, 1985). The indicator medium of choice for mutant selection was MacConkey agar base (Difco). The cooled medium was supplemented with filter sterilized (0.22-µm-pore-size Millex filter; Millipore Corp., Bedford, Mass.) carbon source.

2.1.6.1. EMS mutagenesis.

The method used was that described by Kotoujansky et al. (1982). EC-C was grown overnight (30°C, 200 rpm) in minimal A medium supplemented with 0.2% (w/v) glucose, diluted 10-fold into fresh medium and incubated for a further 4 h at 30°C until the cell density reached 5×10^8 cells/ml (OD_{550} = 0.85). The cells were centrifuged, washed twice in sterile double distilled water, and resuspended in half the initial volume of minimal A medium without glucose. After equilibrating in a shaking waterbath at 30°C for 5 min, EMS was added at 15 µl/ml and incubation continued for 2 h. The cultures were diluted 100-fold in Luria Broth and grown overnight (30°C, 200 rpm), to allow for phenotypic expression of the mutants.

2.1.6.2. Ampicillin treatment of EMS-mutagenized cultures.

Ampicillin treatment was similar to the method employed by Kotoujansky et al. (1982). After phenotypic expression, the cultures were centrifuged, washed twice in 5 ml 0.1 M citrate buffer pH 5.5, and resuspended in 5 ml of the same buffer. The cell suspension was diluted 100-fold in minimal M63 medium, supplemented with either melibiose, raffinose or sucrose at

0.2% (w/v). The cultures were grown (30°C, 200 rpm) for 4 h and ampicillin added to a final concentration of 200 µg/ml. After an overnight incubation (30°C, 200 rpm), the cells were centrifuged (150 x g, 20 min, 4°C). The pellets were suspended in 10 ml Luria Broth and the cultures further grown (30°C, 200 rpm) until they reached stationary phase (9 h).

2.1.6.3. ICR 191 mutagenesis.

Since ICR 191 is sensitive to light and unstable at room temperature, it is always stored in the dark at -20°C and thawed immediately before use. To prepare a 1 mg/ml stock solution of ICR-191 (obtained from the Department of Microbiology, University of Iowa, U.S.A.), the powder was weighed out to the desired amount and added to a screw-cap bottle covered with aluminium foil. The appropriate amount of sterile double distilled water was added to dissolve the powder (the covering foil can be periodically removed for short periods to ensure that the powder has dissolved). The stock solution, still wrapped in foil, was stored at -20°C .

(i) Endpoint titration with ICR 191

The method was carried out as described by Miller (1972), with the following modifications:

(1) Minimal medium A was supplemented with 0.5% glycerol; 0.02% $MgSO_4$ and 2 ml Luria Broth per 100 ml medium.

(2) EC-C mutants selected for were those that were defective in melibiose, raffinose and sucrose metabolism.

Three milliliter aliquots of the supplemented medium A were dispensed into 13 x 100 mm test tubes. The test tubes were covered with aluminium foil and labeled according to the ICR 191 concentration. The ICR 191 stock solution (1 mg/ml) was thawed and used to prepare a new 100 µg/ml stock solution in sterile double distilled H_2O. Only sufficient diluted stock solution for the experiment was prepared. The fresh stock solution was kept in an Eppendorf tube covered with foil. The tubes containing 2, 4 and 6 µg/ml ICR 191 were supplemented from the

100 µg/ml stock and the tubes containing 8, 10, 12, 14, 16, 20, 30, 40 and 50 µg/ml were supplemented from the 1 mg/ml stock solution.

Aliquots of 0.1 ml of a 10^{-4} dilution of an overnight EC-C culture grown in Luria Broth were dispensed into each of the test tubes. The absorbance at 600 nm was determined for all tubes, using medium A as a blank. The tubes, still covered with aluminium foil, were incubated overnight with shaking (200 rpm) at 30°C. The absorbance of the overnight mutagenized cultures was measured before plating.

2.1.6.4. Isolation of mutants.

Serial dilutions (10^{-5} to 10^{-7}) of the mutagenized cultures in Luria Broth were spread on MacConkey agar plates supplemented either with melibiose, raffinose or sucrose. The plates were incubated at 30°C for 72 h and individual mutant colonies picked and checked for purity by routinely streaking on fresh MacConkey agar plates supplemented with the same sugar. The phenotype of each mutant was determined by streaking them on MacConkey agar supplemented with lactose, melibiose, sucrose or raffinose. They were also streaked on crystal violet-pectate (CVP) medium (Gray, 1985) using *E. coli* and EC-C as negative and positive controls respectively.

2.1.6.5. Isolation of Lac$^+$ mutants.

Individual EC-C mutants (Lac$^-$) were streaked on YS lactose agar plates and left to incubate at 30°C for 3 days. Large slimy colonies were isolated, purified by routinely restreaking on fresh YS lactose agar and tested for their Lac$^+$ phenotype on MacConkey lactose agar with EC-S and EC-C as positive and negative controls respectively.

2.2. RESULTS.

2.2.1. Growth in complex and minimal media.

The complex medium (Luria Broth) supported growth by *E. chrysanthemi* with a doubling time of about 45 min (Fig. 3).

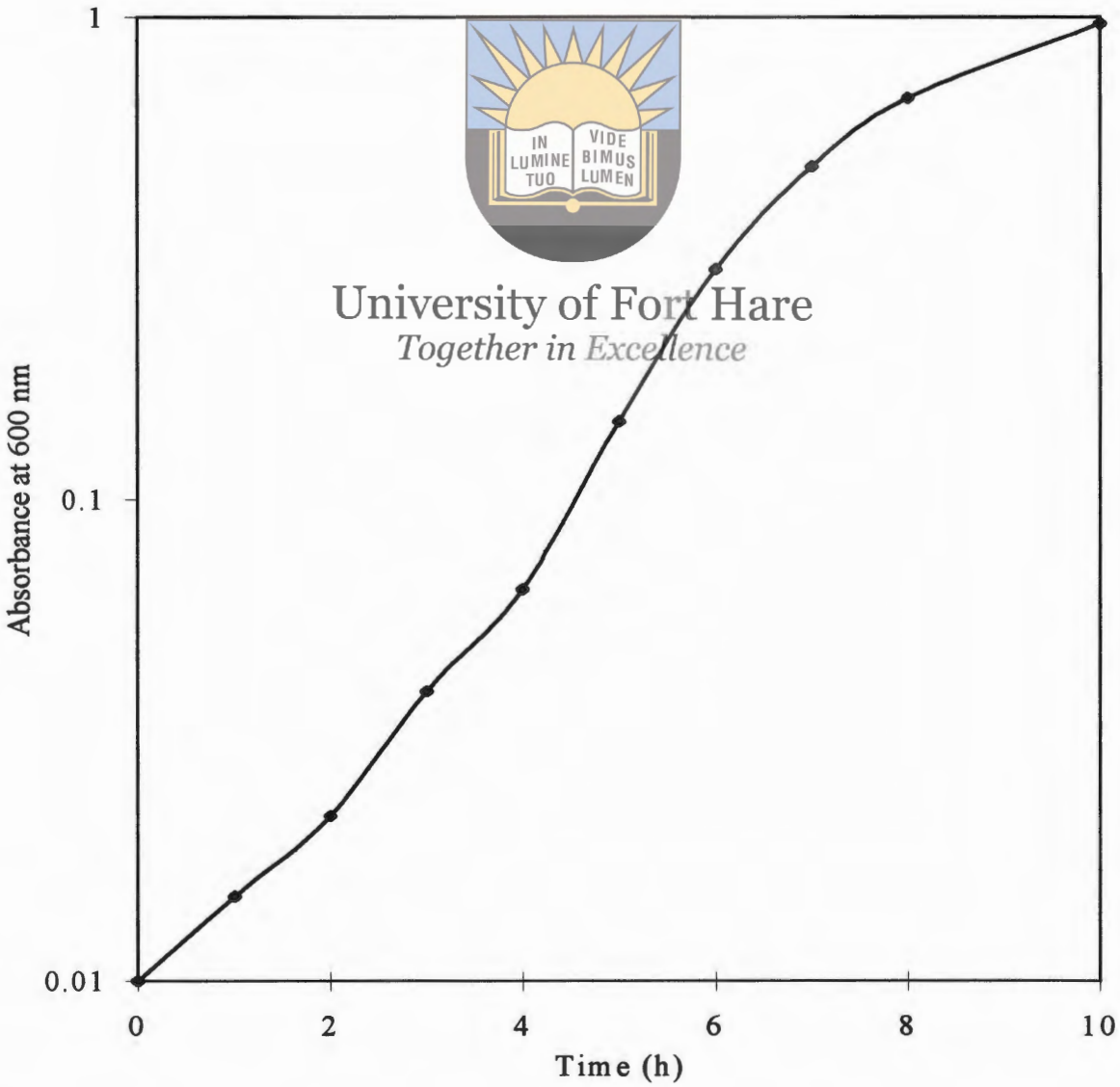


Fig. 3. Growth of EC-C in Luria Broth.

Viable cells were determined by spreading a measured aliquot of a diluted bacterial culture onto LB agar plates and counting the resulting colonies after a period of incubation. Growth was measured in parallel with viable colony count determination (Table 3), and showed some correlation with this (Fig. 4).

Table 3. Growth of EC-C in Luria Broth and colony count on LB agar plates.

Time (min)	OD_{600}	Dilutions [a]	Colony count [b]
0	0.03	10^{-8}	0.45
60	0.035	10^{-8}	0.65
120	0.08	10^{-8}	0.88
180	0.17	10^{-8}	1.35
240	0.42	10^{-8}	2.5
300	0.75	10^{-8}	3.7
360	1.05	10^{-8}	4.3

[a] Serial dilutions of 100 µl cell suspension in 0.9 ml of Luria Broth were made in 13 x 100 mm culture tubes.

[b] Each value represents the mean of three determinations.

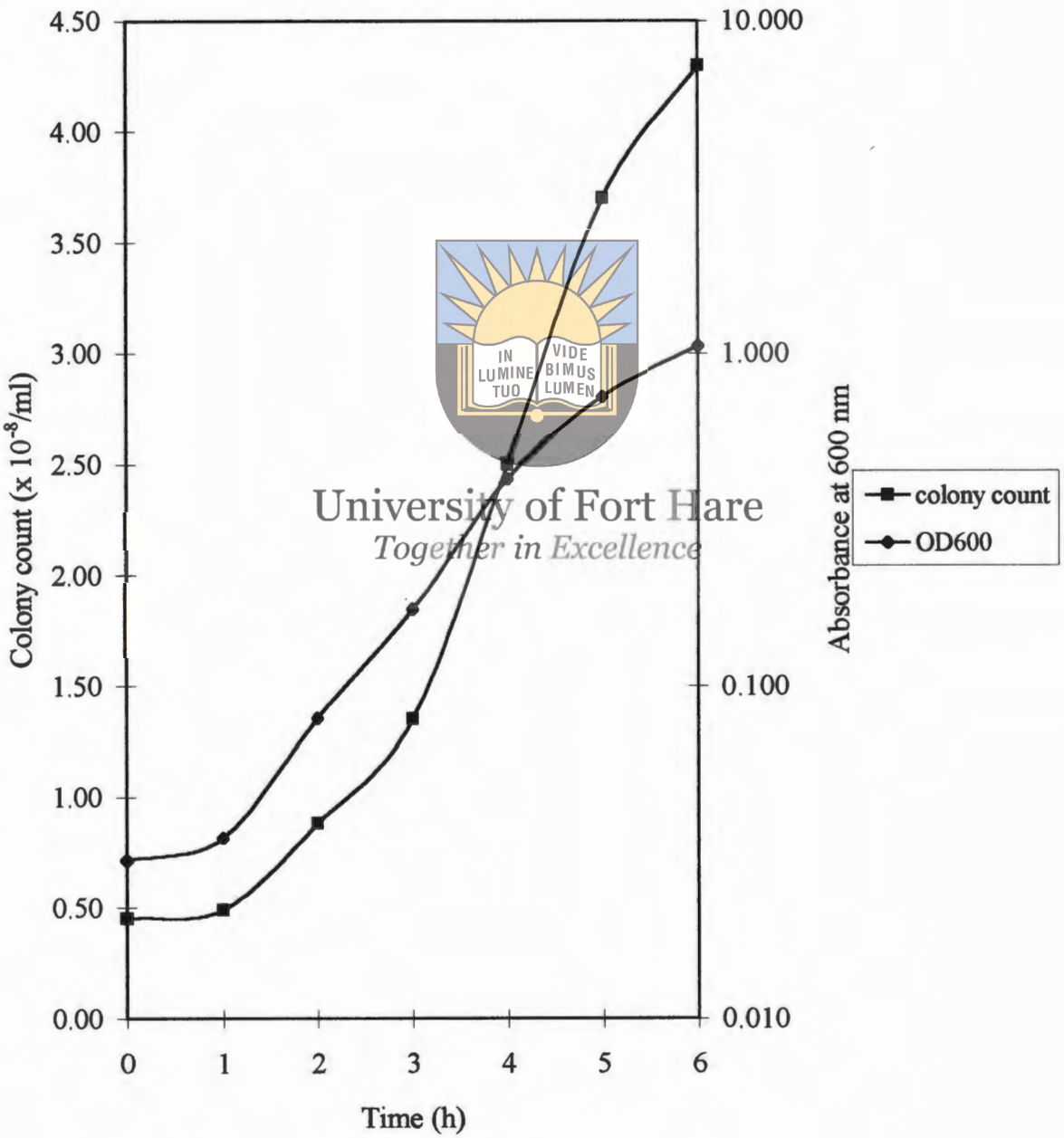


Fig. 4. Growth curve and viable colony counts of EC-C in Luria Broth.

Glycerol, melibiose and sucrose all supported growth at a similar rate while raffinose was much less effective in doing so (Fig. 5).

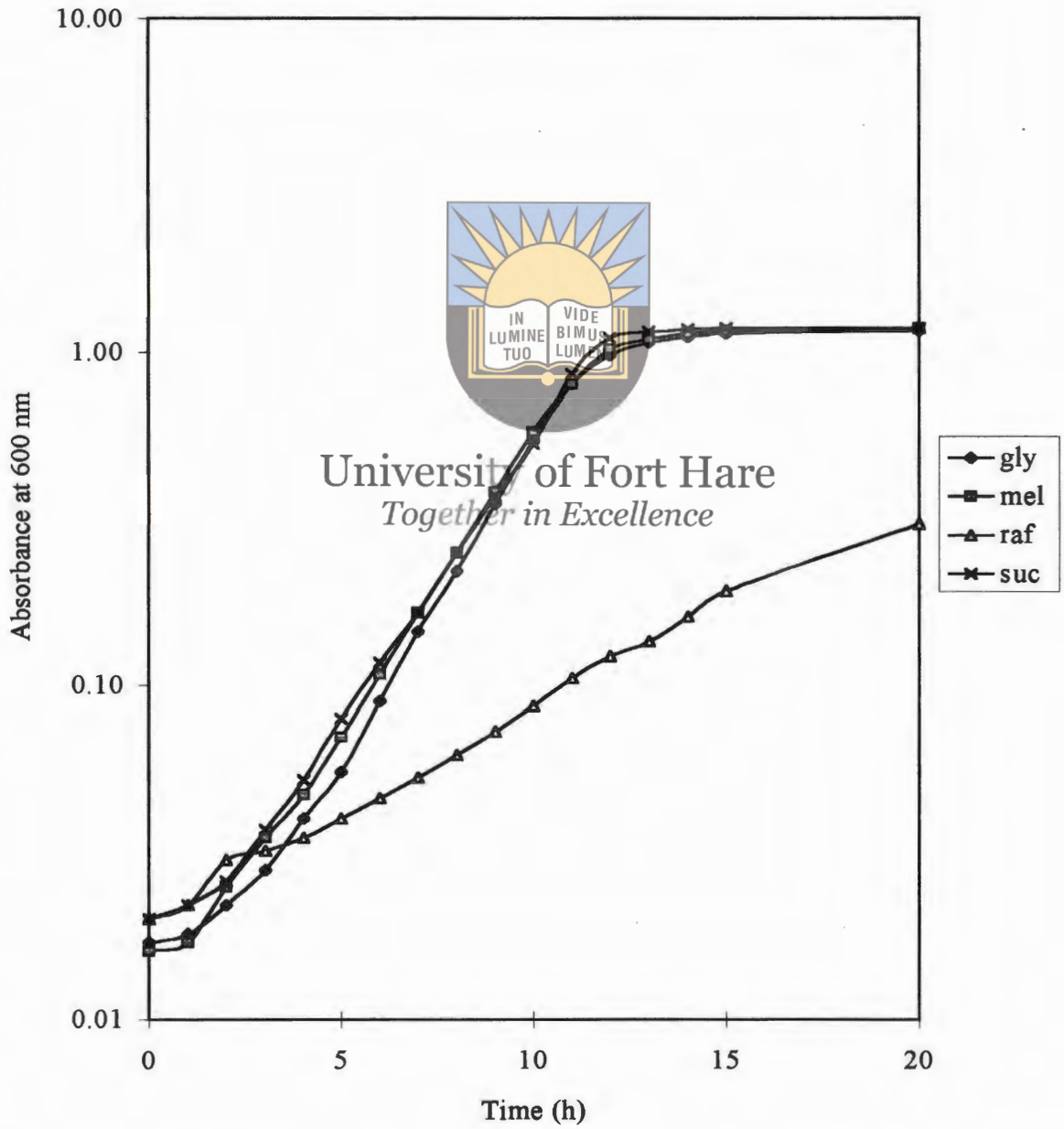


Fig. 5. Growth of EC-C in minimal M63 medium supplemented with different carbon sources.

Sucrose and melibiose, with doubling times of 1.4 h and 1.7 h respectively, seem to be the preferred carbon sources as compared to raffinose (Fig. 5). An extended lag phase is observed with raffinose as a carbon source even when it is present in limiting amounts (Fig. 6).

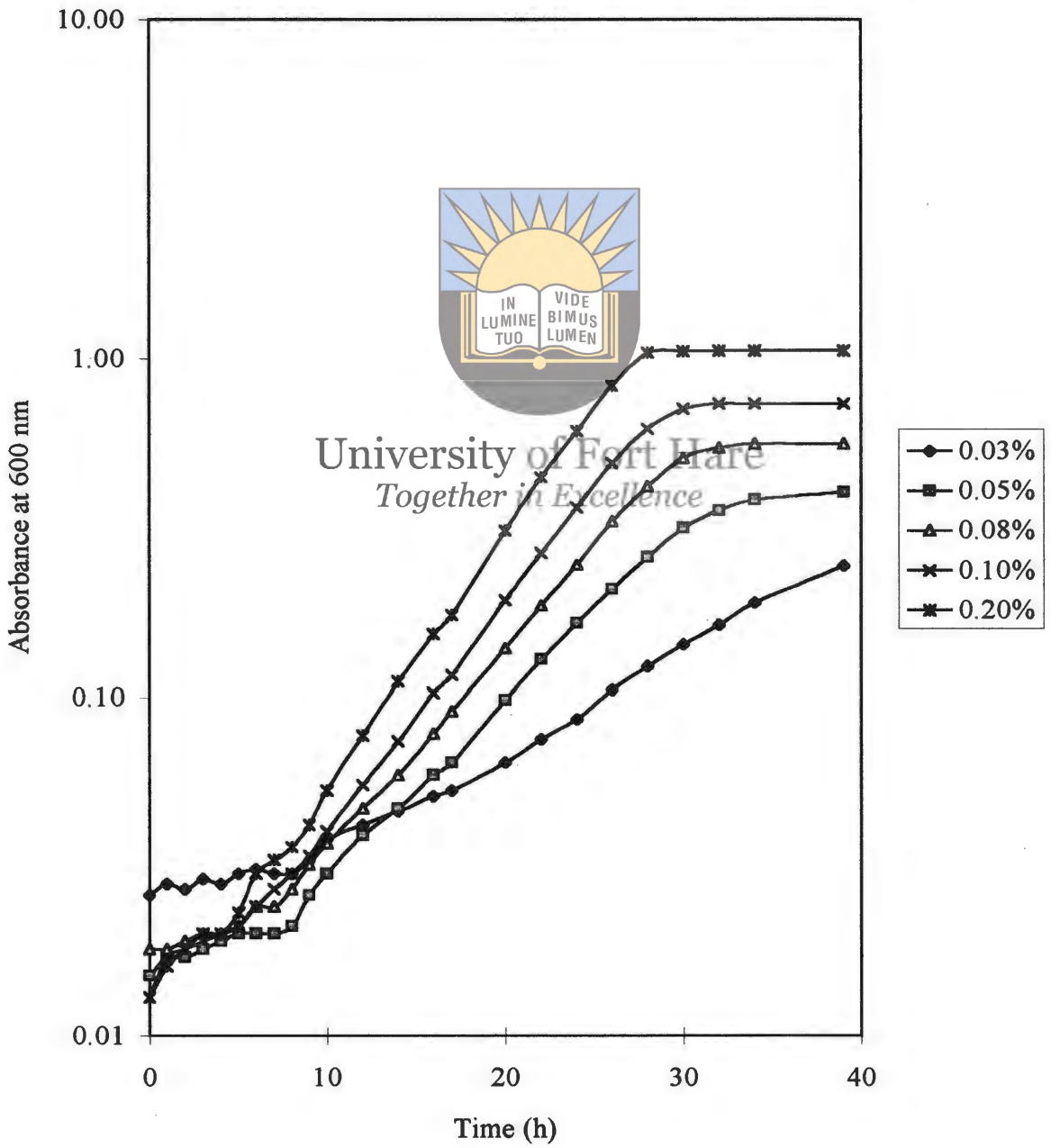


Fig. 6. Growth of EC-C in minimal M63 medium supplemented with varying amounts of raffinose.

2.2.2. Reactions of EC-C on indicator plates.

Different media were tested for their suitability as indicators for phenotypic expression *by E. chrysanthemi* on different carbon sources.

EMB seems to support spreading of colonies and slime production by EC-C. This feature renders this indicator medium unsuitable for EC-C growth since it becomes very difficult to pick individual colonies (Table 4).

Precise, clear reactions were obtained with tetrazolium agar as an indicator medium (Table 4). Nevertheless, the type and colour of colonies given by EC-C in this particular medium was very similar to that given by EC-S, particularly with sucrose and melibiose as carbon sources.

MacConkey was the indicator medium of ch[illegible]t not only revealed precise clear reactions, but gave different colony types and colours for EC-C and EC-S (Table 4).

Table 4. Reactions of EC-C and EC-S on indicator plates.

Medium [b]	Colony type [a]	
	EC-C	EC-S
EMB + sucrose	slimy, large, shiny spreading colonies with a pale blue sheen	slimy, large, spreading colonies with a pale blue sheen
EMB + lactose	small, white colonies with a blue centre	small colonies with a shiny pale blue sheen
EMB + raffinose	small, shiny colonies with a pale blue centre	large, slimy, pale blue spreading colonies
EMB + melibiose	large, slimy spreading colonies with a pale blue sheen	large, slimy spreading colonies with a pale blue sheen
TETRAZOLIUM + sucrose	large, red colonies with white shiny edges	large, red colonies with white shiny edges
TETRAZOLIUM + lactose	small, deep red colonies	large, red colonies
TETRAZOLIUM + raffinose	small, deep red colonies	large, red colonies
TETRAZOLIUM + melibiose	large, red colonies	large, red colonies
MacCONKEY + sucrose	small, red colonies	small, red colonies
MacCONKEY + lactose	large, white spreading colonies	small, red colonies
MacCONKEY + raffinose	large, red colonies	small, red colonies
MacCONKEY + melibiose	large, red colonies	small, red colonies

[a] An overnight culture of EC-C or EC-S grown in Luria Broth was diluted 10^{-7} fold in Luria Broth. Diluted culture (100 µl) was spread on each indicator plate in triplicate. The plates were incubated at 30°C for 48 h prior to scoring for their reactions in the different media.

[b] Each sugar was added to a final concentration of 0.5%.

2.2.3. Mutagenesis.

The frequency of induction mutations by ICR 191 differs with different bacterial strains and experimental conditions (Miller, 1972). For ICR 191 mutagenesis to be effective, a slow growing culture in the presence of this mutagen is preferred (Miller, 1972). A concentration of 10 µg/ml ICR 191 was found to be the most suitable for mutagenesis in EC-C (Table 5), as bacterial cells could not survive concentrations higher than this value (Fig. 7).

Table 5. Endpoint titration of EC-C with ICR 191.

ICR 191 [a] (µg/ml)	OD_{600}		Plate counts (CFU/ml)
	0 hrs	17 hrs	
0	0.00	0.42	
2	0.00	0.37	0.38×10^7
4	0.00	0.34	0.28×10^7
6	0.00	0.27	0.18×10^7
8	0.00	0.22	0.09×10^7
10	0.00	0.08	0.04×10^7
12	0.00	0.01	0.00
14	0.00	0.00	
16	0.00	0.00	
20	0.00	0.00	
30	0.00	0.00	
40	0.00	0.00	
50	0.00	0.00	

[a] A stock solution of ICR 191 was made in sterile deionized H_2O and stored at -20°C.

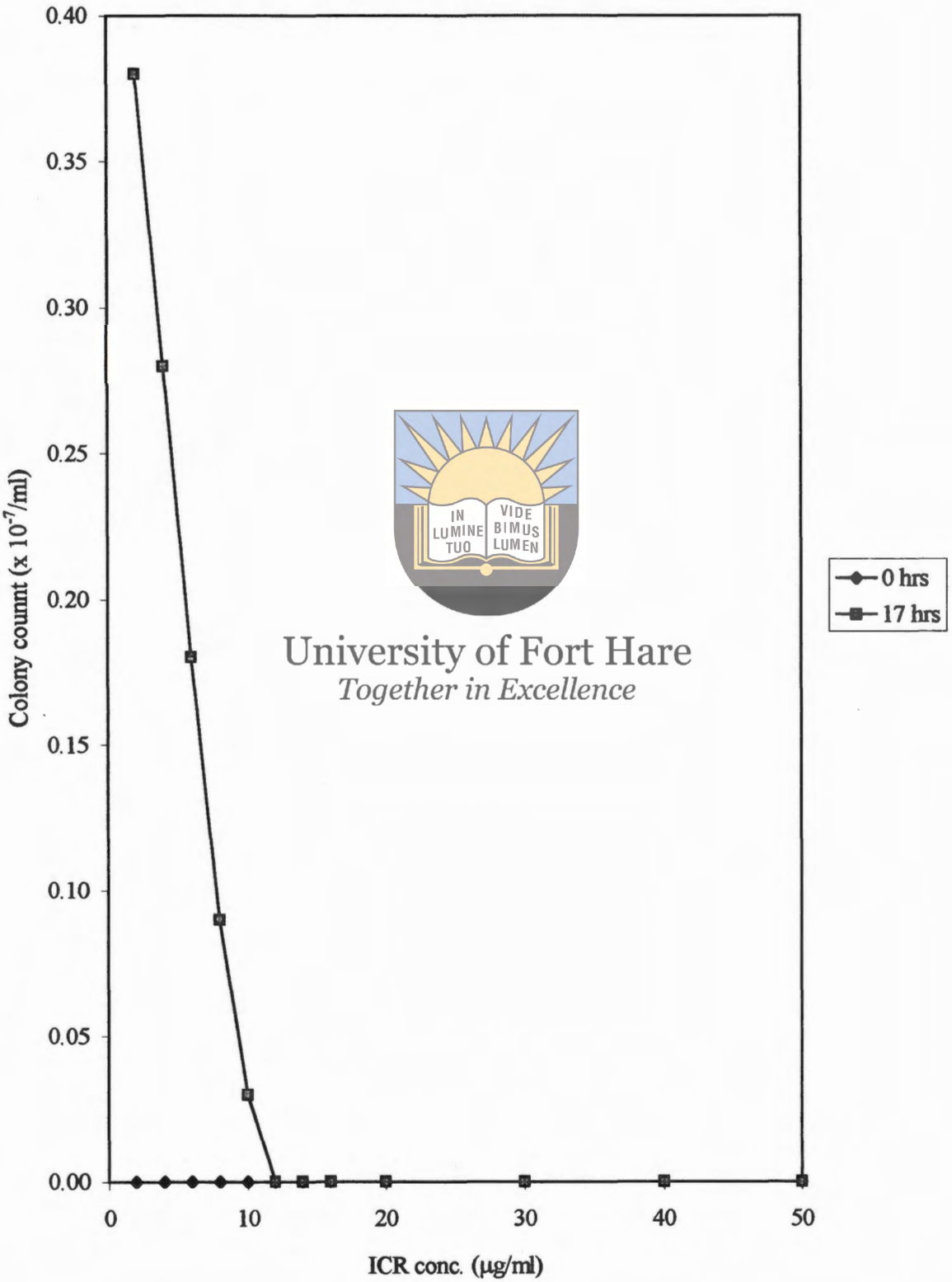


Fig. 7. End point titration of EC-C with ICR 191.

The production of mutants was achieved by the use of two mutagens, ethyl methyl sulfonate (EMS) and ICR 191. A collection of mutants unable to grow on one or more of the sugars was isolated (Table 6). Each of these mutants isolated after EMS or ICR 191 mutagenesis was scored for its ability or inability to ferment each one of these sugars. Based on their phenotypes, the mutants were divided into seven and five classes after EMS and ICR 191 mutagenesis respectively. Each category was characterized by a defect in the metabolism of one or more of the sugars. It is not clear why only five classes were obtained with ICR 191.

Table 6. Properties of isolated mutant strains.

Mutagen				
EMS	ICR 191	Phenotypes [a]	CVP [b]	Class
FH 10		Raf$^-$ Mel$^+$ Scr$^+$ Lac$^-$	+	1
FH 11	FH 100	Raf$^-$ Mel$^-$ [illegible]	+	2
FH 12	FH 101	Raf$^-$ Mel$^-$ Scr$^+$ Lac$^-$	+	3
FH 13	FH 102	Raf$^-$ Mel$^-$ Scr$^-$ Lac$^-$	+	4
FH 14	FH 103	Raf$^-$ Mel$^-$ Scr$^+$ Lac$^+$	+	5
FH 15	FH 104	Raf$^+$ Mel$^+$ Scr$^-$ Lac$^-$	+	6
FH 16		Raf$^-$ Mel$^+$ Scr$^-$ Lac$^+$	+	7
EC-S		Raf$^+$ Mel$^+$ Scr$^+$ Lac$^+$	+	
EC-C		Raf$^+$ Mel$^+$ Scr$^+$ Lac$^-$	+	
E. coli			-	

[a] Individual mutant colonies were picked and cross-streaked on MacConkey agar supplemented with different sugars at 1% (w/v). The plates were incubated at 30°C for 72 h before scoring for positive (+) and negative (-) reactions.

[b] A positive reaction on CVP was characterized by the formation of a well as a result of the hydrolysis of the polypectate by the pectic enzymes produced by the *Erwinia* species. EC-C and EC-S were included as positive controls and *E. coli* as a negative one.

CHAPTER 3

ENZYME PRODUCTION AND SUCROSE UPTAKE BY *ERWINIA CHRYSANTHEMI*

The survival of *E. chrysanthemi* in the host plant depends, among other things, on its ability to degrade and assimilate the host tissue. This requires the presence of specific degradative enzymes and the existence of various transport systems.

In this chapter the presence or absence of enzymes known to be involved in the metabolism of these sugars is determined. In addition, the kinetic properties and localization of these enzymes within the bacterial cell are investigated. Studies on the uptake of sucrose by *E. chrysanthemi* are also reported.

3.1. MATERIALS AND METHODS.

3.1.1. Chemicals.

o-Nitrophenyl-α-D-galactopyranoside (α-ONPG), *o*-Nitrophenyl-β-D-galactopyranoside (β-ONPG) and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Sigma Chemical Co. (St. Louis, MO.). [U-^{14}C] Sucrose (specific activity, 540 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, England).

3.1.2. Reagents.

3.1.2.1. α-ONPG.

α-ONPG (120.52 mg) was dissolved in 20 ml of distilled water to give a 20 mM solution. A fresh solution was prepared every week and stored in the dark at 4°C.

3.1.2.2. β-ONPG.

β-ONPG (100 mg) was dissolved in 25 ml of distilled water to give a 13.2 mM solution. A fresh solution was prepared every week and stored in the dark at 4°C.

3.1.2.3. 5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-gal) agar.

X-gal agar was prepared according to Gray (1985) and contained:

1 M $MgSO_4.7H_2O$	0.25 ml
X-gal (40 mg/ml in dimethyl formamide)	1.0 ml
YS agar	1 l

The medium was supplemented with either g[redacted].2%), glycerol (0.05%) + melibiose (0.2%) or glycerol (0.05%) + raffinose (0.2%) before it was dispersed into glass Petri dishes, allowed to dry for at least 24 h, and stored in plastic bags in the dark.

3.1.3. Buffers.

3.1.3.1. Acetate,phosphate and tris-HCl.

The following 0.1 M buffer solutions at different pH values were prepared in double distilled water and supplemented with $MgSO_4.7H_2O$ to a final concentration of 0.1%:

Sodium acetate	pH's 3.7; 4.8 and 5.6
Sodium phosphate	pH's 6.4; 7; 7.4 and 8
Tris-HCl	pH 9

3.1.2.2. β-ONPG.

β-ONPG (100 mg) was dissolved in 25 ml of distilled water to give a 13.2 mM solution. A fresh solution was prepared every week and stored in the dark at 4°C.

3.1.2.3. 5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-gal) agar.

X-gal agar was prepared according to Gray (1985) and contained:

1 M $MgSO_4.7H_2O$	0.25 ml
X-gal (40 mg/ml in dimethyl formamide)	1.0 ml
YS agar	1 l

The medium was supplemented with either g[illegible].2%), glycerol (0.05%) + melibiose (0.2%) or glycerol (0.05%) + raffinose (0.2%) before it was dispersed into glass Petri dishes, allowed to dry for at least 24 h, and stored in plastic bags in the dark.

3.1.3. Buffers.

3.1.3.1. Acetate, Phosphate and Tris-HCl.

The following 0.1 M buffer solutions at different pH values were prepared in double distilled water and supplemented with $MgSO_4.7H_2O$ to a final concentration of 0.1%:

Sodium acetate	pH's 3.7; 4.8 and 5.6
Sodium phosphate	pH's 6.4; 7; 7.4 and 8
Tris-HCl	pH 9

3.1.3.2. Polyacrylamide Gel Electrophoresis

(i) Running buffer.

The running buffer contained:

0.025 M Tris-base pH 8.5

0.2 M Glycine

SDS (0.1% w/v)

(ii) Loading buffer.

The loading buffer contained:

0.0625 M Tris-HCl pH 6.8

Glycerol (12.5% v/v)

β-Mercaptoethanol (1.25% v/v)

Bromo phenol blue (0.0013% w/v)

Both SDS and mercaptoethanol were replaced with distilled H_2O for the Disc gel system.

(iii) Staining solution.

The staining solution contained:

Ethanol (25% v/v)

Glacial acetic acid (10% v/v)

Commassie brilliant blue (0.03% v/v)

3.1.4. α-Galactosidase and β-Galactosidase assays.

3.1.4.1. Growth of cells.

An overnight EC-C or mutant culture grown in Luria Broth (30°C, 200 rpm), was used to inoculate minimal M63 medium supplemented with either 0.05% glycerol and 0.4% melibiose, 0.05% glycerol and 0.4% raffinose for α-galactosidase activity or 0.05% glycerol and 0.5 mM IPTG for β-galactosidase activity. The cultures were grown at 30°C with shaking (200 rpm) until an absorbance at 600 nm of about 0.8 was reached. Cultures supplemented with glycerol only (0.4%), were used as controls. After determining the absorbances at 420 nm and 550 nm respectively in a Milton Roy spectronic 1201 spectrophotometer, a correction for turbidity was made by subtracting 1.711 x OD_{550} from the OD_{420} reading. Enzyme activities for both assays were calculated according to Miller (1972), as follows:

Activity units = (1000 x Nett OD_{420}) / (t x v x OD_{600})

Nett OD_{420}= Ave. OD_{420} - Ave. OD_{550} x 1.711

t = time of the reaction in min.

v = volume (ml) of culture used in the assay

3.1.4.2. α-Galactosidase assay.

The assay method for α-galactosidase activity was adapted from that reported by Gray (1985) and contained:

Cell suspension	0.1 ml
Tris-HCl buffer pH 7.5	0.5 ml
Distilled H_2O	0.3 ml
Toluene	25 µl

After mixing by vortexing, the tubes were equilibrated at 30°C for 5 min prior to adding 0.1 ml of α-ONPG. The tubes were further incubated at 30°C until a definite yellow colour developed (15 to 20 min) before the reaction was stopped by the addition of 1ml of 1 M Na_2CO_3.

3.1.4.3. β-Galactosidase assay.

β-Galactosidase was assayed by the method of Miller (1972) as follows:

Cell suspension	0.5 ml
Z-buffer pH 7	0.5 ml
Toluene	25 µl

The flasks were incubated in a shaking waterbath at 37°C for 40 min before they were equilibrated at 30°C for 5 min. After adding 0.2 ml of a 13.2 mM stock β-ONPG solution, incubation was continued at 30°C until a definite yellow colour developed (10 min). The reaction was stopped by the addition of 1 ml of 1 M Na_2CO_3.

3.1.5. Invertase activity and sucrose transport.

3.1.5.1. Cell suspension preparation.

EC-C and mutant cultures grown overnight in Luria Broth (30°C, 200 rpm) were diluted 50-fold in minimal M63 medium supplemented with glycerol and sucrose at 0.05% and 0.2% respectively. Minimal M63 medium supplemented with glycerol only was included as a control. After growing to late log phase (30°C, 200 rpm), the cells were centrifuged (Eppendorf microcentrifuge), washed twice in phosphate buffer (100 mM, pH 6.6), and resuspended in the same buffer to an OD_{600} of 2.

3.1.5.2. Invertase assay.

Invertase activity was determined by a modification of the methods reported by Schmid et al. (1982) and Hardesty et al. (1987) as follows:

The assay mixture contained:

Cell suspension	250 µl
Phosphate buffer pH 6.6	250 µl
Toluene	10 µl

After mixing and equilibrating the tubes at 30°C, 50 µl of a 500 mM sucrose solution was added to each tube and incubation continued for a further 30 min. The reaction was stopped by boiling the tubes for 3 min. For a blank, sucrose was added after boiling. The samples were centrifuged and the supernatant fluid collected for determining glucose concentration with a commercially available glucose-dehydrogenase (E. Merck, Federal Republic of Germany) test kit as follows:

Supernatant	100 µl
Gluc-DH reagent	1 ml

After a 15 min incubation at 30°C, the OD_{340} of the assay tubes and the appropriate blanks was measured in a Milton Roy Spectronic 1201 spectrophotometer.

Enzyme unit = µmol/min/ml/OD_{600}

3.1.6. Transport of ^{14}C-labelled sucrose.

Sucrose uptake assays (Schmid et al., 1982; Gray et al., 1986) were carried out at room temperature in Eppendorf microfuge tubes. After equilibrating 0.3 ml cells for 5 min in phosphate buffer, uptake was initiated by the addition of 50 µl of a 10-fold diluted labelled sucrose

(1 μCi/mmol) solution. Samples (0.1 ml) were removed after 10 s and 60 s incubation periods, filtered through a 0.45 μm pore-size HAWP filter (Millipore Corp.) on a Millipore filtration manifold, and washed with three 0.2 ml aliquots of suspension medium using a Hamilton microlab P micropipettor.

The filtration and wash process was completed within 5 s. The filters were counted in either 10 ml of Packard Filter count (Packard Instruments International, Zurich, Switzerland) or 10 ml of Beckman EP scintillation fluid (Beckman Instruments International, Geneva, Switzerland) in a Beckman model 8100 liquid scintillation counter. The uptake over 10 s was used as a control and subtracted from the total uptake over 60 s.

Uptake activity = picomol/50 s/OD_{600}

3.1.7. Kinetic properties of α-galactosidase and invertase

The kinetic properties of α-galactosidase and invertase were determined using α-ONPG and sucrose as substrates respectively. These properties include the pH optimum, the pH stability, the temperature optimum, the temperature stability, the Michaelis constant (K_m) and the maximum reaction rate (V_{max}) of these enzymes.

3.1.7.1. α-Galactosidase.

(i) Progress curve.

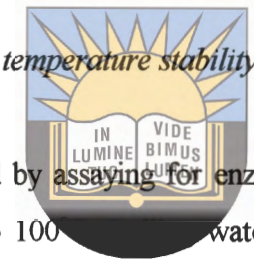
A linear progress curve was established by mixing phosphate buffer, pH 7.4 (2.5 ml), with distilled water (2.2 ml) and α-ONPG (0.25 ml) in a 16 x 100 mm test tube. The mixture was equilibrated at 30°C for 5 min prior to the addition of 0.25 ml of cell suspension. Samples (0.5 ml) were taken at 5 min intervals over a period of 45 min and immediately mixed with 1 M Na_2CO_3 (1 ml) to stop the reaction. Absorbance was determined at 420 nm in a Milton Roy Spectronic 1201 spectrophotometer. A blank was prepared by replacing the substrate with distilled water. All subsequent kinetic determinations were done in accordance with the findings of the progress curve.

(ii) pH optimum and pH stability

The determination of enzyme activity was done in triplicate at each pH. The assay procedure was similar to the one used in establishing the progress curve but scaled down in volume and carried out in Eppendorf microfuge tubes.

The reaction mixtures were incubated for 30 min at 30°C before determining the absorbance at 420 nm. For pH stability, the crude enzyme was incubated with individual buffers in equal volumes and left to stand at room temperature for 3 h before its activity was assayed in phosphate buffer (pH 7.4) as described for pH optimum.

(iii) Temperature optimum and temperature stability



Temperature optimum was determined by assaying for enzyme activity at different temperatures ranging from 0°C (ice-water slurry) to 100 [illegible] water bath). The assay procedure was as described previously for pH optimum except that only phosphate buffer at pH 7.4 was used. For temperature stability, 0.5 ml of cell suspension was dispensed into Eppendorf microfuge tubes and pre-incubated at various temperatures for 1 h before assaying for enzyme activity in phosphate buffer (pH 7.4) at 30°C as described for pH optimum.

(iv) K_m and V_{max}

The K_m and V_{max} were measured at 30°C in phosphate buffer (pH 7.4) over a substrate concentration range of 0.24 mM to 0.96 mM. Details of the assay procedure were similar to those reported for determining other kinetic parameters.

3.1.7.2. Invertase.

(i) Progress curve.

The progress curve was established by mixing 50 mM sucrose solution (0.25 ml) with phosphate buffer, pH 6.6 (2.5 ml) in a 16 x 100 mm test tube and pre-incubating the resulting mixture at 30°C for 5 min. The reaction was initiated by the addition of 1 ml of cell suspension.

Samples (0.1 ml) were taken at 5 min intervals over a period of 60 min and immediately immersed in a boiling water bath for 3 min to stop the reaction. A blank was treated in the same manner except that sucrose was added after the 3 min immersion.

Glucose dehydrogenase reagent (1 ml) was added to each tube, mixed by inversion and incubated at 30°C for 15 min before reading the absorbance at 340 nm in a Milton Roy Spectronic 1201 spectrophotometer. All other kinetic determinations were done in accordance with the findings of the progress curve.

(ii) pH optimum and pH stability.

The determination of enzyme activity was done in triplicate at each pH. The assay procedure described for the determination of the progress curve was scaled down in volume and the assay carried out in 1.5 ml Eppendorf microfuge tubes. The reaction mixtures were incubated for 15 to 20 min at 30°C in accordance with the findings of the progress curve and the reaction stopped by immersing in a boiling water bath for 3 min prior to measuring the absorbance at 340 nm. For pH stability, equal volumes of cell suspension and various buffers were mixed and pre-incubated at room temperature for 3 h before enzyme activity was measured in phosphate buffer (pH 6.6) as described for pH optimum.

(iii) Temperature optimum and temperature stability.

The temperature optimum was determined by assaying for enzyme activity at different temperatures ranging from 0°C to 100°C. The assay procedure was as described previously for pH optimum except that only phosphate buffer at pH 6.6 was used. For temperature stability, cell suspension was dispensed into 1.5 ml Eppendorf microfuge tubes and pre-incubated at various temperatures for 1 h before assaying for enzyme activity in phosphate buffer (pH 7.4) at 30°C.

(iv) K_m and V_{max}

The K_m and V_{max} were measured in phosphate buffer (pH 6.6) at 30°C over a substrate concentration range 3.33 mM to 33.35 mM using the assay procedure described for pH optimum.

3.1.8. Characterization of EC-C mutants with X-gal.

EC-C mutant strains, defective either in melibiose or raffinose metabolism, were individually streaked on X-gal plates. The plates were incubated at 30°C for 48 h prior to recording the colour of the mutant colonies. The plates were then exposed to chloroform for 30 min at room temperature before recording any further changes in the colour of the colonies.

3.1.9. Effect of sodium dodecyl sulphate (SDS) and deoxycholate (DOC) on enzyme activity.

Enzyme assays for both α-galactosidase and invertase were conducted using the methods which have been described previously except that both SDS and DOC were added to a final concentration ranging from 0.01% to 0.1%. The reaction mixtures were equilibrated at appropriate temperatures before the reaction was initiated by the addition of the enzyme. Any suspension that was present after the reaction was stopped was removed by centrifugation before the absorbance was determined.

3.1.10. Thin Layer Chromatography (TLC).

An overnight EC-C culture grown in 100 ml of M63 medium supplemented with 0.4% raffinose was washed in the same volume of sterile double distilled water and the pellet resuspended in 10 ml of 0.1 M phosphate buffer pH 6.6. The suspended cells were disrupted by sonication in the cold (ice-water slurry) for 2 min at 10 s intervals. The disrupted cells were centrifuged in the cold for 20 min and the supernatant dispensed into clean Eppendorf microfuge tubes and used as an enzyme source. The reaction mixture was prepared by adding together 100 µl of phosphate buffer, 200 µl of enzyme, raffinose to a final concentration of 0.1% and SDS to a final concentration of 0.03%. A control using commercial invertase (Boehringer, 10 µl of a 100-fold dilution in double distilled water) without SDS was also prepared. The enzyme digests were incubated in a water bath at 30°C for 2 h and 5 µg of the standard sugars and 10 µl of the enzyme digests loaded onto a Kieselgel 60 F_{254} TLC plate (E. Merck, Federal Republic of Germany). The plate was developed in a solution of ethyl acetate-glacial acetic acid-water (3 : 3 : 1) to within 2 cm from the top. After drying in an oven at 100°C for 10 min, the spots were detected by spraying with ethanolic H_2SO_4 without anisaldehyde and drying in an oven at 100°C for 10 min according to the method of Randerath (1963).

3.1.11. Protein determination and enzyme localization studies.

The localization of periplasmic proteins (Willis et al., 1974; Ames et al., 1984) was investigated after growing the cells in M63 medium supplemented with $MgSO_4$ (0.001 M) and various carbon sources (0.4 %), to an absorbance value of about 0.6 at 600 nm. The cells were conditioned for osmotic shock by adding 1 M NaCl and 1 M Tris-HCl buffer (pH 7.3) to a final concentration of 0.03 M each. The conditioned cells were harvested by centrifugation in a bench top centrifuge for 10 min (4500 rpm) at room temperature and the pellet resuspended in 7.5 - 10 volumes of 0.03 M Tris-HCl buffer pH 7.3..

After mixing by inversion, an equal volume of a mixture of 0.03 M Tris-HCl, 40% sucrose, and 2 mM Na-EDTA was added with stirring and the cells pelleted by centrifugation. The cell pellet was resuspended in 20 ml of double distilled water and 1 M $MgCl_2$ was added within 1 min to a

final concentration of 1 mM. The shocked cells were removed by centrifugation and the supernatant represented the periplasmic fraction.

The pellet was washed twice in 0.03 M Tris-HCl buffer and resuspended in 1 ml of double distilled water prior to adding a drop of toluene and incubating at 37°C for 1 h. After harvesting the cells by centrifugation, the supernatant was retained for the assay of cytoplasmic proteins.

To fractionate the membrane bound proteins, the pellet was washed twice in double distilled water, resuspended in 1 ml of sterile double distilled water and the cells disrupted by sonication in the cold at 10 s bursts for 3 min with intervals of 20 s in between. After centrifuging in an Eppendorf microfuge for 20 min at room temperature, the supernatant was retained and assayed for membrane bound enzymes.

Total protein was determined by using the Bio-Rad protein micro-assay procedure with bovine serum albumin (BSA) as the standard protein.

3.1.12. Polyacrylamide Gel Electrophoresis (PAGE)

PAGE of α- and β-galactosidases was performed using both discontinuous (Disc) and sodium dodecyl sulphate (SDS) gel systems. Commassie brilliant blue R-250 (E. Merck, Federal Republic of Germany) was used to stain for general proteins. To stain specifically for α- and β-galactosidase, 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, prepared in dimethyl formamide (40 mg/ml), were used respectively.

3.1.12.1. Preparation of gels.

Polyacrylamide gels were prepared as follows:

(i) Running gel.

Stock solution	SDS gel	Disc gel	Final conc.
1.5 M Tris - HCl pH 8.7	18.3 ml	18.3 ml	0.55 M
Acrylamide (33% w/v)	16.7 ml	16.7 ml	11%
Bis-acrylamide (3% w/v)	5 ml	5 ml	0.3%
Glycerol (80% v/v)	6.25 ml	6.25 ml	10%
SDS (10% w/v)	0.5 ml	-	0.1%
Distilled H_2O	3.15 ml	3.65 ml	-
Amm.persulphate (10% w/v)	[illegible]	0.1 ml	0.02%
100% Temed	20 µl	20 µl	0.04%

(ii) Stacking gel.

Stock solution	SDS gel	Disc gel	Final conc.
0.5 M Tris - HCl pH 6.8	2.5 ml	2.5 ml	0.125 M
Acrylamide (33% w/v)	1.5 ml	1.5 ml	5%
Bis - acrylamide (3% w/v)	0.4 ml	0.4 ml	0.12%
Glycerol (80% v/v)	1.25 ml	1.25 ml	10%
SDS (10% w/v)	0.1 ml	-	0.1%
Distilled H_2O	4.25 ml	4.35 ml	-
Amm.persulphate (10% w/v)	20 µl	20 µl	0.02%
100% Temed	7.5 µl	7.5 µl	0.075%

EC-C and mutant cultures were grown in minimal M63 medium containing glycerol to a final concentration of 0.05% and supplemented with either melibiose (0.4%), raffinose (0.4%) or IPTG (0.5 mM). For a control, glycerol only (0.4%) was used as the carbon source.

The cultures were grown (30°C, 200 rpm) to late log phase and then centrifuged (10.000 x g, 15 min, 4°C). The pellet was washed once in 10 ml of 10 mM Tris-HCl pH 7.8 and 5 mM $MgCl_2$ (1:1) and resuspended in 2 ml of the same mixture. The cell suspensions, kept in ice-water slurry, were sonicated (MSE 150 Watt Ultrasonic Disintegrator Mk2), with 10 s bursts for 90 s with 20 s intervals in between and re-centrifuged at (10.000 x g, 15 min, 4°C). One part of the supernatant was mixed with 5 parts of loading buffer, boiled for 2 min (SDS-PAGE) and duplicate 100 µl samples loaded onto the gel. The gel was run at constant current (35 mA per gel) at 4°C (Disc-PAGE) for 7 h and cut in two; one half was stained with Commassie blue and the other half incubated with the appropriate enzyme activity stain. To remove Commassie blue, the gels were incubated in 10% acetic acid and shaken at 37°C until all the background stain had cleared.

3.2. RESULTS

3.2.1. Enzyme production

3.2.1.1. α- and β-Galactosidase activities.

The isolated mutants exhibited different levels of α-galactosidase. Both melibiose and raffinose induced α-galactosidase activity in strains which utilized these sugars as carbon sources, but induction with raffinose resulted in lower levels of this enzyme (Table 7).

Table 7. α-Galactosidase activity of isolated mutants.

Strain	Phenotype	Activity [a] (U/OD$_{600}$) Inducer [b]			Class
		none	melibiose	raffinose	
FH 15	Mel$^+$ Raf$^+$	7.1	20.6	11.5	6
FH 104		10.4	31.7	17.6	
FH 13	Mel$^+$ Raf$^-$	2.5	41.3	2.7	4
FH 102		6.9	53.8	6.6	
FH 12	Mel$^-$ Raf$^-$	0.0	2.3	2.8	3
FH 101		0.0	0.4	0.0	
FH 14	Mel$^-$ Raf$^-$	4.4	2.5	3.5	5
FH 103		6.5	6.8	6.8	
FH 16	Mel$^+$ Raf$^-$	1.6	64.9	6.2	7
FH 10	Mel$^+$ Raf$^-$	9.3	15.2	10.6	1
FH 11	Mel$^-$ Raf$^-$	7,6	28,9	10.2	2
FH 100		9.5	18.8	12.9	
EC-C	Mel$^+$ Raf$^+$	9.4	33.3	13.4	

[a] The assay mixture contained 0.1 ml cells, 0.5 ml Tris buffer, 0.3 ml H_2O and 25 ml toluene. After vortexing and equilibrating at 30°C for 5 min, the reaction was started by the addition of 0.1 ml α-ONPG (20 mM). The reaction was stopped after 15 min by the addition of 1 ml of a 1 M Na_2CO_3 solution. After determining the OD_{420} and OD_{550}, a correction for turbidity was made by subtracting 1.711 x OD_{550} from the OD_{420}. Each value represents a mean of 3 determinations.

[b] Cells were grown to late log phase in minimal M63 supplemented either with 0.4% glycerol (no inducer), 0.05% glycerol + 0.4% melibiose or 0.05% glycerol + 0.4% raffinose.

Basal levels of β-galactosidase activity were obtained in all strains with glycerol as the only carbon source, however, high enzyme activities were recorded for all mutants tested when IPTG was used as an inducer (Table 8).

Table 8. β-Galactosidase activity of isolated mutants.

Activity [a] (U/OD_{600})				
Strain	Phenotype	Inducer [b]		Class
		none	IPTG	
FH 14	Lac $^+$	74.4	867.1	5
FH 103		62.1	437.4	
FH 16	Lac $^+$	15.5	481.9	7
FH 10	Lac $^-$	43.2	496.0	1
FH 13	Lac $^-$	40.8	444.6	4
FH 102		51.5	362.9	
FH 11	Lac $^-$	27.3	505.1	2
FH 100		37.8	284.9	
FH 12	Lac $^-$	63.2	770.1	3
FH 101		46.7	304.6	
FH 15	Lac $^-$	28.5	187.4	6
FH 104	Lac $^-$	33.1	263.5	
EC-C	Lac $^-$	33.7	234.0	

[a] The reaction mixture contained 0.25 ml cells, 0.75 ml Z-buffer and 25 ml toluene. After shaking in a water bath at 37°C for 40 min, and equilibrating at 30°C for 5 min, the reaction was started by the addition of 1 ml of β-ONPG. OD_{420} and OD_{550} were determined and a correction for turbidity was made by subtracting 1.711x OD_{550} from OD_{420}. Each value represents a mean of 3 determinations.

[b] Cells were grown to late log phase in minimal M63 medium supplemented either with 0.4% glycerol (no inducer) or 0.05% glycerol + 0.5 mM IPTG.

EC-C is normally unable to metabolize lactose but reversion of EC-C to a Lac^+ phenotype was reported by Gray et al. (1986). However, Lac^+ mutants are readily isolated by selective pressure on a poor medium containing lactose as a sole carbon source. The only difference between the Lac^+ and the Lac^- strains was found to be the presence and absence of the lactose permease (Gray et al., 1984, 1986). All the revertants were found to be consistently Lac^+ on MacConkey agar and exhibited high levels of β-galactosidase on induction with IPTG (Table 9).

Table 9. Enzyme activities of lactose-utilizing (Lac^+) colonies isolated from EC-C mutants [a].

	α-Galactosidase (U/OD_{600})			β-Galactosidase (U/OD_{600})	
	Inducer [b]			Inducer [b]	
strain	none	melibiose	raffinose	IPTG	none
FH 10 Lac^-	48.0	60.2	61.2	546.6	29.7
FH 10 Lac^+	40.6	57.6	50.6	758.7	36.3
FH 11 Lac^-	15.2	54.4	20.4	560.5	33.3
FH 11 Lac^+	59.9	53.9	50.4	585.6	40.1
FH 13 Lac^-	7.5	79.3	7.2	735.6	57.8
FH 13 Lac^+	111.9	128.9	53.7	979.5	63.6
FH 12 Lac^-	2.1	5.3	5.8	529.9	51.2
FH 12 Lac^+	51.3	46.5	54.2	540.8	59.8
EC-C	9.4	33.3	13.4	829.8	58.9
EC-S	9.8	52.6	60.8	914.5	62.4

[a] Lactose non-utilizing EC-C mutants were streaked on YS agar plates supplemented with 0.5% lactose. After incubating the plates at 30°C for 3 days, large slimy colonies (an indication of a Lac^+ phenotype) were isolated.

[b] Cells were grown to late log phase in minimal M63 medium supplemented either with 0.4% glycerol (no inducer), 0.05% glycerol + 0.4% melibiose, 0.05% glycerol + 0.4% raffinose for α-galactosidase activity or 0.4% glycerol + 0.5 mM IPTG for β-galactosidase activity.

The *mel* operon has two genes, *mel* A which codes for a melibiose hydrolyzing enzyme (α-galactosidase), and *mel* B which codes for a melibiose transport system (permease). On incubation with X-gal, the presence of the two functions is indicated by the appearance of blue to deep blue colonies. A defect in either of the two genes results in the appearance of pale blue colonies. In the absence of both the transport system and the hydrolyzing enzyme, white colonies appear. Qualitative assays for α-galactosidase of isolated mutants were carried out using X-gal as substrate, supplemented with either melibiose or raffinose as enzyme inducers. The results suggest a defect either in the transport of these sugars into the cell or in their subsequent hydrolysis. Some mutants showed defects in both these mechanisms (Table 10).

Table 10. Characterization of EC-C mutants using X-gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside [a]).

	α-Galactosidase activity [b]				
	Before $CHCl_3$ treatment		After $CHCl_3$ treatment [c]		Class
Strain	X-gal + melibiose	X-gal + raffinose	X-gal + melibiose	X-gal + raffinose	
FH 13	++	-	+++	-	4
FH 101	-	-	-	-	3
FH 14	-	-	-	-	5
FH 15	++	++	++	++	6
FH 104	++	++	++	+++	
FH 16	++	-	+++	+	7
FH 10	++	+	++	+	1
FH 11	-	-	+	+	2
EC-C	+	+	+	+	
EC-S	++	++	+++	+++	

(-) = negative reaction (white)

(+) = weakly positive (pale blue)

(++) = positive (blue)

(+++) = strongly positive (deep blue)

[a] X-gal (4% in dimethyl formamide) was mixed with 1 l of sterile YS agar to give a final concentration of 0.004%. The carbon source was added to a final concentration of 0.2% before pouring the agar into glass Petri dishes. The plates were stored in plastic bags in the dark.

[b] Each plate was divided into 8 segments, and mutants were individually streaked, including EC-C and EC-S as positive controls. After incubating the plates at 30°C for 48 h, the plates were scored (colour of colonies recorded) before exposing the plates to chloroform.

[c] Glass microfibre filter paper (Whatman GF/A) was placed inside the lid of the Petri dish, soaked in chloroform, and the plate inverted and incubated at room temperature for 30 min, after which the change in the previous colour of the colonies was noted.

3.2.1.2. Invertase activity and sucrose uptake.

Studies on sucrose uptake and the localization of invertase in the mutant strains were carried out. Results reveal the absence of both sucrose transport and invertase activity. In many bacteria sucrose enters the cell as a phosphorylated derivative via the PEP:PTS transport system. The uptake of sucrose by EC-C showed linearity over 60 s (Fig. 8).

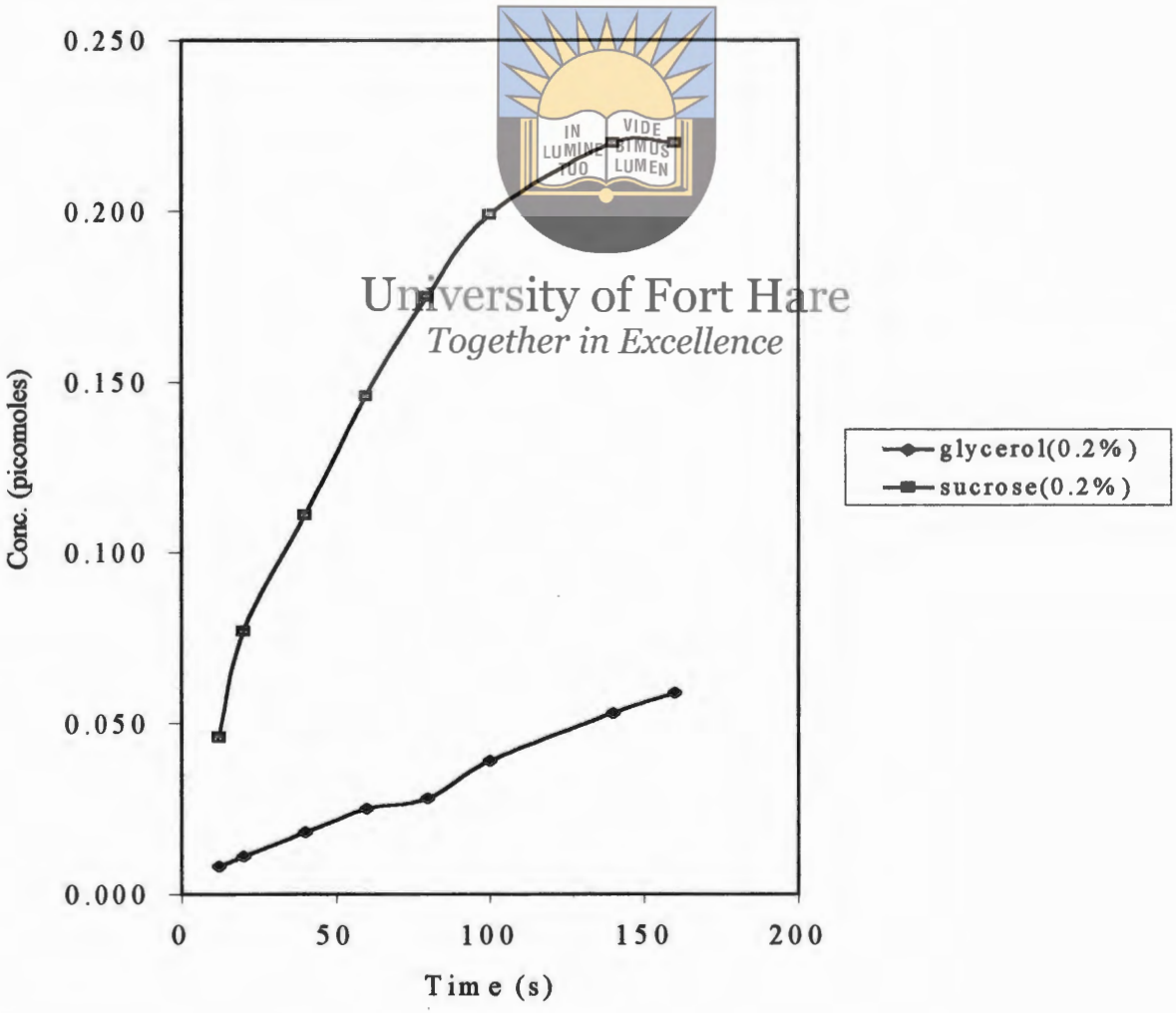


Fig. 8. Time course assay of ^{14}C-Sucrose uptake by *Erwinia chrysanthemi*.

All the sucrose utilising mutants (Table 6) showed a sucrose inducible invertase activity. Raffinose induced invertase activity in both raffinose positive strains (FH 15 and FH 104) and in some raffinose non-utilizing strains (Table 11).

Table 11. Invertase activity of isolated mutants.

	Invertase activity [a] (U/OD_{600})				
strain	Phenotype	Inducer [b]			
		raffinose	sucrose	fructose	none
FH 10	$Scr^+ Raf^-$	16.9	27.3	6.8	0.8
FH 12	$Scr^+ Raf^-$	2.3	34.9	3.5	3.3
FH 101		2.5	27.4	3.7	0.5
FH 14	$Scr^+ Raf^-$	1.8	30.5	4.6	0.3
FH 103		2.8	18.6	3.0	0.5
FH 15	$Scr^- Raf^+$	10.7	0.9	0.0	0.0
FH 104		16.5	0.9	7.3	0.7
FH 100	$Scr^- Raf^-$	12.4	33.1	4.5	0.3
FH 11	$Scr^- Raf^-$	16.6	0.0	2.7	0.4
FH 102	$Scr^- Raf^-$	0.0	0.0	0.04	0.4
FH 13		0.18	0.0	0.0	0.7
FH 16	$Scr^- Raf^-$	0.0	0.0	2.5	0.6
EC-C	$Scr^+ Raf^+$	8.9	23.7	3.9	1.4

[a] The assay mixture contained 0.25 ml cells, 0.25 ml phosphate buffer and 10 µl toluene. After equilibration at 30°C for 10 min, the reaction was initiated by the addition of 50 µl of 0.5 M sucrose. The reaction was stopped 30 min later by boiling for 3 min. After sedimenting the cells by centrifugation in an Eppendorf microcentrifuge, 100 µl of supernatant was mixed with 1 ml of Gluc-DH reagent. The OD_{340} was determined after a 15 min incubation at 30°C.

[b] Cells were grown at 30°C in minimal M63 medium containing either 0.05% glycerol + 0.4% raffinose, 0.05% glycerol + 0.4% sucrose, 0.05% glycerol + 0.4% fructose, or 0.4% glycerol (no inducer).

The inability of the other isolated mutants to utilize sucrose was shown to be due to the absence of, or a defect in, both the sucrose specific transport protein (Table 12) and the hydrolyzing enzyme (Table 11). Fructose, did not induce invertase activity in EC-C and the mutant strains (Table 11), even though it has been reported to induce both the sucrose transport system and invertase in *B. subtilis* (Robeson et al., 1983) and in *S. mutans* (St. Martin and Wittenberger, 1979; Russell et al., 1985).

Table 12. Characterization of sucrose non-utilizing EC-C mutants by sucrose transport and invertase activity.

Strain	Phenotype	Inducer [a]	Sucrose uptake [b] (pmol/50s/OD_{600})
FH 11	Scr $^-$	none	0.00
		sucrose	0.11
FH 102	Scr $^-$	none	0.01
		sucrose	0.13
FH 13	Scr $^-$	none	0.02
		sucrose	0.03
FH 16	Scr $^-$	none	0.01
		sucrose	0.03
FH 104	Scr $^-$	none	0.00
		sucrose	0.02
EC-C	Scr $^+$	none	0.02
		sucrose	0.15

[a] Cells were grown at 30°C in minimal M63 medium supplemented with either 0.4% glycerol (no inducer) or 0.05% glycerol + 0.4% sucrose.

[b] The cell suspension (0.6 ml) was dispensed into Eppendorf microfuge tubes and incubated at room temperature for 5 min. The reaction was initiated by the addition of 100 µl of a 100 fold diluted ^{14}C-sucrose solution (Amersham). Samples were withdrawn after 10 s and 60 s incubation. The 10 s was used as a control and subtracted from the 60 s assay.

Selected mutants were characterized with respect to their ability to hydrolyse α- and β-galactosides, and to transport sucrose (Table 13).

Table 13. Enzyme activities of selected mutants..

Strain	β-Galactosidase	α-Galactosidase	Sucrose invertase	Sucrose transport
FH 15	+	+	-	-
FH 104	+	+	-	-
FH 10	+	+	+	+
FH 100	+	+	+	+
FH 11	+	+	-	+
FH 102	+	+	-	+
FH 13	+	+	-	-
FH 12	+	+	+	+
FH 101	+	+	+	+
FH 14	+	-	+	+
FH 103	+	-	+	+
FH 16	+	+	-	-
EC-C	+	+	+	+

3.2.1.3. Kinetic properties of α-galactosidase and invertase.

Kinetic studies of α-galactosidase were carried out using *o*-Nitrophenyl-α-D-galactopyranoside as substrate.

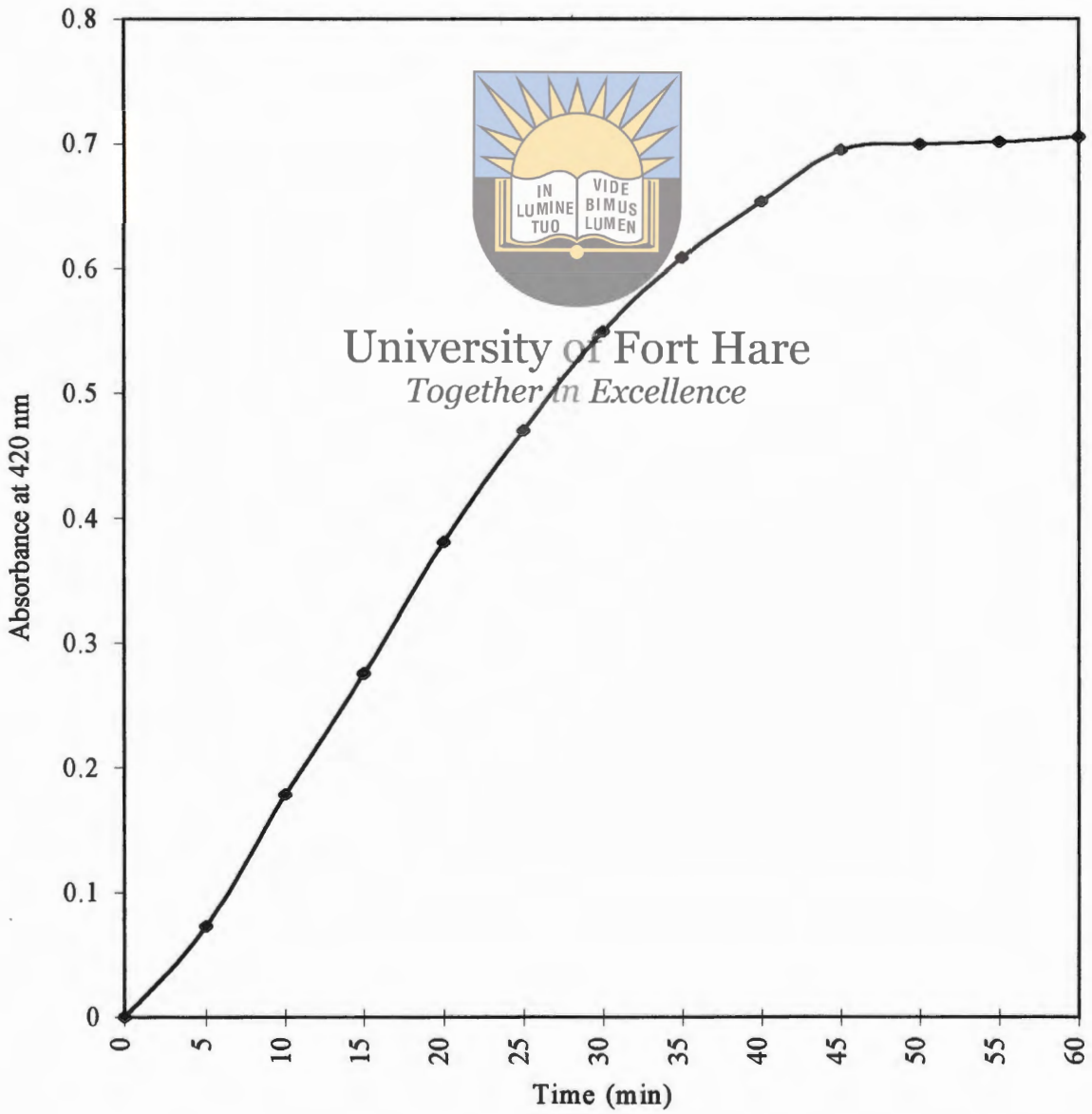


Fig. 9. Time course assay of EC-C α-Galactosidase using α-ONPG as substrate.

The pH profile of α-galactosidase was determined over the pH range 3.7 to 9 and showed a pH optimum of 7.4 (Fig. 10).

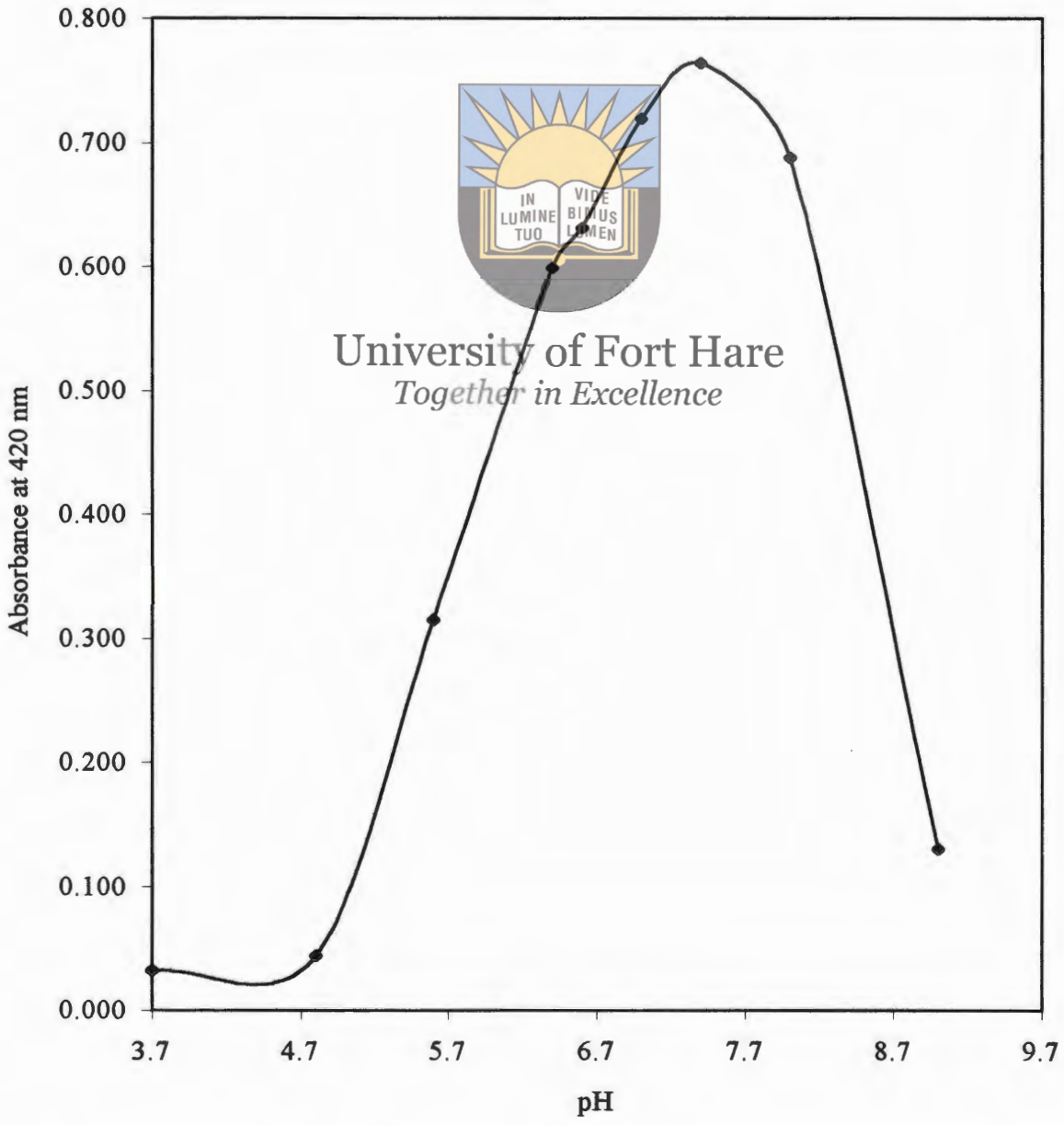


Fig. 10. pH optimum of EC-C α-Galactosidase.

The temperature optimum of α-galactosidase was found to be 50°C (Fig. 11) and the temperature stability was determined after an incubation period of 1 h.

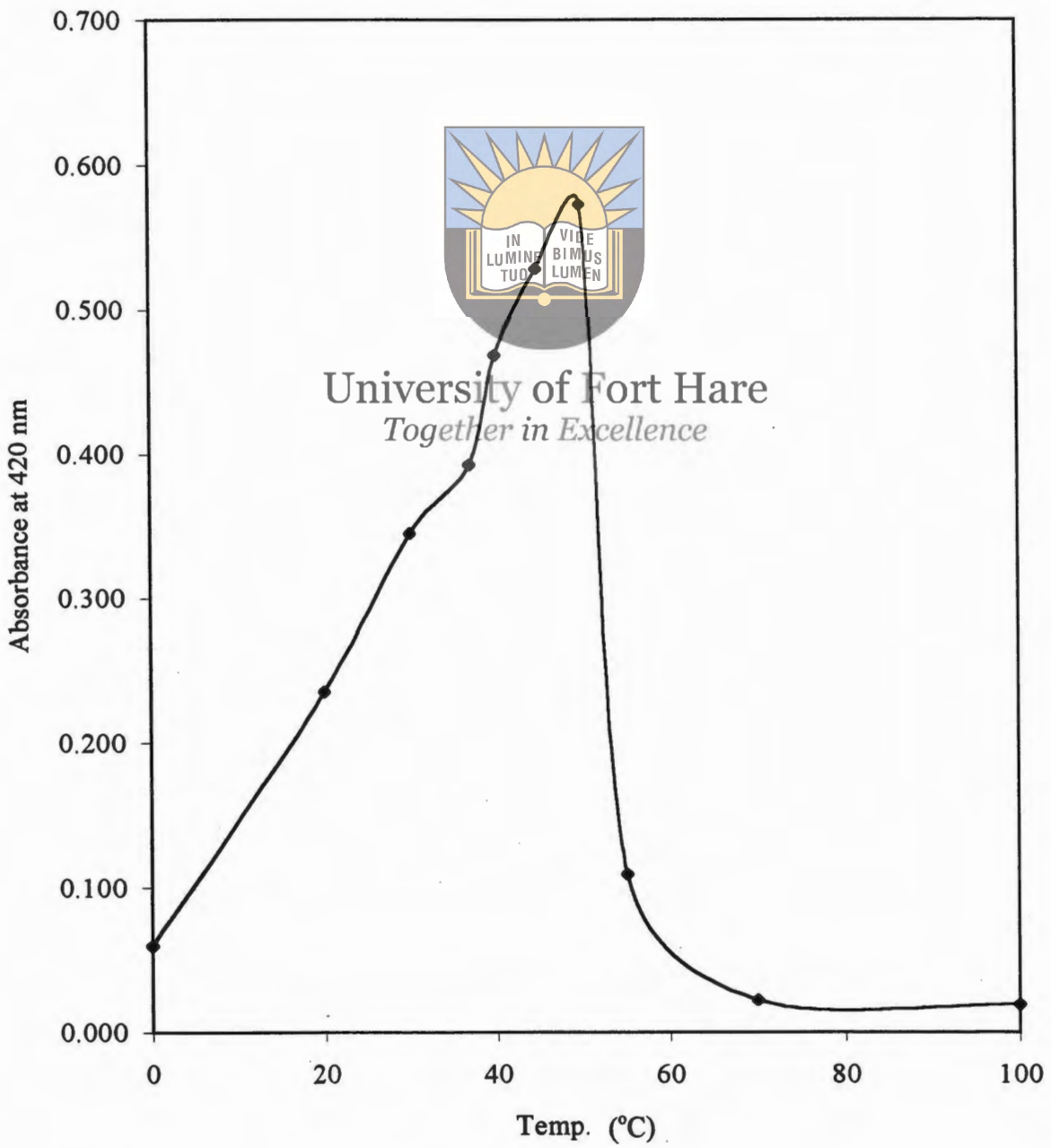


Fig. 11. Temperature optimum of EC-C α-Galactosidase.

Kinetic studies of invertase were carried out using sucrose as substrate.

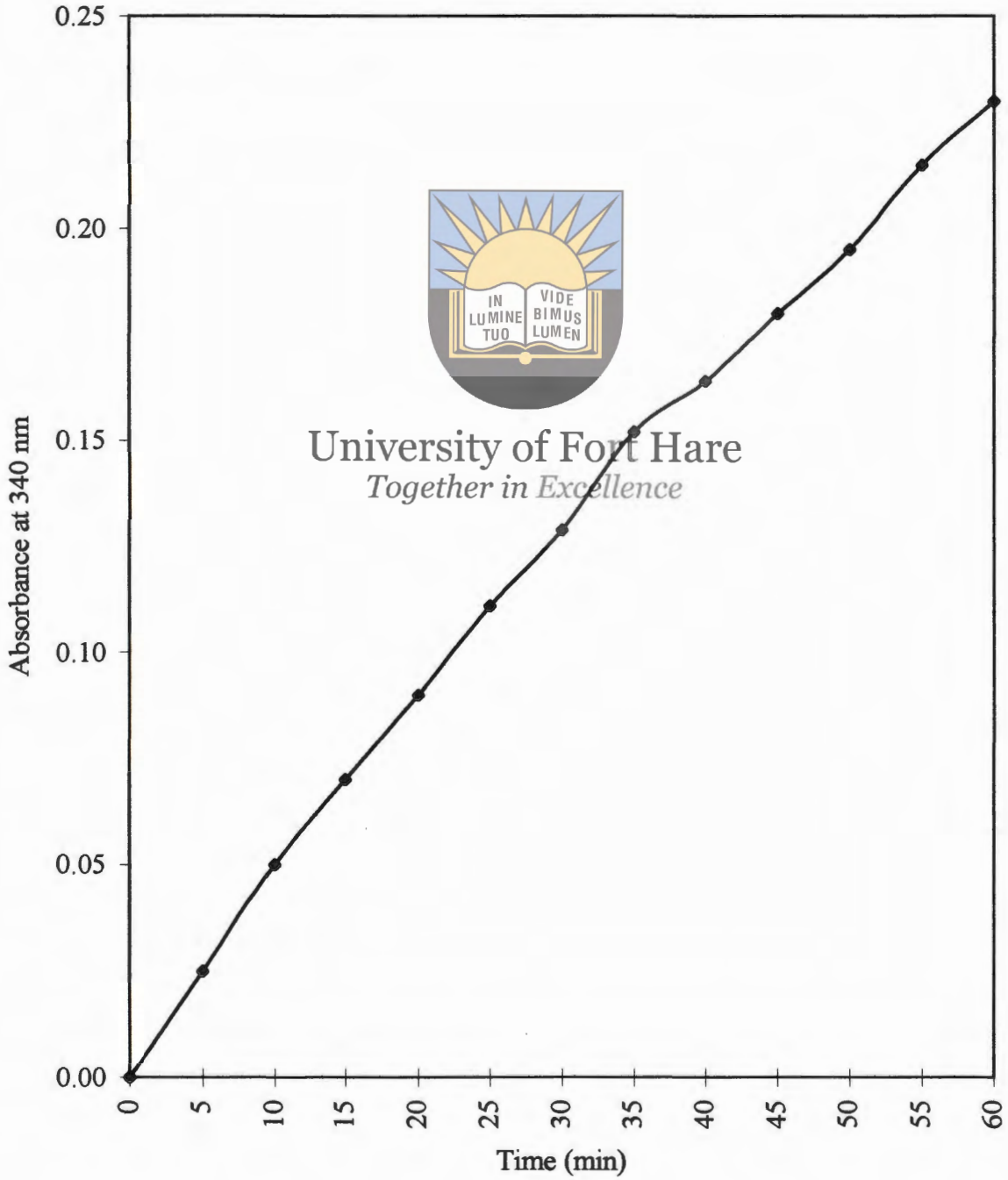


Fig. 12. Time course assay of EC-C invertase using sucrose as substrate.

Invertase had a pH optimum of 6.6 (Fig. 13) and a broad pH stability ranging between 6.4 and 8 (Table 15).

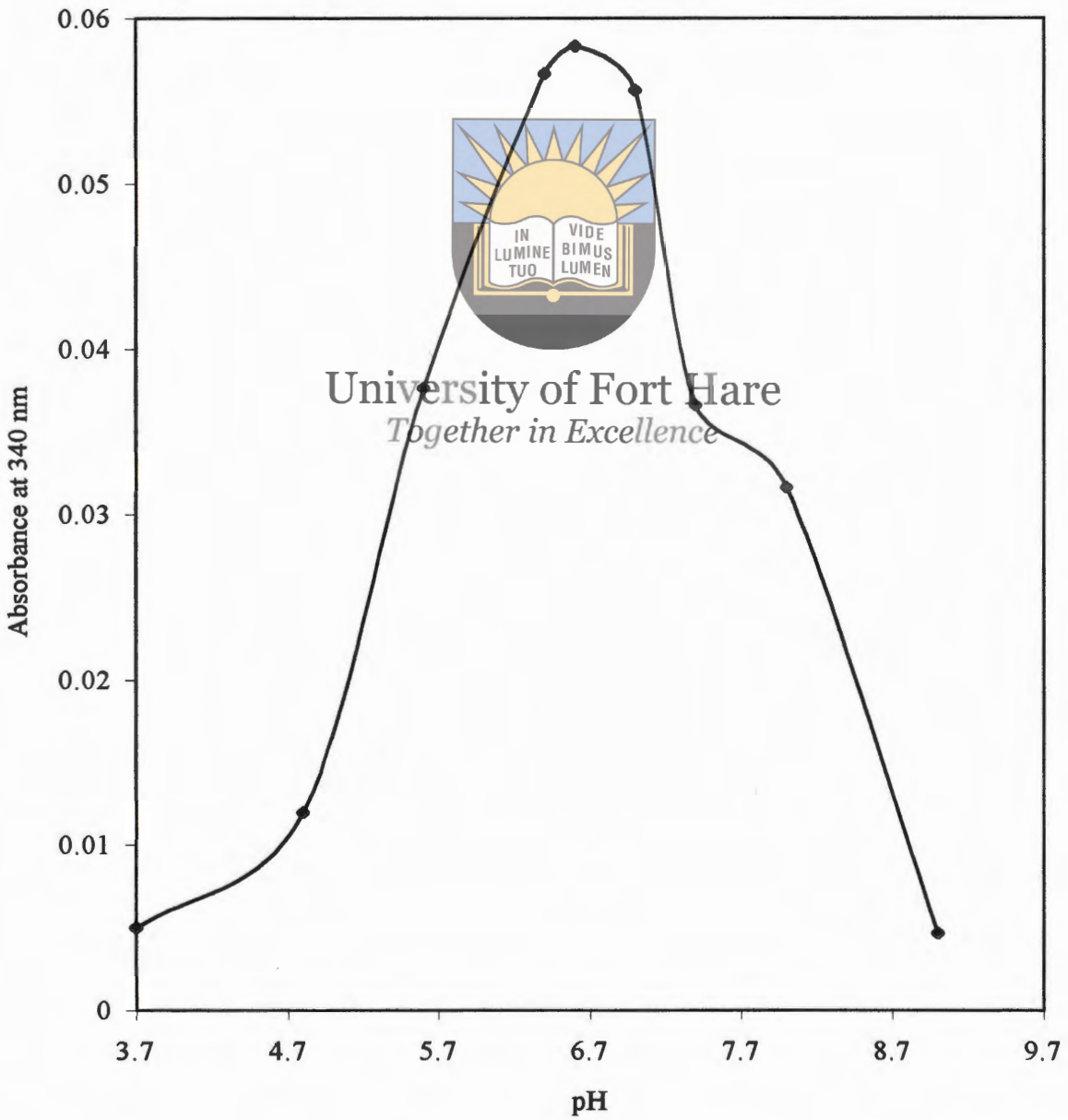


Fig. 13. pH optimum of EC-C invertase.

Invertase exhibited a temperature optimum of 30°C (Fig. 14) and a pH stability ranging from 6.4 to 8 (Table 15).

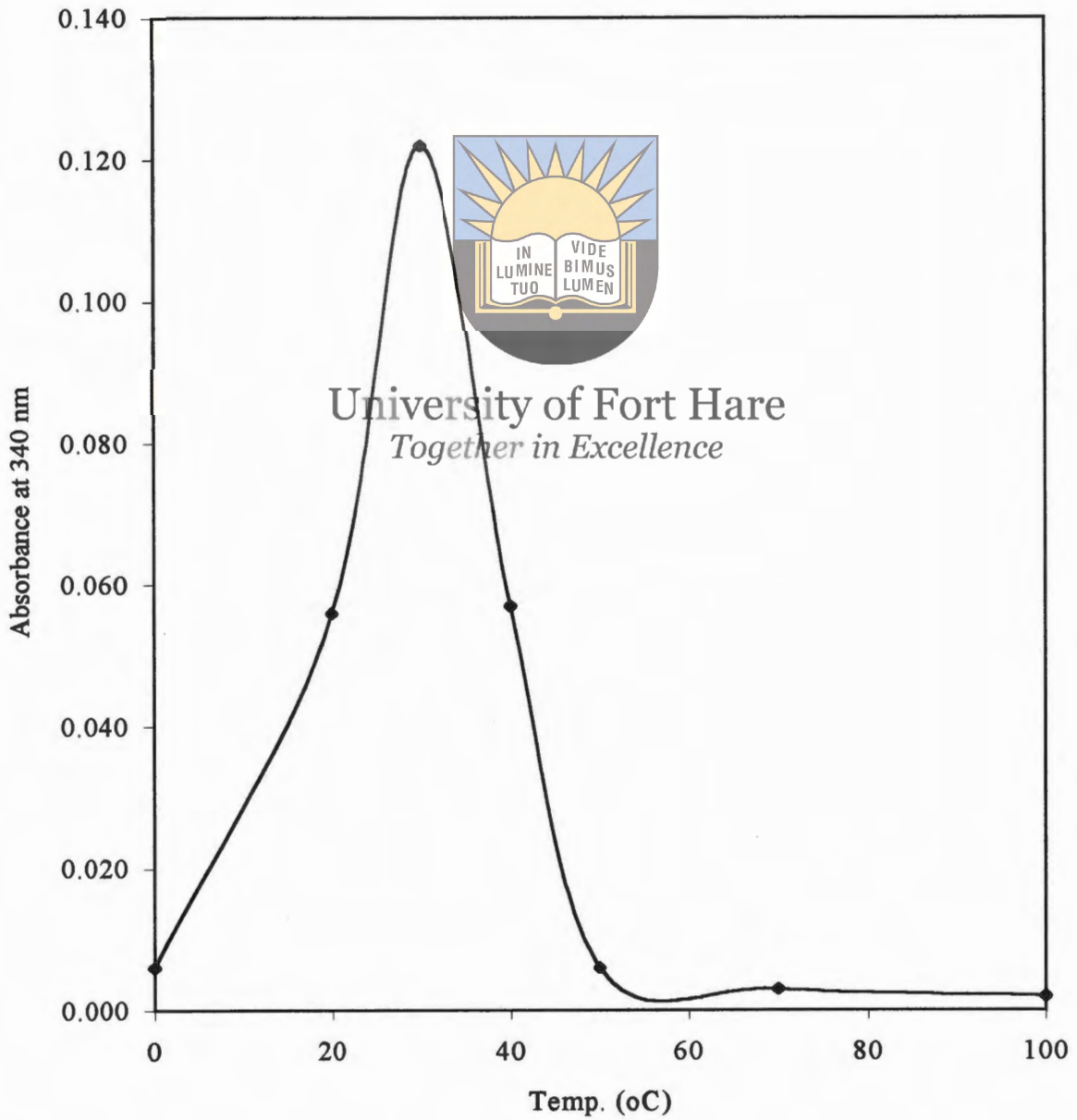


Fig. 14. Temperature optimum of EC-C invertase.

The Km's of α-galactosidase in EC-C were determined using *o*-Nitrophenyl-α-D-galactopyranoside as substrate (Table 14), whereas that of invertase was determined using sucrose as substrate (Table 15).

Table 14. Determination of the kinetic properties of melibiose- and raffinose-induced EC-C α-galactosidase using ONPG as substrate.

Inducer	Temperature (°C)		pH		K_m (mM)	V_{max} (U/OD_{600})
	optimum	stability	optimum	stability		
melibiose	50	0 - 50	7.4	6.4 - 8.0	0.32	0.39
raffinose	50	0 - 50	7.4	6.4 - 8.0	0.35	0.41

Table 15. Kinetic properties of sucrose- and raffinose-induced EC-C invertase using the Glucose Dehydrogenase assay with sucrose as substrate.

Inducer	Temperature (°C)		pH		K_m (mM)	V_{max} (U/OD_{600})
	optimum	stability	optimum	stability		
sucrose	30	0 - 30	6.6	6.4 - 8.0	17.8	0.45
raffinose	40	0 - 40	5.6	5.6 - 8.0	27.3	0.59

Table 16. Comparison of the kinetic properties of sucrose- and raffinose-induced EC-C invertase using the Nelson-Somogyi assay and either sucrose or raffinose as substrates.

Sucrose induced						
Substrate	Temperature (°C)		pH		K_m (mM)	V_{max} (U/OD_{600})
	optimum	stability	optimum	stability		
sucrose	30	0 - 30	6.6	6.4 - 8.0	19.84	0.99
raffinose	30	0 - 30	6.6	6.4 - 8.0	26.53	2.39
Raffinose induced						
sucrose	40	0 - 40	5.6	5.6 - 8.0	6.17	0.11
raffinose	40	0 - 40	5.6	5.6 - 8.0	9.78	0.62

3.2.1.4. Effect of SDS and DOC on enzyme activity.

Both α-galactosidase and invertase activities were inhibited by SDS but not by DOC. Higher concentrations of SDS (0.05%) were required to inhibit invertase (Fig. 16) whereas α-galactosidase was more sensitive (0.02%) to SDS (Fig. 15). This finding made it possible to assay for invertase in a crude enzyme preparation using raffinose as a substrate.

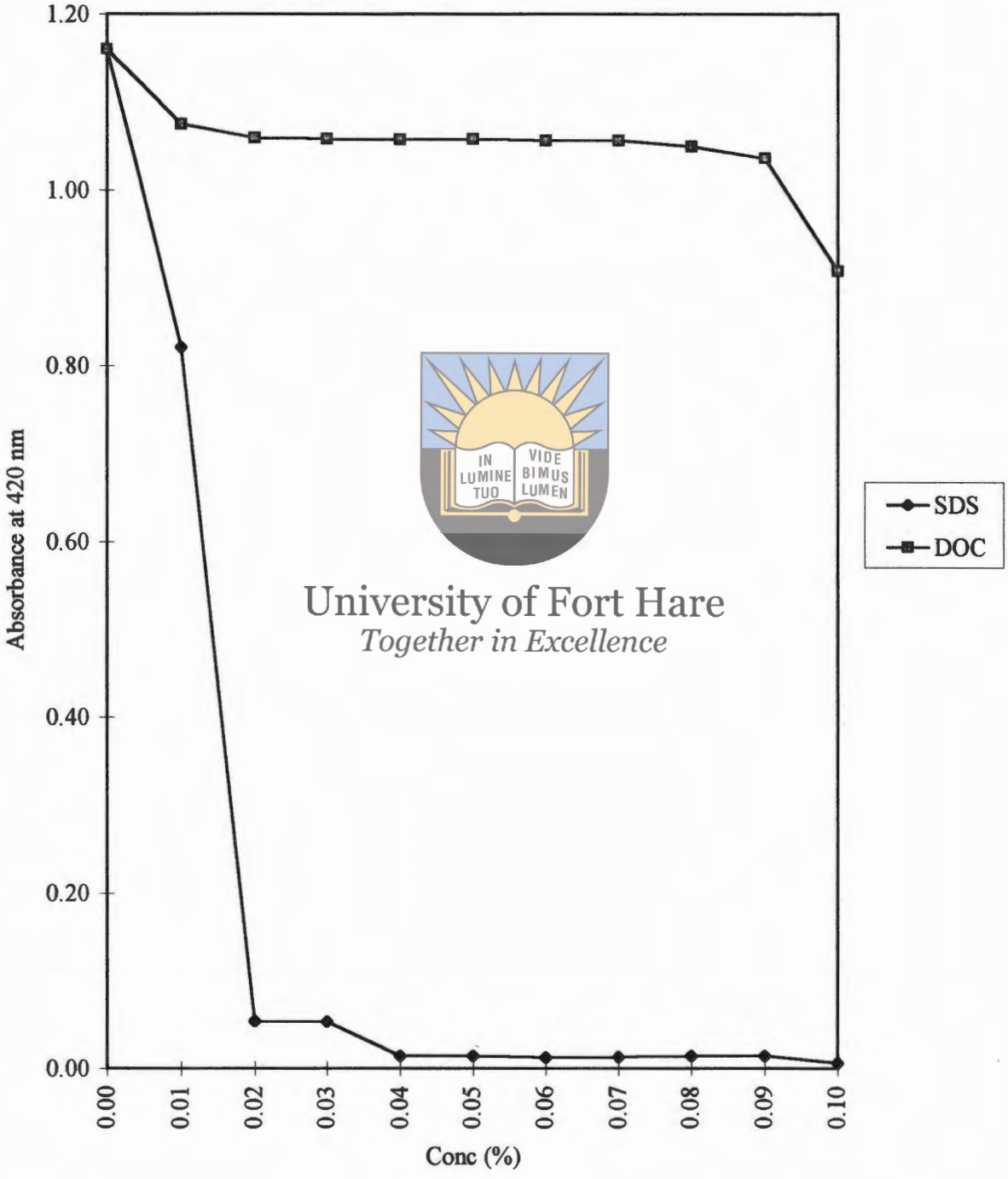


Fig. 15. Effect of SDS and DOC on EC-C α-Galactosidase.

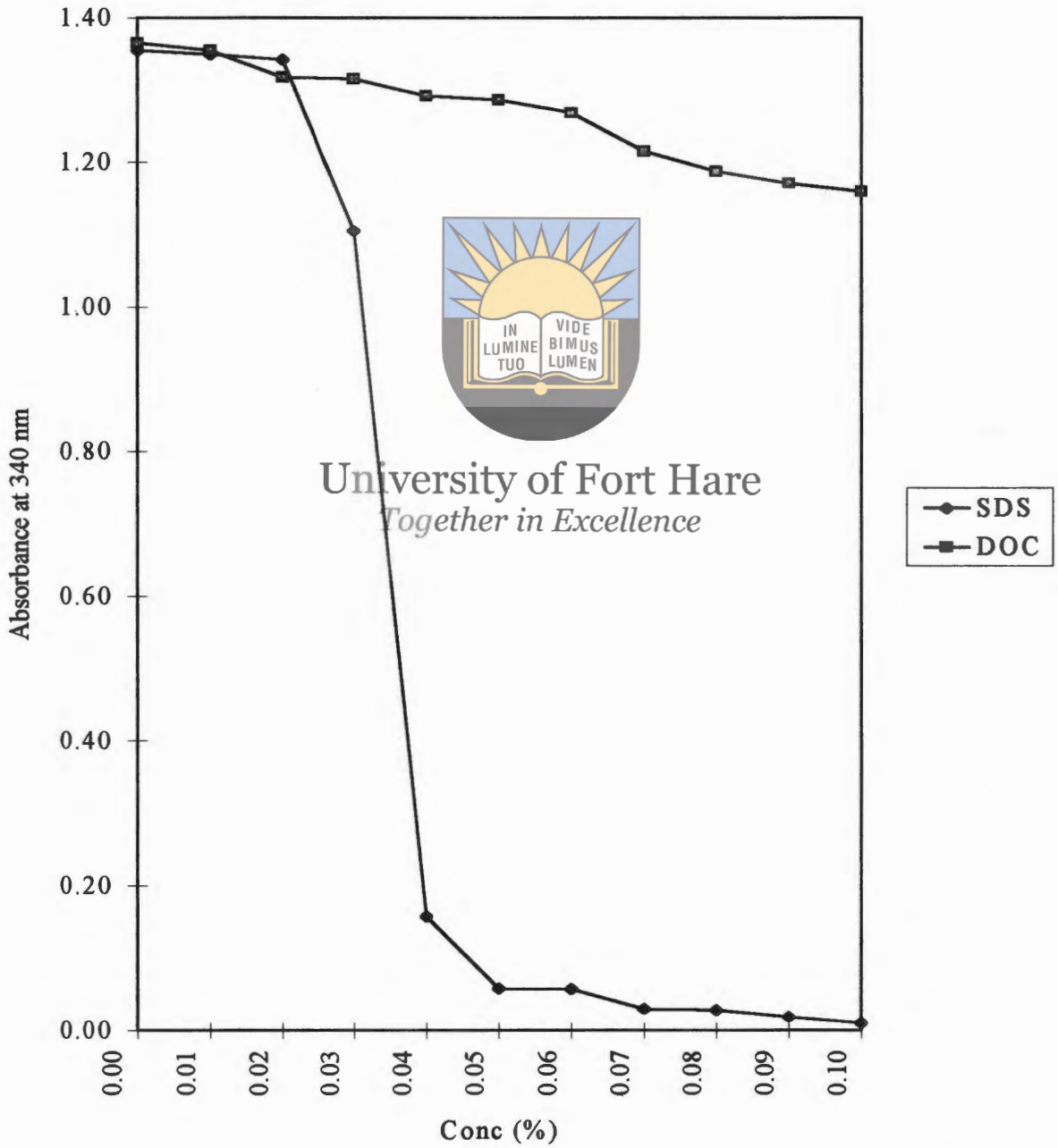


Fig. 16. Effect of SDS and DOC on EC-C invertase.

3.2.1.5. Thin layer chromatography.

To determine the mode of action of EC-C invertase against raffinose, the reaction end products of raffinose were analyzed by thin layer chromatography (Fig. 17).

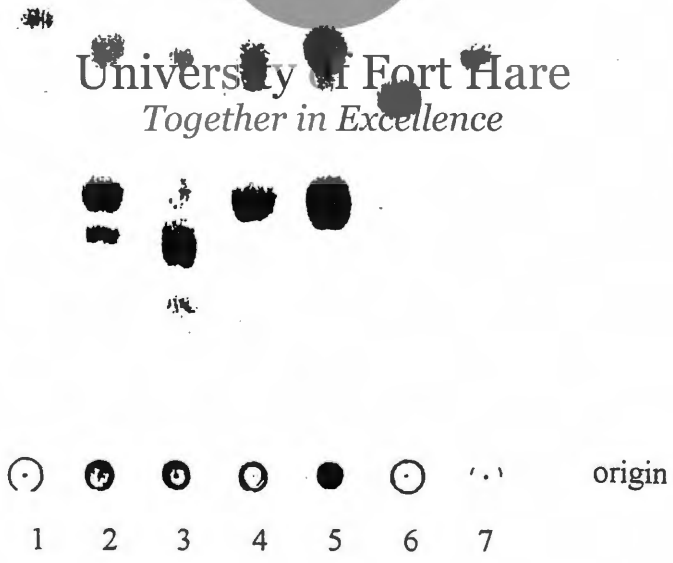


Fig. 17. Thin layer chromatography reaction products of raffinose hydrolysis by EC-C invertase. Lanes: 1 = glucose, 2 = standards (raffinose-lower; melibiose-middle; and fructose-top), 3 = raffinose hydrolysis by EC-C invertase, 4 = standards (melibiose-lower; and fructose-top), 5 = raffinose hydrolysis by Boehringer invertase, 6 = sucrose, 7 = galactose.

3.2.2. Enzyme localization studies.

The localization of these enzymes was investigated using both the osmotic shock method described by Willis et al. (1974) and the chloroform shock method described by Ames et al. (1984).

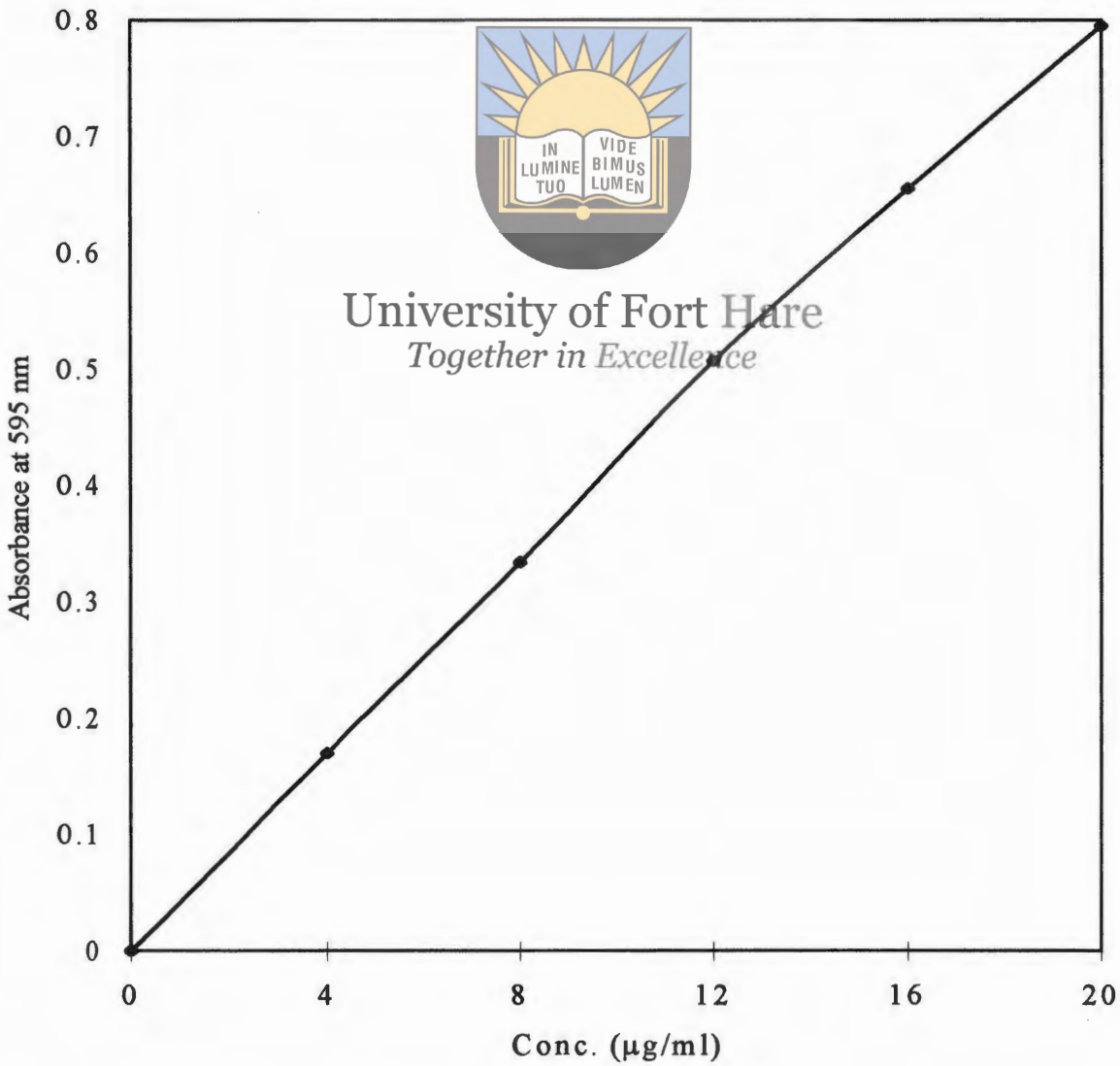


Fig. 18. BSA standard curve using Bio-Rad micro-assay.

Protein was generally found to be localized in the cytoplasm in both the parent strain and the isolated mutants (Figs. 19 and 20).

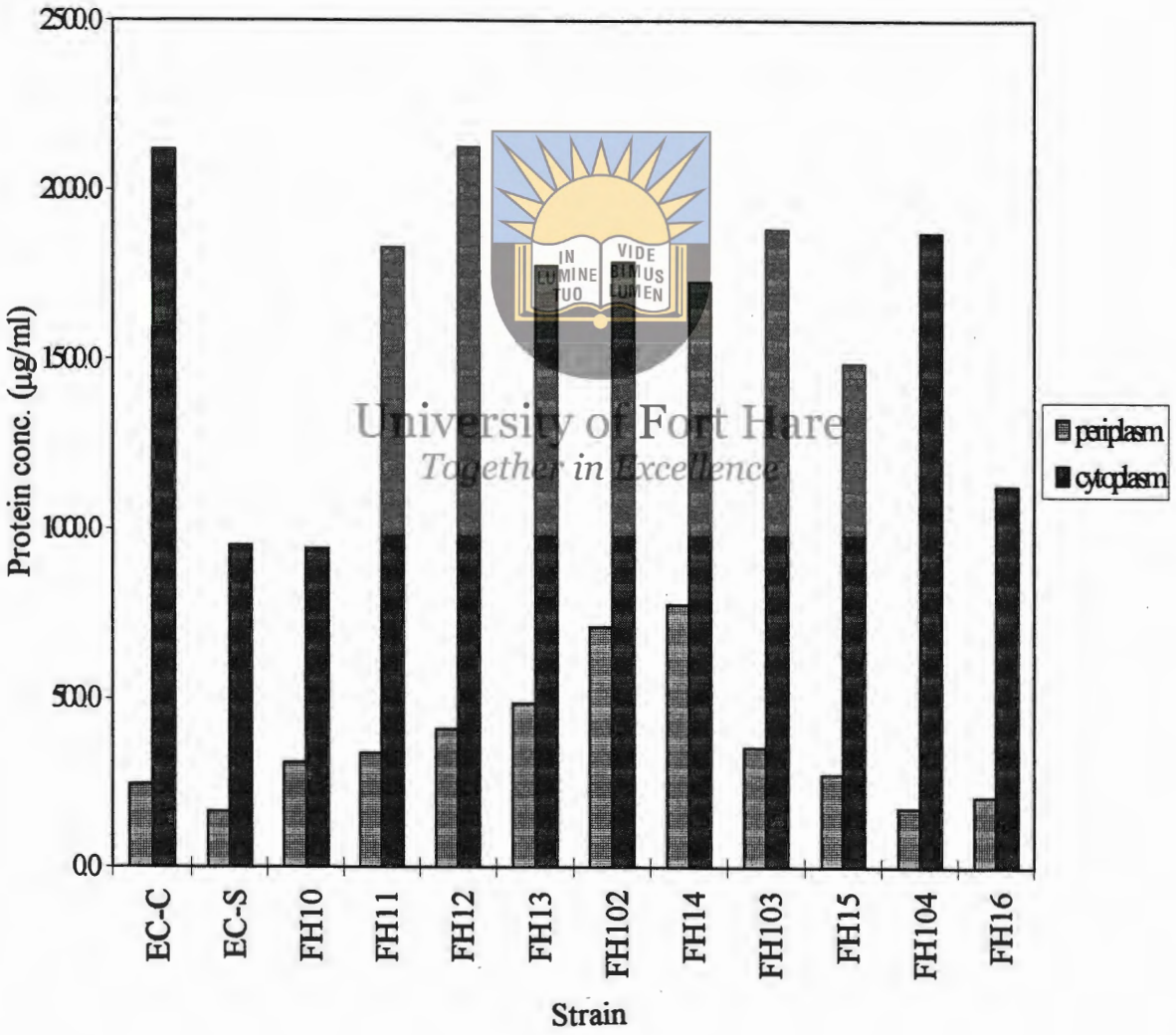


Fig. 19. Determination of total protein of IPTG-induced EC-C and mutant strains.

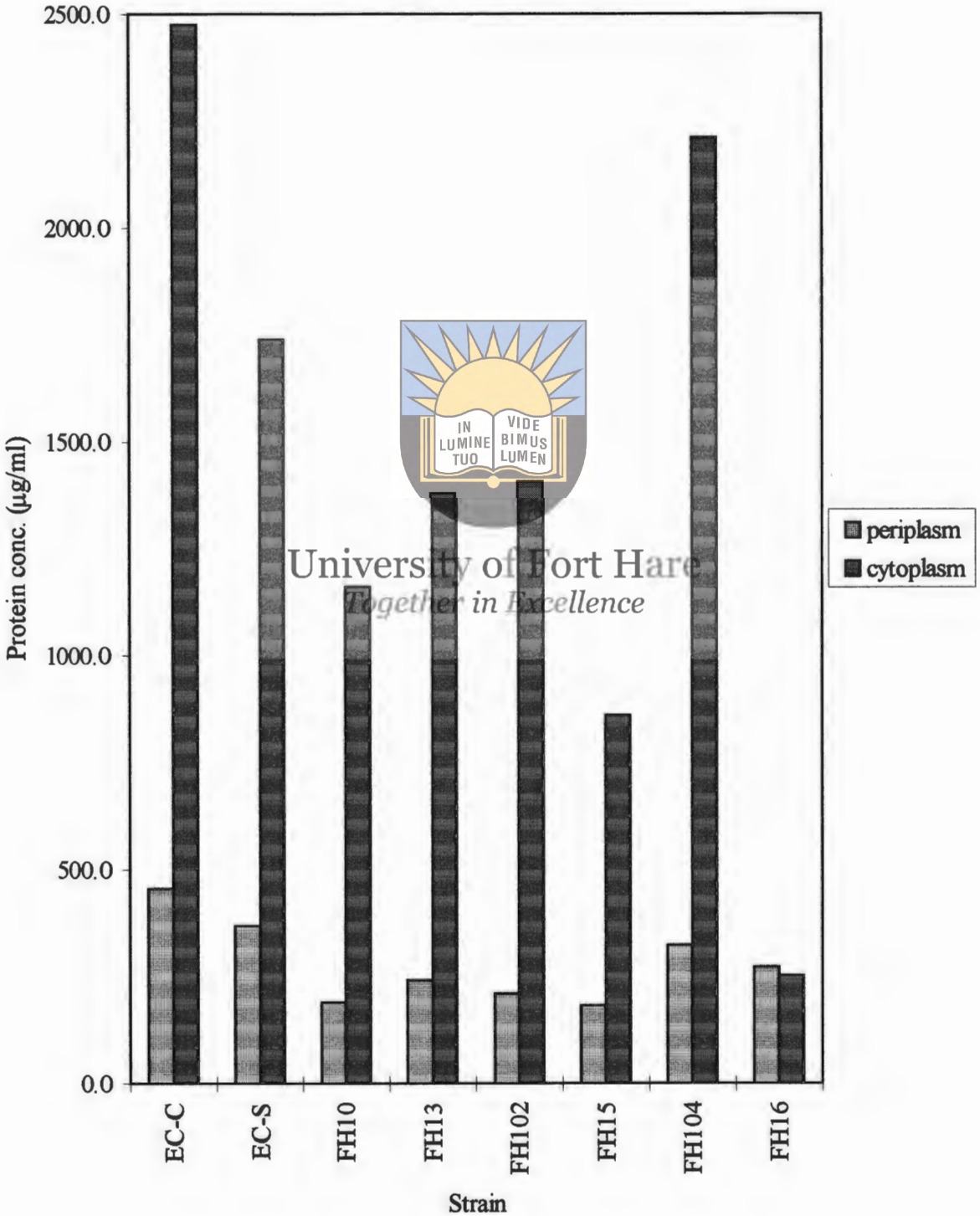


Fig. 20. Determination of total protein of EC-C and melibiose-utilizing mutant strains.

The majority of both α- and β-galactosidase was detected in the cytoplasm (Figs. 21 and 22).

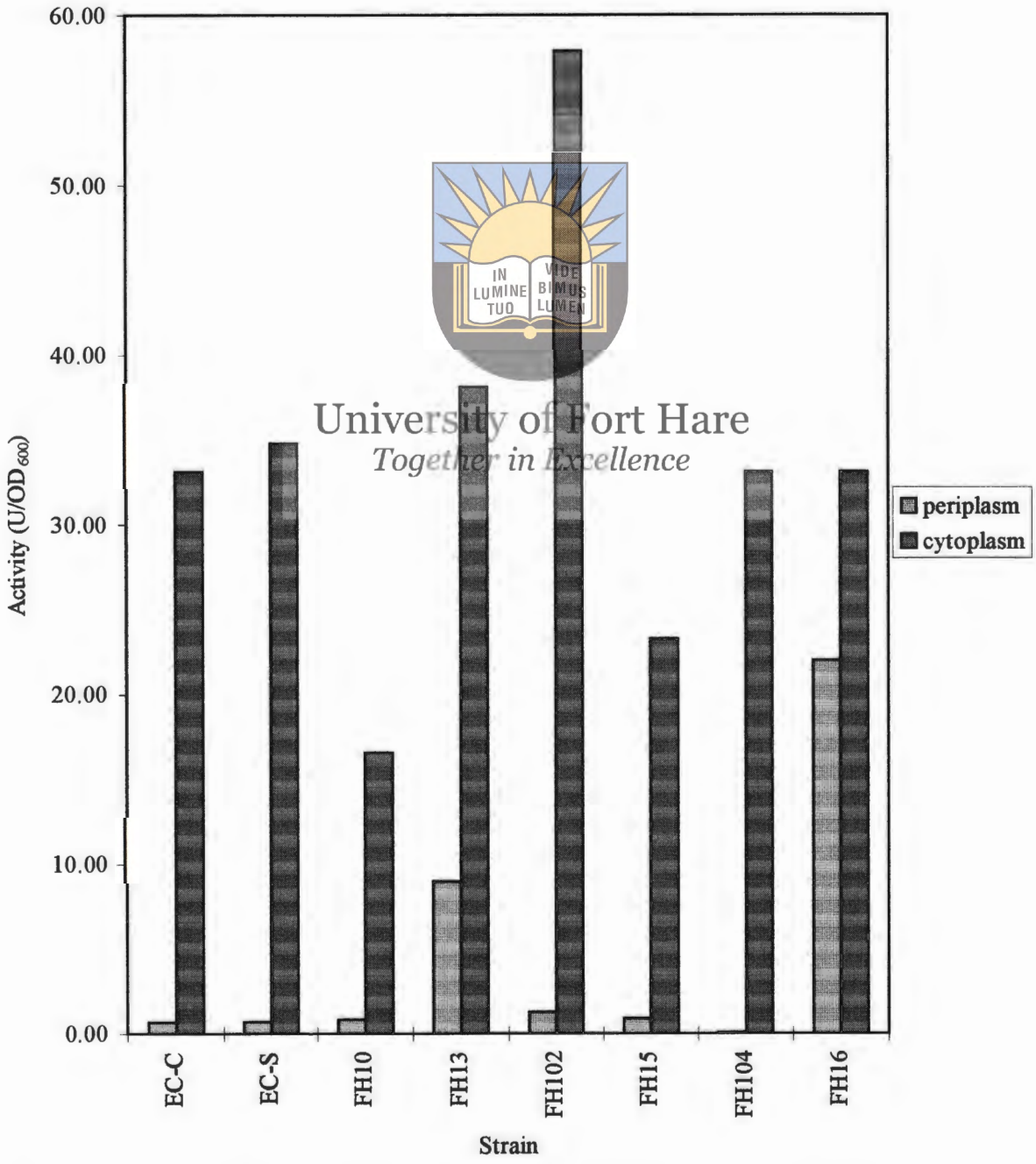


Fig. 21. Localization of α-Galactosidase in EC-C and melibiose-utilizing mutant strains.

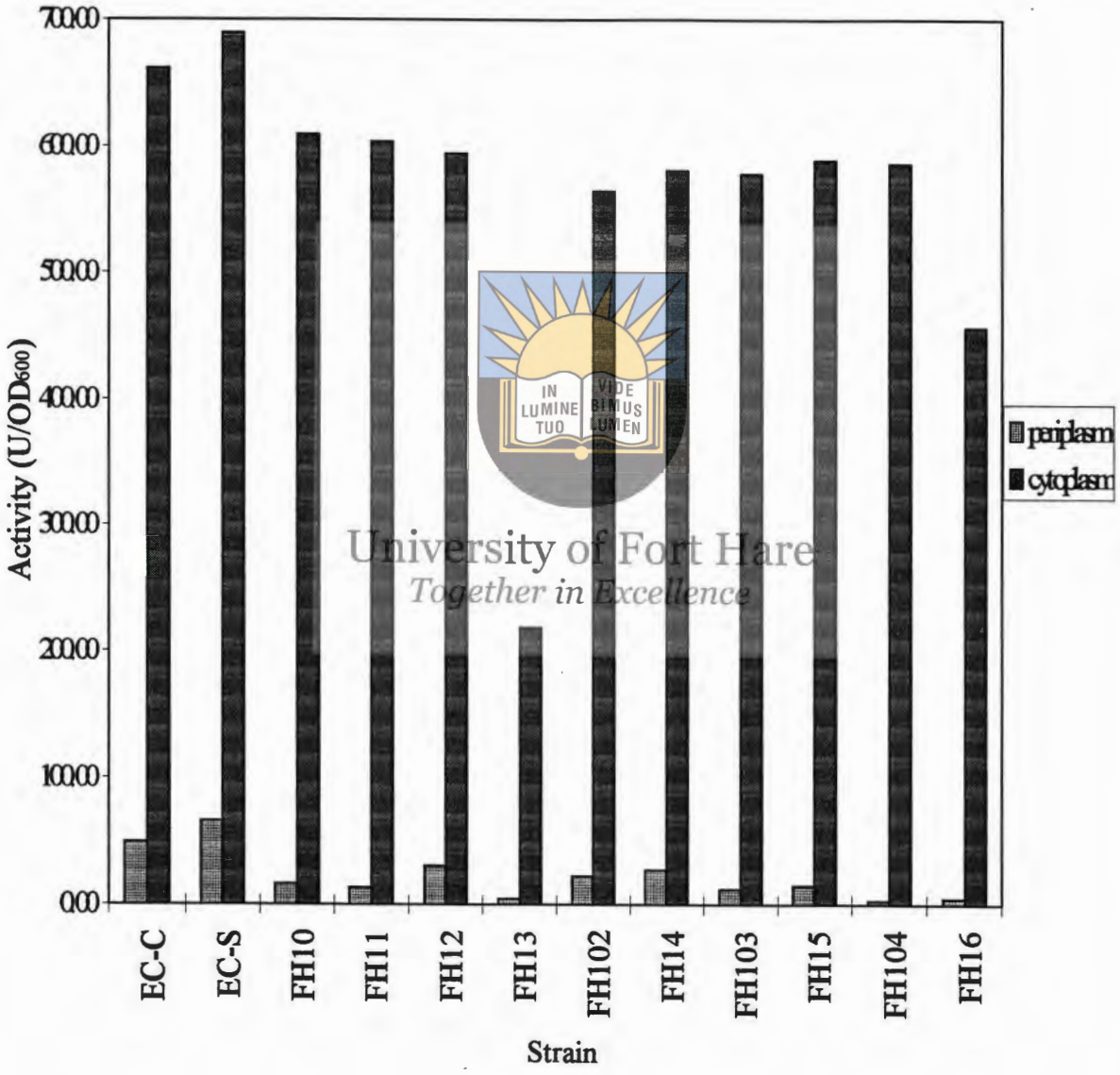


Fig. 22. Localization of IPTG-induced β-Galactosidase in EC-C and mutant strains.

In addition, a small fraction of protein was detected outside the cell whilst substantial amounts were found to be membrane bound. (Fig. 23).

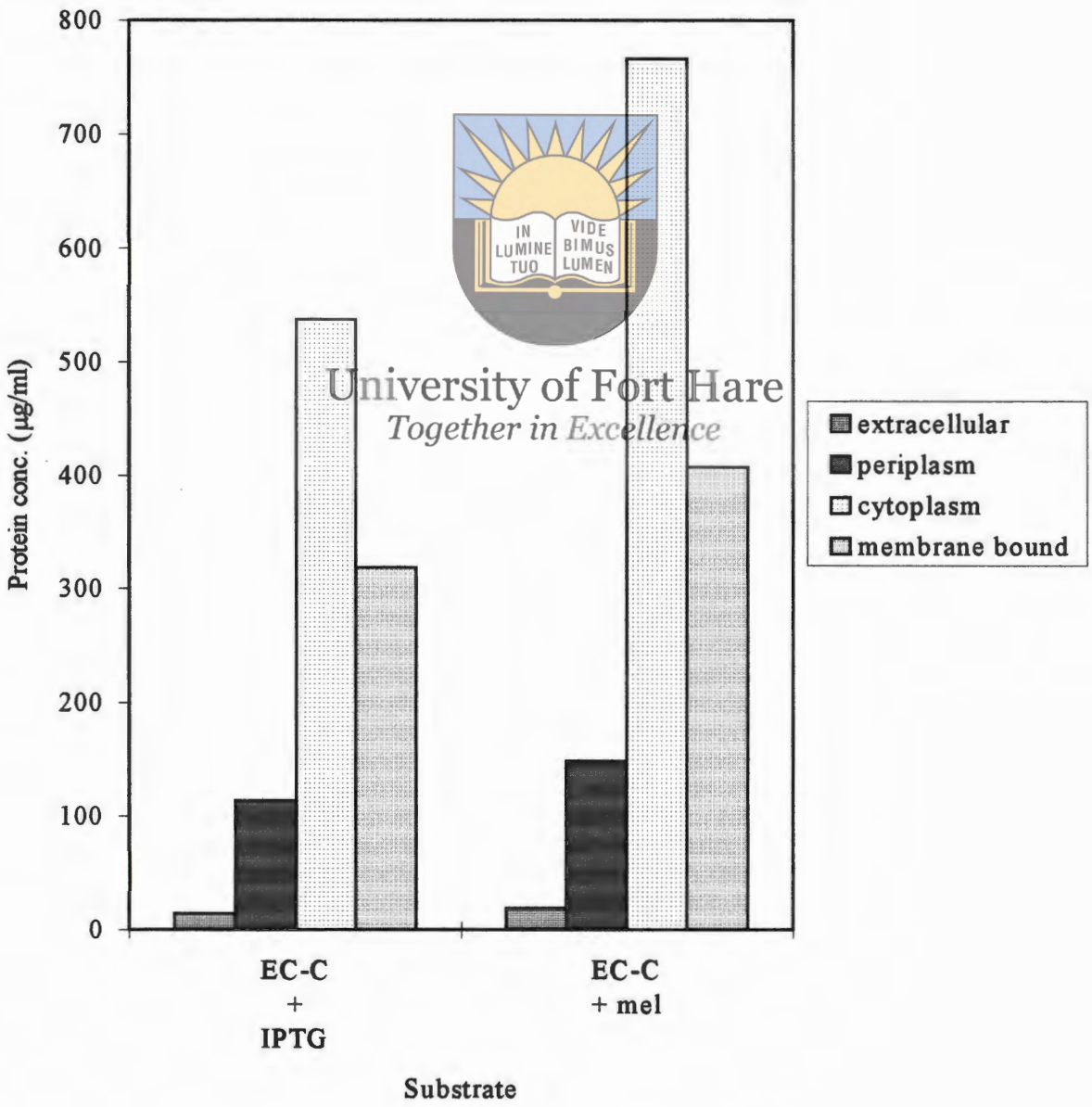


Fig. 23. Distribution of total protein in EC-C.

3.2.2.1. Specific activity stain and PAGE.

An *in situ* method was developed for the screening of α- and β-galactosidase on Polyacrylamide gels. Success was obtained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (β-X-Gal) but not 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (α-X-Gal) as specific activity stains for α- and β-galactosidase respectively. *E. coli* β-galactosidase was used as a standard and gave two distinct bands; one of higher molecular weight and the other of lower molecular weight. *E. chrysanthemi* β-galactosidase corresponded with the lower molecular weight *E. coli* β-galactosidase (Fig. 24). Polyacrylamide gel electrophoresis failed to reveal the absence of any major bands in the mutants (Fig. 25).

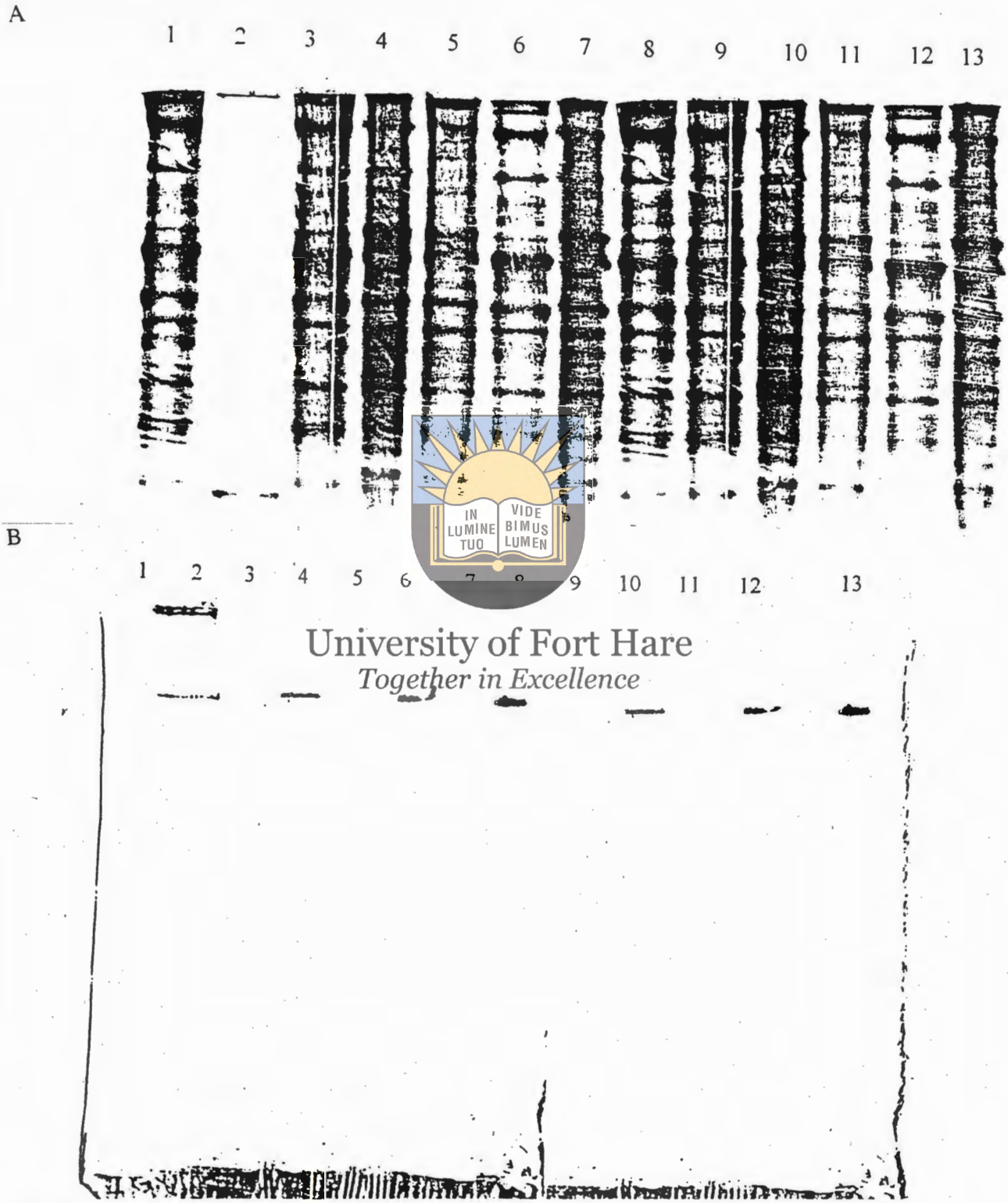


Fig. 24. Discontinuous PAGE of EC-C β-Galactosidase. (A) Commassie blue; (B) Specific activity stain. Lanes: 1, 3, 5, 7, 9 and 11 = controls; Lanes: 4, 6, 8, 10,12 and 13 = IPTG induced and lane 2 = *E. coli* β-galactosidase (Boehringer).

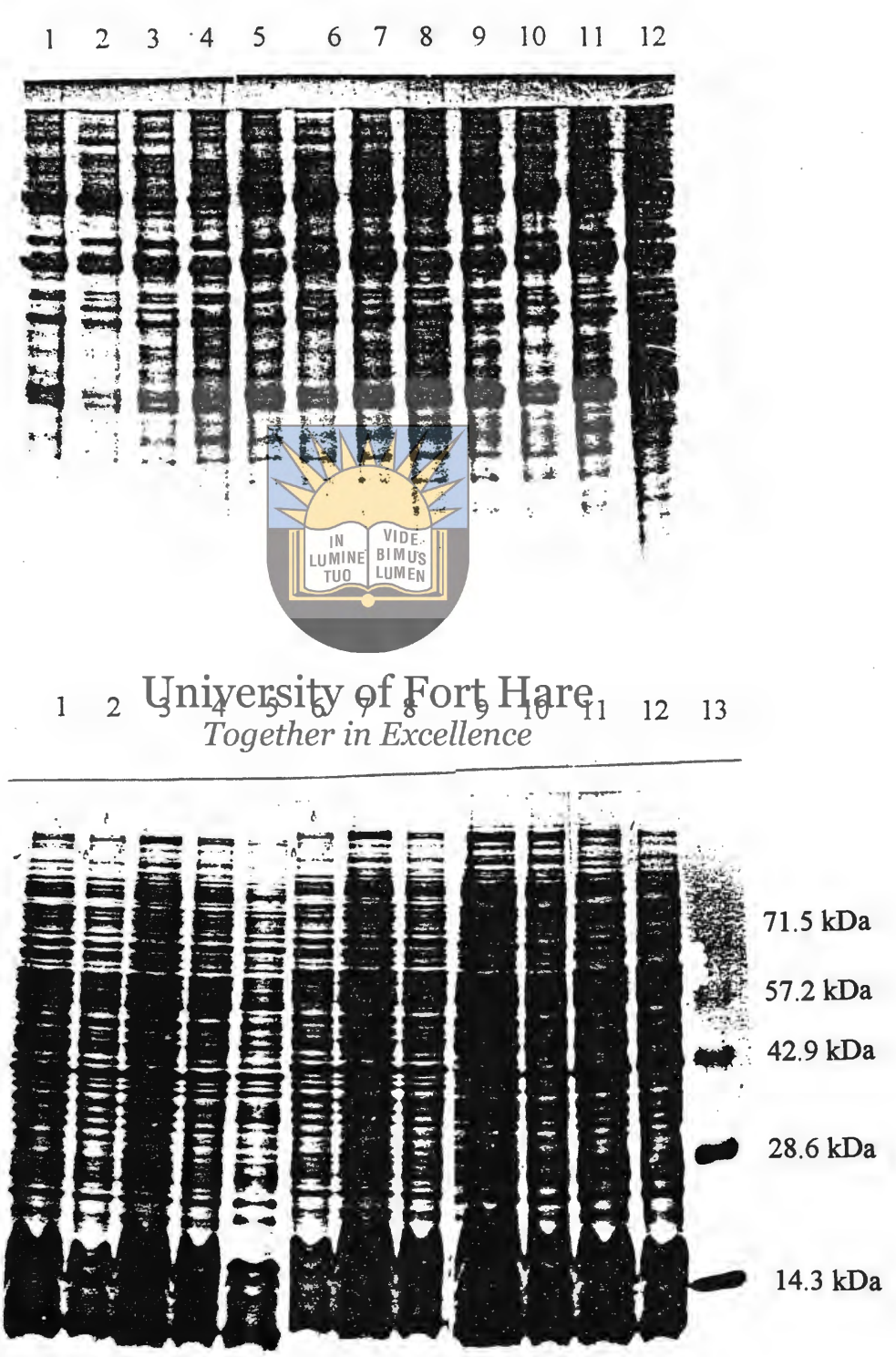


Fig. 25. PAGE of EC-C α-Galactosidase stained with Commassie brilliant blue. (A) Disc, (B) SDS. Lanes: 1, 3, 5, 7, 9 and 11= controls; Lanes: 2, 4, 6, 8, 10 and 12 = melibiose-induced and lane 13 = Mol. wt. stds (range: 14.300 - 71.500).

CHAPTER 4

DISCUSSION

E. chrysanthemi is normally unable to metabolize lactose. However, Lac^+ mutants are readily isolated by selective pressure on a poor medium containing lactose as a sole carbon source. This mutation was finally established to be a regulatory mutation allowing constitutive expression of the *mel* operon. It was demonstrated that lactose transport occurred via the melibiose permease (Gray et al., 1986).

The growth of *E. chrysanthemi* in both a complex and a minimal medium supplemented with different sugars was followed spectrophotometrically (Figs. 3 and 5) and the plate count method has been used to detect the number of viable bacterial colonies (Table 3). Growth was measured in parallel with viable colony counts determination. A corresponding but steady increase in the number of viable cells with growth rate (Fig. 4), indicated that a significant fraction of culture cells were actively growing and multiplying. Non viable bacterial cells are incapable of division because a sizeable fraction of these cells are dead cells (ghosts) and are therefore not recorded as viable by the plate count method. They can, however, maintain metabolic activities until they are lysed (Zweifel and Hagstrom, 1995). It is thought that a large proportion of the growing culture consists of these bacterial ghosts and that this was the major explanation for the low number of viable cells. These cells however, did contribute positively to the measure of growth spectrophotometrically (Fig. 4).

E. chrysanthemi can grow on melibiose, sucrose and raffinose. However, growth on raffinose was significantly slower than that achieved with melibiose and sucrose (Fig. 5). On the basis of doubling times and the K_m value, EC-C seems to have a low affinity for raffinose. Even lowering the concentration of raffinose did not alter this pattern (Fig. 6). It is known that raffinose is usually not a substrate of *E. coli* although the *raf* operon may be plasmid-borne in certain pathogenic strains (Schmid and Schmitt, 1976; Hardesty et al., 1987).

The low affinity displayed by this enzyme for raffinose was also reported for α-galactosidases produced by fungi (Rios et al., 1993; Zeilinger et al., 1993). Rios et al. (1993) noted that the secretion of α-galactosidase by a filamentous fungus *Aspergillus nidulans*, seems to be regulated by the carbon source used for growth.

Several media were tested for their suitability as indicator media for EC-C (Table 4). It was observed that using EMB as an indicator medium, colony spreading and slime production by EC-C was common (Table 4). Consequently, EMB was considered unsuitable as an indicator medium since it became very difficult to pick individual colonies. Although Tetrazolium agar supported clear and precise reactions, it was noted that the type and colour of colonies given by both EC-C and EC-S in this medium were very similar, particularly with sucrose and melibiose as carbon sources (Table 4). The most suitable indicator medium for EC-C was found to be MacConkey because it not only revealed precise clear reactions but gave different colony types and colours for EC-C and EC-S (Table 4).

The production of mutants has been achieved by the use of two mutagens, ICR 191 and EMS. High concentrations of ICR 191 are known to prevent growth of *E. coli* and low concentrations are insufficient for good mutagenesis (Miller, 1972). A concentration of 10 μg/ml was found to be the most effective dose for mutagenesis in EC-C since it allowed growth whilst inducing mutations (Table 5). An increase above this value resulted in total death of cells (Fig. 7). A collection of mutants unable to grow on either lactose, melibiose, sucrose or raffinose was isolated (Table 6) and each mutant scored for its ability to metabolize each of the other three sugars. Based on their phenotypes, the mutants were divided into a number of different classes, five after ICR 191 mutagenesis and seven after EMS mutagenesis. Each mutant was characterized by a defect in the metabolism of one or more of these sugars (Table 6). It is not clear why ICR 191 gave rise to five classes of mutants whereas EMS gave rise to seven classes of mutants.

Barletta and Curtiss III (1993) observed that α-galactosidase levels might be an important parameter in melibiose and raffinose use by *S. mutans* and measured the enzyme activity in both the wild-type and isolated mutants. A similar approach was adapted for *E. chrysanthemi*.

The isolated mutant strains exhibited different levels of α-galactosidase activity. All the melibiose and raffinose utilizing mutant strains showed an increase in α-galactosidase activity when these two sugars were used as inducers (Table 7). Both the wild-type and class 6 expressed similar melibiose and raffinose fermentation properties and displayed approximately the same level of increase (about 1.6 to 3.5 times the basal levels) of α-galactosidase activity in the presence of raffinose or melibiose with melibiose being a slightly better inducer (Table 7). The galactose-containing oligomers, melibiose and raffinose were also discovered to be good inducers of α-galactosidase in the soft-rot fungus, *Trichoderma reesei* (Zeilinger et al., 1993).

The inability of classes 3, 4 and 5 to utilize raffinose (Table 6) was consistent with low α-galactosidase levels obtained when raffinose was used as an inducer (Table 7). The mutant strains of these classes (3, 4 and 5) have defects in both the raffinose transport system and its hydrolyzing mechanisms (Table 10). In addition, melibiose did not induce α-galactosidase in strains FH 12, FH 101, FH 14 and FH 103 (Table 7). The two classes, 3 and 5 (Table 6), seem to have lost the mechanisms for the transport and hydrolysis of melibiose (Table 10). Both melibiose and raffinose induced significant levels of α-galactosidase activity in mutant strains FH 15 and FH 104 (Table 7). The positive phenotypes of the two strains when grown in the presence of these two sugars (Table 6), were indicative of this observation. In this particular class, both the *mel* and the *raf* operons seemed to be present (Table 10).

The mutant strain, FH 16 could utilize melibiose but not raffinose (Table 6), a result confirmed by low levels of α-galactosidase detected when raffinose was used as an inducer (Table 7). Further characterization of this mutant strain in the presence of X-gal and subsequent treatment with chloroform showed that the absence of a raffinose specific transport system was the reason for its inability to utilize raffinose (Table 10). This observation suggested the existence of different transport systems for raffinose and melibiose in EC-C. Schmid and Schmitt (1976), also reported the presence of different uptake mechanisms for melibiose and raffinose in *E. coli* mutants with an active presence of the *raf* system but defective in the melibiose uptake system.

The levels of α-galactosidase activity exhibited by the mutant strain FH 10 pointed to the constitutive expression of this enzyme in this particular strain (Table 7). Despite the fact that this strain could not utilize raffinose (Table 6), this observation was supported by the results which showed the presence of both the raffinose transport and hydrolyzing enzymes on incubation with X-gal (Table 10).

Both strains FH 11 and FH 100 have shown an inability to utilize either melibiose or raffinose (Table 6), but it is not clear why significant levels of α-galactosidase activity were obtained (Table 7), when these strains were grown on these sugars nor is it possible to explain why this class showed the presence of α-galactosidase but a lack of the transport system for these sugars (Table 10). In *E. coli*, raffinose metabolism was shown to be activated by a plasmid-encoded gene (Konishi et al., 1985; Muiznieks and Schmitt, 1994).

All of the strains tested were able to hydrolyze β-ONPG (Table 8), although only class 5 and class 7 mutants were Lac^+ (Table 6). This phenotype appears to require the constitutive expression of a melibiose permease that also recognizes and transports lactose (Gray et al., 1986). Therefore, the inability to metabolize lactose by the majority of the mutant strains could be attributed to a defect in the gene coding for the lactose transport system (permease). Gray et al. (1984; 1986), also showed that IPTG induced high levels of β-galactosidase activity in both the lactose non-utilizing EC-C and the lactose metabolizing EC-S strain. The reversion of EC-C to a Lac^+ phenotype was reported by Gray (1985). These Lac^+ revertants were isolated, routinely tested and found to be consistently Lac^+ on MacConkey lactose agar plates. Increased β-galactosidase activity was obtained with IPTG as an inducer (Table 9). It was also noted that the reversion to a Lac^+ phenotype had a bearing on the expression of α-galactosidase by these mutant strains. All showed a constitutive expression of α-galactosidase (Table 9).

In a recent study, Chadwick et al. (1995) discovered some possible errors in the assay of β-glycosidases activity when using glycosides of *p*-nitrophenol as substrates. It was discovered that under normal growth conditions enzyme activity was appreciably reduced compared to incubation under anaerobic conditions in the presence of excess 3,4-dichloronitrobenzene, a substrate for

nitroreductase, which significantly enhanced enzyme activity. Consequently, Chadwick et al. (1995), recommended that for the analysis of microbial β-glycosidases, an alternative substrate (other than glycosides of *p*-nitrophenol) whose metabolite is not susceptible to degradation by other enzymes, be selected.

Sucrose, raffinose and fructose have all been reported to induce both invertase and sucrose uptake activities in *B. subtilis* and *S. mutans* (Schmid et al., 1988; Steinmetz et al., 1989). In both these strains, sucrose was found to be transported by the PEP:PTS system and subsequently cleaved by invertase yielding glucose-6-phosphate and fructose as intracellular products (St. Martin and Wittenberger, 1979; Lunsford and Macrina, 1986). In addition, extracellular levansucrase has been purified from both the gram-positive (Igarashi et al., 1992; Burne and Penders, 1994) and the gram-negative (Hettwer et al., 1995) bacteria. In both strains, the purified enzyme was capable of degrading not only levan but also sucrose.

All sucrose utilizing mutants (Table 6) showed sucrose inducible invertase activity (Table 11). In contrast, fructose, unlike in *B. subtilis* (Robeson et al., 1983) and *S. mutans* (St. Martin and Wittenberger, 1979; Russell et al., 1985), did not induce invertase activity in the parent (EC-C) and the mutant strains (Table 11). In strains FH 15 and FH 104 (Table 6), invertase activity was induced by raffinose (Table 11), despite the fact that both of these strains were sucrose negative (Table 6). This finding indicated that the invertase induced by raffinose is different from the one induced by sucrose. Raffinose was taken up into the cell where it was broken down by a raffinose specific invertase into melibiose and fructose. Furthermore, the inability of these two strains to transport sucrose (Table 13), also implied that raffinose and sucrose were taken up by different transport systems in *E. chrysanthemi*. As with α-galactosidase (Table 7), invertase activity seemed to be constitutively expressed in strain FH 10 (Table 11).

Strain FH 100 showed an inability to utilize both raffinose and sucrose (Table 6). It cannot be explained why significant levels of invertase activity were induced by these sugars in this particular strain (Table 11).

The inability to metabolize sucrose in strains FH 11 and FH 102 (Table 11), could be attributed to the absence of a sucrose specific invertase only, since the presence of a sucrose uptake system in both strains was established (Table 12). All the other sucrose non-utilizing strains tested showed the absence of not only invertase (Table 11), but also the sucrose uptake system (Table 12). In a study of the role played by plasmid-encoded genes in the metabolism of sucrose by *E. coli* K-12, Schmid et al. (1982) suggested that fructose or a phosphorylated derivative was the actual inducer of the sucrose metabolic genes and that both sucrose and raffinose required hydrolysis by invertase before becoming inducers.

The characterization of all the mutant strains isolated, with respect to their ability to utilize the different sugars, showed defects in one or another of the enzymes required for the metabolism of the sugars (Table 13).

The enzyme, α-galactosidase catalyzes the hydrolysis of melibiose to glucose and galactose and raffinose to sucrose and galactose. However, in practice, it is more convenient to assay the enzyme using the synthetic substrate ONPG. Hydrolysis of ONPG results in the release of the coloured product, *o*-nitrophenol which, unlike the products of melibiose and raffinose hydrolysis, can be determined directly by spectrophotometry at 420 nm.

Kinetic properties of both EC-C α-galactosidase and invertase were determined using ONPG and sucrose as substrates respectively. These properties included pH optimum and stability, temperature optimum and stability, K_m and V_{max}. In order to obtain an accurate measure of the initial reaction rate (velocity), progress curves were constructed and their linearity with time determined (Figs. 9 and 12).

As with most enzymes, a characteristic bell-shaped curve with a pH optimum of 7.4 (Fig. 10) was obtained for α-galactosidase. The pH stability of this enzyme ranged between pH 6.4 and 8.0.

This enzyme exhibited a temperature optimum of 50°C and was stable at this temperature for an hour. Rapid inactivation of this enzyme occurred at temperatures above 50°C (Fig. 11). Both raffinose- and melibiose-induced α-galactosidase showed very similar kinetic properties (Table 14).

Invertase showed pH and temperature optima of 6.6 and 30°C respectively (Figs. 13 and 14), and was found to be stable over a pH range of 6.4 to 8.0 (Table 15).

The K_m and V_{max} were determined using the Lineweaver-Burk double reciprocal plot. The K_m values obtained for melibiose-induced (0.32 mM) and raffinose-induced (0.35 mM) α-galactosidase (Table 14), suggested that the same enzyme was responsible for the hydrolysis of both melibiose and raffinose in EC-C. The differences observed in invertase K_m values for sucrose and raffinose (Table 16) confirmed a higher degree of affinity for sucrose than for raffinose. This finding further explains the slow growth rate of EC-C obtained with raffinose as a carbon source (Fig. 5) and is consistent with the observation made by Rios et al. (1993) that the affinity decreases as the length of the substrate molecule increases.

The kinetic data of sucrose- and raffinose-induced invertase exhibited differences (Tables 15 and 16), indicating that the invertase induced by sucrose may be different from that induced by raffinose.

Both α-galactosidase and invertase activities were inhibited by SDS but not by DOC. Higher concentrations (0.05%) of SDS were required to inhibit invertase (Fig. 16), whilst α-galactosidase was sensitive to much lower concentrations (0.02%) of SDS (Fig. 15). This finding on the behaviour of these two enzymes with respect to SDS made it possible to assay for invertase while inhibiting α-galactosidase with SDS. This was evident when invertase from a raffinose grown EC-C culture was assayed for by TLC.

The detection of melibiose and fructose but not sucrose (lane 3 of Fig. 17), confirmed the inhibition of α-galactosidase but not invertase by SDS (Fig. 17). Hettwer et al. (1995) purified and characterized an extracellular levansucrase from *Pseudomonas syringae* which hydrolyzed levan and sucrose but not raffinose and was highly tolerant of denaturing agents such as SDS.

The periplasmic proteins of gram-negative bacteria usually comprise 10 to 15% of the total cell protein and are defined as those proteins which are released into the medium by mild osmotic shock. The separation of these proteins from the bulk of the cell protein is a very important step in characterizing many mutations which affect periplasmic proteins (Willis et al., 1974; Ames et al., 1984).

In *E. coli*, α-galactosidase is a cytoplasmic enzyme inducible by melibiose and raffinose and is subject to catabolite repression by other simple sugars. However, Wong (1990) reported an α-galactosidase in *Azotobacter vinelandii* which was found to be located outside the cell membrane (exocellularly). In addition, the expression of this enzyme was induced by monosaccharides such as galactose (Wong, 1990).

The localization of α- and β-galactosidase in the parent and mutant strains was investigated by employing the chloroform shock method described by Ames et al. (1984), and a modified osmotic shock method described by Willis et al. (1974). To release cytoplasmic proteins, toluene was used to permeabilize the cell membrane, and the permeabilized cells were subjected to sonication to free the membrane-bound proteins. The majority of proteins were found to be cytoplasmic in both the parent and the mutant strains (Figs. 19 and 20). High levels of both α- and β-galactosidase were detected in the cytoplasm with a fair amount found in the periplasm, with the exception of strain FH 16 (Figs. 21 and 22). These data are consistent with reports that β-galactosidase is known as a cytoplasmic protein in gram negative bacteria (Ames et al., 1984). In strain FH 16, a high proportion of α-galactosidase was localized in the periplasm (Fig. 21). It was also observed that a significant amount of protein in EC-C was membrane-bound (Fig. 23).

A comparison of the protein composition of the various mutant strains by PAGE failed to reveal the absence of any major protein bands in the strains tested (Fig. 25). An *in situ* method was developed for the screening of α- and β-galactosidase on Polyacrylamide gels using α-X-Gal and β-X-Gal to stain specifically for α- and β-galactosidase activity respectively. *E. coli* β-galactosidase was used as a standard and gave two distinct bands; one of higher molecular weight and the other of lower molecular weight. The mobility of the *E. chrysanthemi* β-galactosidase corresponded to the lower molecular weight *E. coli* β-galactosidase (Fig. 24).

All attempts to prove the presence or absence of α-galactosidase activity by incubating the gel with α-X-Gal failed even in the presence of both Mn^{2+} and NAD^{+}. An attempt to purify α-galactosidase by ion-exchange column chromatography was also unsuccessful as no active fractions could be recovered. The possibility exists that, despite being very unstable, this enzyme may be present in minute amounts in EC-C. In support of [illegible]gs, Zeilinger et al. (1993) encountered difficulties in trying to purify α-galactosidase produced by *Trichoderma reesei* because of its tendency to elute in broad rather than sharp peaks, resulting in low levels of recovery. In *E. coli*, α-galactosidase is reported to require the presence of both Mn^{2+} and NAD^{+} for its stability and activity enhancement (Burnstein and Kepes, 1971). Furthermore, Nagao et al. (1988) failed to achieve the purification of this enzyme due to its instability. Wong (1990) also discovered that the exocellular α-galactosidase found in *A. vinelandii* rapidly lost activity once isolated.

The various results obtained indicate that there is a great deal of overlap between the various pathways that are responsible for the metabolism of lactose, melibiose, raffinose and sucrose in *E. chrysanthemi*. A further complication is the apparent presence of these enzymes, viz. α-galactosidase, in both the cytoplasm and the periplasm (Figs. 21 and 23). The potential pathways for the metabolism of these sugars by *E. chrysanthemi*, which are supported by the data obtained on the various mutants, are outlined in Fig. 26.

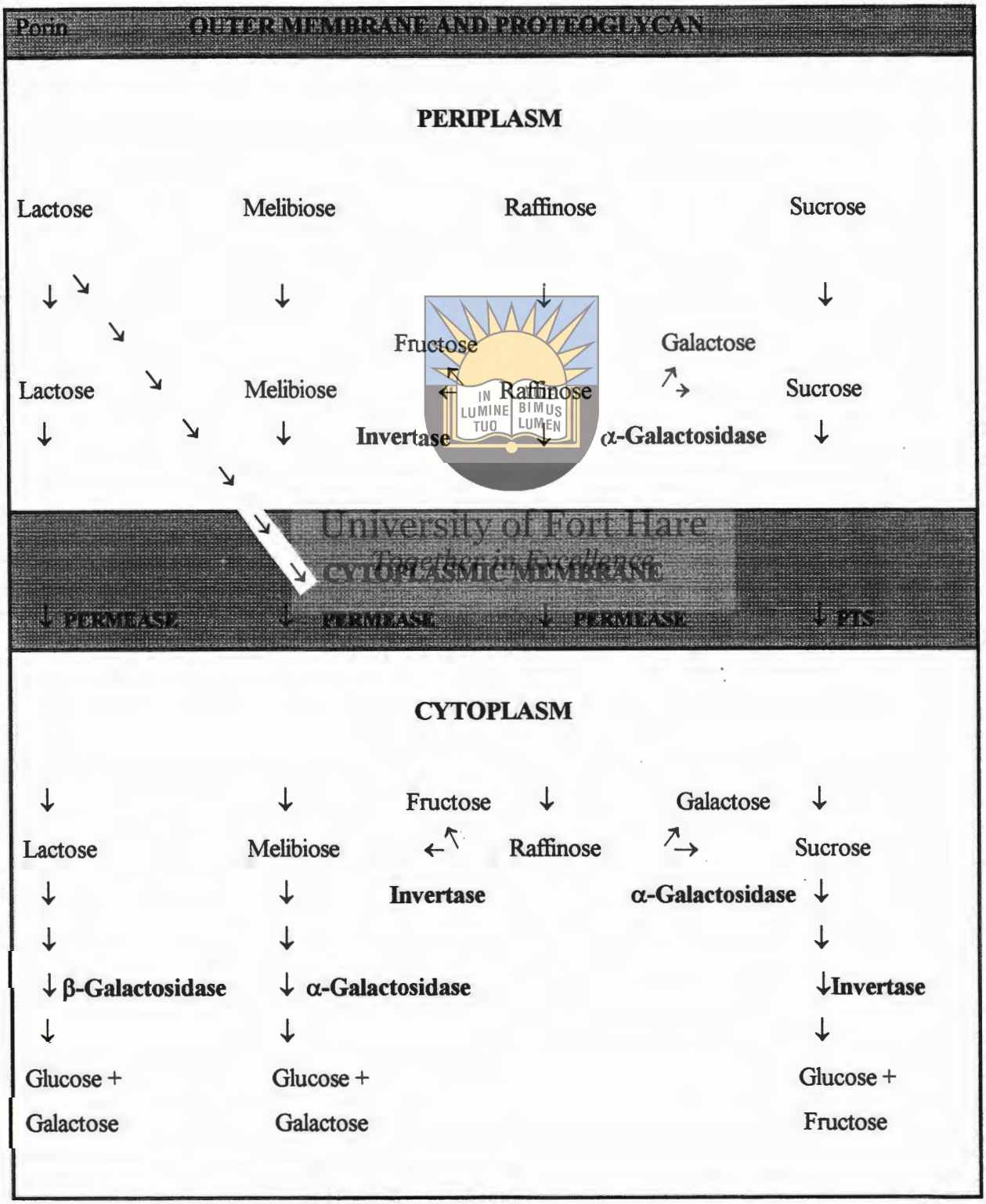


Fig. 26. Potential pathways for sugar metabolism by *E. chrysanthemi*.

SUMMARY

1. Growth in complex and minimal media.

i. Sucrose and melibiose seem to be preferred carbon sources as compared to raffinose (Fig. 5). It is possible that raffinose may first have to be broken down into its component disaccharides or monosaccharides which are fermented more readily by EC-C.

2. Reactions of EC-C on indicator plates.

i. EMB seems to support spreading of colonies and slime production by EC-C. This feature renders this indicator medium unsuitable for EC-C growth since it becomes very difficult to pick individual colonies (Table 4).

ii. Precise, clear reactions were obtained with Tetrazolium agar as an indicator medium (Table 4). Nevertheless, the type and colour of colonies given by EC-C in this particular medium was very similar to that given by EC-S, particularly with sucrose and melibiose as carbon sources.

iii. MacConkey was the indicator medium of choice since it not only revealed precise clear reactions, but gave different colony types and colours for EC-C and EC-S (Table 4).

3. Mutagenesis.

i. Different bacteria are affected differently by ICR 191 (Miller, 1972). For ICR 191 mutagenesis to be effective, a slow growing culture in the presence of this mutagen is preferred (Miller, 1972). A concentration of 10 μg/ml ICR 191 was found to be the most effective for mutagenesis in EC-C (Fig. 7).

ii. A collection of mutants unable to grow on either lactose, melibiose, sucrose or raffinose was isolated (Table 6). Each of these mutants was scored for its ability to ferment each of the other three sugars. Based on their phenotypes, the mutants were divided into seven and five classes after EMS and ICR 191 mutagenesis respectively. Each category was characterized by a defect in the metabolism of one or more of the sugars. It is not clear why only five classes were obtained with ICR 191.

4. Enzyme activities of isolated mutants.

The *mel* operon has two genes, *mel* A which codes for a melibiose hydrolyzing enzyme (α-galactosidase), and *mel* B which codes for a melibiose transport system (permease). On incubation with X-gal, the presence of the two functions is indicated by the appearance of blue to deep blue colonies. A defect in either of the two genes results in the appearance of pale blue colonies. In the absence of both the transport system and the hydrolyzing enzyme, white colonies appear.

4.1. α-Galactosidase activity.

i. The isolated mutant strains exhibited different levels of α-galactosidase activity (Table 7). Strains FH13, FH16 and FH102 showed an induction of α-galactosidase activity by melibiose, a characteristic which also confirmed the positive phenotypes of these mutants on melibiose (Table 6).

ii. The inability of class 4 to utilise raffinose (Table 6) is consistent with low α-galactosidase levels obtained when raffinose was used as an inducer (Table 7). This suggests the absence of both a raffinose inducible α-galactosidase and the transport system for this sugar (Table 10).

iii. Both melibiose and raffinose did not induce α-galactosidase in FH12, FH101, FH14 and FH103 (Table 7). The two classes, 3 and 5 (Table 6), seem to have lost the mechanisms required for transport and hydrolysis of the two sugars (Table 10).

iv. In strain FH104, the induction of α-galactosidase activity by both melibiose and raffinose was similar to the enzyme levels obtained with EC-C (Table 7). In this particular class, both melibiose and raffinose support growth due to the presence of the operons coding for the transport and hydrolysis of the two sugars (Table 10).

v. FH11 and FH100, which both belong to class 2, inexplicably exhibit significant levels of α-galactosidase activity (Table 7), although both mutants have shown an inability to utilise both melibiose and raffinose (Table 6).

4.2. β-Galactosidase activity.

i. Basal levels of β-galactosidase were obtained with glycerol as the only carbon source. However, high enzyme activities were recorded for all mutants tested when IPTG was used as an inducer (Table 8).

ii. The majority of both α- and β-galactosidase was detected in the cytoplasm (Figs. 21 and 22). In addition, substantial amounts of α-galactosidase were also detected in the periplasm (FH 16), whilst significant amounts of protein was found to be membrane bound (Fig. 23).

4.3. Lac^+ mutants.

i. The reversion of EC-C to a Lac^+ phenotype was reported by Gray (1985). EC-C mutants (Lac^-) were streaked on YS lactose agar plates and left to incubate at 30°C for 3 days. Large slimy colonies were isolated, purified by restreaking on YS lactose agar with EC-S and ECC as positive and negative controls respectively. All the mutants were found to be consistently Lac^+ on MacConkey lactose agar and the reversion to EC-S was also confirmed by assaying for β-galactosidase activity (Table 9).

4.4. Invertase activity and sucrose uptake.

i. All the sucrose utilizing mutants (Table 6) showed a sucrose inducible invertase activity (Table 11). Raffinose, when added as an inducer, also resulted in an increase in invertase activity in strain FH10 (Table 11).

ii. The absence of either sucrose transport or invertase activity or both in those strains unable to utilize sucrose (Table 13) is evident.

iii. The data obtained also indicated that the invertases induced by sucrose and raffinose are different.

iv. Fructose, although it has been reported to induce both the sucrose transport system and invertase in *B. subtilis* (Robeson et al., 1983) and in *S. mutans* (St. Martin and Wittenberger, 1979; Russell et al., 1985), did not induce invertase activity in EC-C and the mutant strains (Table 10).

v. Both α-galactosidase and invertase activities were inhibited by SDS but not by DOC (Figs. 15 and 16).

vi. Invertase showed a pH optimum of 6.6 (Fig. 16), whereas the pH optimum for α-galactosidase was 7.4 (Fig. 15). Both enzymes exhibited a broad pH stability.

4.5. Polyacrylamide gel electrophoresis (PAGE).

i. PAGE, undertaken to compare the protein composition of the various mutants, failed to reveal the absence of any major protein bands in the strains tested (Fig. 25).

ii. Incubation of the gel with a specific activity stain confirmed the presence of IPTG induced β-galactosidase activity in all the mutant strains isolated (Fig. 24). This further confirmed the results obtained with β-ONPG assays (Table 8).

iii. All attempts to prove the presence or absence of α-galactosidase activity by incubating the gel with an α-galactosidase specific activity stain failed even in the presence of both Mn^{2+} and NAD^{+}. A possibility exists that, besides being unstable, this enzyme may also be present in minute amounts in EC-C.

iv. *E. coli* α-galactosidase has been reported to require both Mn^{2+} and NAD^{+} for its stability and activity enhancement (Burnstein and Kepes, 1971). In addition, Nagao et al. (1988) failed to achieve the purification of this enzyme because of its instability.

CONCLUSION

E. chrysanthemi produces α-galactosidase, invertase and β-galactosidase in order to degrade melibiose, raffinose, sucrose and lactose.

The same α-galactosidase catalyzes the hydrolysis of melibiose to glucose and galactose and raffinose to sucrose and galactose. However, melibiose is transported by a different system from that of raffinose in this organism.

On the other hand, the enzymes that catalyze the uptake and subsequent degradation of sucrose are not the same as those that are involved in raffinose metabolism by EC-C.

The Lac ⁻ phenotype of EC-C is not due to the inability of β-galactosidase to hydrolyze lactose but can be attributed to a defect in the gene coding for the lactose transport system (permease).

Both α-galactosidase and invertase produced by *E. chrysanthemi* are sensitive to inhibition by sodium dodecyl sulphate (SDS) and insensitive to deoxycholate (DOC). α-Galactosidase is more susceptible to SDS inhibition than invertase. In addition, α-galactosidase produced by this organism has a low affinity for raffinose compared to melibiose.

The majority of α- and β-galactosidase are localized in the cytoplasm in *E. chrysanthemi* and this observation is indicative of gram-negative bacteria.

In addition to other plant carbohydrates, sucrose, melibiose, lactose and raffinose provide a nutritional resource for *E. chrysanthemi* and the ability of this organism to metabolize these sugars contributes to its pathogenecity.

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