CRANFIELD UNIVERSITY

INSTITUTE OF BIOSCIENCE AND TECHNOLOGY

Ph.D Thesis

Academic Year 2000-2004

SHANNON MOLLOY

Sugar transport and water relations of *Agaricus bisporus*

Supervisors:
Prof. Naresh Magan, Cranfield University
Dr. Kerry Burton, HRI Wellesbourne,

February 2004

ABSTRACT

The *A. bisporus* fruit body can develop from a 0.5 cm primordium to a 7 cm fruit body within 6 days and it is this rapid growth and expansion which was investigated. The mycelium and fruit body extract water and solutes from the compost and casing soil. Water and solute translocation is thought to occur by osmotically derived pressure driven mass flow whereby the accumulation of the polyol mannitol in the fruit body lowers the water potential and allows for an influx of water which increases the turgor pressure and creates a turgor gradient thus driving water and solutes into the mycelium. This work investigated the mechanism of water and sugar translocation through the mycelium and into the developing sporophore and the factors which affect this translocation path. The effect of different water stresses (osmotic and matric) on mycelial growth, osmotic, turgor and water potential and the accumulation of endogenous sugars and polyols when grown on two different media: a defined malt extract and compost derived was investigated. The concentration of various sugars and polyols was measured in the following sporophore tissue: cap, gills, skin and upper and lower stipe. A sugar transporter gene *SUT1* which had previously been identified in *A. bisporus* was fully characterised.

The mycelium was most sensitive to changes in matric than osmotic potential on malt extract agar (MEA) medium and there was no mycelial growth below -0.98 MPa on matrically modified medium but the mycelium continued to grow at water stresses of -1.48 and -2.48 MPa on osmotically modified MEA medium. However on compost derived medium less difference was found between osmotic and matric stresses. The following sugars: glucose and trehalose and the polyols: mannitol, glycerol and erythritol were quantified in the mycelium and mannitol was found to be the dominant polyol which increased with increasing water stress in the mycelium. However in mycelium grown on matrically modified compost media at -0.48 and -0.98 MPa the mannitol levels didn't increase with increasing water stress and this was reflected by the mycelium having a

lower internal water potential than the external medium indicating that the mycelial tolerance to matric stresses is linked to the components of the compost as no such tolerance was found on MEA medium.

Mannitol was the main polyol detected in all the sporophore tissues but there were no significant differences between the stages of sporophore development indicating that there was no change in the levels of mannitol. Previous reports by Hammond and Nichols (1976) and Wannet *et al*. (2000) have shown that the level of mannitol increased during sporophore development but the difference could be attributed to the casing soil which is now wetter and therefore the sporophore is not under increasing water stress and can extract water without having to constantly adjust its polyol concentration.

The SUT1 protein had a predicted molecular weight of 61.2 kDa and according to topology predictions had 12 transmembrane domains and a number of highly conserved regions which place it in the sugar transporter family of the major facilitator superfamily. The level of *SUT1* transcript increased during sporophore development and there were higher but equal levels of *SUT1* transcript in the upper and lower stipe compared to the cap, gills and skin tissue (indicated by Northern analysis). However no *SUT1* transcript could be detected in the mycelium supporting stage 2 and 4 sporophore development indicating that it is possibly involved in translocation in the fruit body but not in the mycelium. The detection of the SUT1 protein by Westerns was attempted using an antibody raised to the SUT1 C-terminal region. However the extraction and solubilisation of the membrane bound SUT1 protein proved very difficult and was not successfully detected by Westerns.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor Dr. Kerry Burton for his untiring enthusiasm, support and guidance throughout this project, without which this would never have been possible, thank you Kerry. I would also like to thank Dr. Sreenivasaprasad for his wonderful patience and teaching me so many techniques and Prof. Naresh Magan for his support and guidance throughout this project. I am very grateful to all the support staff at HRI including Margaret Jones in the graphics department, Rodney Edmondson for help with statistical analysis, Sylvie Elliott and Peter Brooks in the library for sourcing journals and allowing me to use the library as a base to write this thesis, and Neil Willoughby in the mushroom unit.

Finally I would like to thank all the people past and present at HRI who I have befriended and for making HRI such a wonderful environment in which to work. I would especially like to thank my lab group: Dan Eastwood, Emilie Combet and Nicola Browning for their friendship and for being a wonderful group to work with, I will miss you all.

LIST OF CONTENTS

LIST OF FIGURES

introns (in italics) and flanking regions are in lower case. The amino acid sequence is given underneath the coding regions in one letter code. The amino acid sequences of the 12 putative transmembrane domains (TMD) (deduced from the topology prediction model of the *SUT1* cDNA sequence) are boxed and duplicated motifs RFGRR and RFGRK repeated between TMD II and III and VIII and IX respectively are highlighted in red. Conserved motifs PESPRWL and PETSGFALE found immediately after the sixth and twelfth TMDs are highlighted in green. Seven introns italicised in lower case and numbered 1-7 were identified from the comparison of the cDNA (STIIE2) and genomic (20A11) sequences. Putative TAATA and CAAT sites are underlined in the promoter and terminator regions. ⏭ indicates start of the cDNA transcript, ⏵ indicates the poly A tail in the cDNA transcript and ⏴ indicates the end of the cDNA transcript. – indicates the stop codon. **150-152**

Figure 5.7: Southern blots showing the copy number of the *SUT1* gene. Genomic DNA was isolated from *Agaricus bisporus* PC1 homokaryotic strain and approximately 4 μg of PC1 DNA was digested with the following restriction enzymes: *Bam*HI (lane1), *Hin*dIII (lane 2), *Pst*I (lane 3), *Kpn*I (lane 4), *Xho*I (lane 5). The DNA fragments were separated on a 0.8 % agarose gel and transferred onto nylon membrane. Southern blot (A) was probed with ST1P2 (hybridisation temperature 65°C) which is a PCR amplified 0.2 kb region of *SUT1* cDNA STIIE2 which codes for the SUT1 hydrophilic carboxyl-terminus after transmembrane domain (TMD) XII and is highly specific to SUT1. Southern blot (B) was probed with ST1P1 (hybridisation temperature 55°C) which is a PCR amplified 0.11 kb region of STIIE2 that codes for a region from TMD IV to V and is conserved amongst the sugar transporter family of the MFS, therefore it is non-specific region of SUT1. The probes were labelled with ^{32}P α-

LIST OF TABLES

LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
bp	base pairs
cDNA	complimentary deoxyribonucleic acid
CE/CYM	compost extract/complete yeast extract medium
CHAPS	3[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMC	critical micelle concentration
C:M	chloroform: methanol ratio
cm	centimetres
CN	4-chloro-1-napthol
^{14}C	carbon 14
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra acetic acid
EtOH	ethanol
HMM	hidden Markov model
HoAc	acetic acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IMAC	immobilised metal affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
kbp	kilobase pairs
KCl	potassium chloride
kDa	kilodaltons
LB	Luria Bertani
LiCl	lithium chloride
M	molarity: moles per litre
MEA	malt extract agar
MEB	malt extract broth
MeOH	methanol
MFS	major facilitator superfamily
$MgCl_2$	magnesium chloride

$mgml^{-1}$	miligram per millilitre
ml	millilitres (10^{-6} m^3)
mm	millimetres (10^{-3} m)
mM	millimolar (10^{-3} M)
MPa	mega pascals
MtDH	mannitol dehydrogenase
MYPG	malt yeast peptone glucose
NaAC	sodium acetate
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
nM	nanomolar (10^{-9} M)
ng	nanogram (10^{-3} g)
N_2	nitrogen
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pET	plasmid for expression by T7 polymerase
psi	pound-force/sq. in. (lbf $in.^{-2}$)
PPP	pentose phosphate pathway
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SP	sugar porter
SSC	standard saline citrate
SUT1	sugar transporter 1
TBE	tris borate EDTA
TBS	tris buffered saline
TE	tris EDTA
TMD	transmembrane domain
UV	ultraviolet
V	volt

VM	vegetative mycelium
(w/v)	weight per volume
(v/v)	volume per volume
%	percentage
^{32}P α-CTP	alpha phosphate radiolabelled deoxy cytidine triphosphate
$\mu g ml^{-1}$	microgram per millilitre
µl	microlitres (10^{-9} m^3)
µM	micromolar (10^{-6} M)
µCi	microCurie (3.7 X10^7 Bq)
µg	microgram (10^{-6} g)
$^{\circ}$C	degree centigrade
Ψ_w	water potential
Ψπ	osmotic potential
Ψ_m	matric potential
Ψ_p	turgor potential

CHAPTER 1

GENERAL INTRODUCTION

1.1 HISTORY AND ECONOMIC IMPORTANCE OF MUSHROOMS

Mushroom production is one of the few large-scale commercial applications of microbial technology for the bioconversion of agricultural waste to valuable foods (Ohga & Kitamoto, 1997). Mushroom cultivation started in France in 1630 and began in England a century later (Atkins, 1983). In 1779, Abercrombie gave the first detailed information on commercial mushroom production involving the composting of horse dung and spawning (Spencer, 1985). In 1831 Callow showed that production was possible all year round in England if they were produced indoors. Mushroom production soon became established in other European countries and later spread to the USA. In 1997 the annual world production of mushrooms exceeded 4 million tons (Ohga & Kitamoto, 1997), with France, Holland and the United Kingdom being the main producers of mushrooms in Europe and worldwide the US and China are the largest mushroom producing countries (Chang & Miles, 1991).

Agaricus bisporus is the most important mushroom produced and is the largest produced mushroom in the world, representing 37.8% of the world production of cultivated mushrooms in 1990 (Chang & Miles, 1991). The origin of *A. bisporus* is assumed to be Europe (Kurtzman, 1997). The commercial mushrooms of Asia are becoming more popular in the West every year (Kurtzman, 1997) and this is reflected in the fact the world production of *Pleurotus* spp.(oyster mushrooms) increased from 7.7-24.2% from 1986-1989/90 and the world production of *A. bisporus* declined in the same period from 56.2-37.8% (of the total world production of mushrooms) (Chang & Miles, 1991). Mushrooms generate £137 million in the UK which makes them one of the most economically valuable horticultural crops (Table 1.1), however the mushroom industry has declined in the last 4 years and this has been due in part to the increasing imports of low cost product from Eastern Europe, particularly Poland (DEFRA, 2002/3). There has been an overall decline in the value of protected vegetables in the last 5/6 years.

Table. 1.1: Value of the home production market of protected vegetables in the UK (DEFRA, 2002/3)

Value of the Home Production Market (HMP)(£'000)										
Calendar Year	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002
Mushrooms	146,512	151,5321	162,243	171,295	168,741	174,456	170,397	149,545	150,298	137,448
Tomatoes, heated	59,268	68,883	61,864	82,047	60,900	63,144	66,318	84,326	78,740	76,649
Tomatoes, cold	5,391	5,697	4,147	3,691	1,422	823	874	624	350	436
Cucumbers	59,411	49,661	68,310	53,124	46,415	42,370	38,471	43,474	33,603	32,738
Lettuce	33,918	23,565	25,835	37,899	29,259	23,422	23,223	18,964	21,240	18,223
Self-blanching Celery	4,348	5,663	5,042	6,043	4,308	4,766	3,362	2,403	2,704	2,112
Sweet Peppers	3,916	4,628	6,699	5,362	6,215	6,908	8,527	6,566	9,031	7,181
Others	5,467	4,037	7,273	9,857	7,037	8,445	6,638	9,705	11,354	11,598
Total value of HPM	318,230	313,665	341,413	369,318	324,297	324,333	317,807	315,607	307,321	289,384

1.2 THE FUNGAL KINGDOM

Fungi have previously encompassed a single kingdom. However, with the advances of techniques such as molecular biology, biochemical and structural analysis, this classification is no longer accepted. The kingdom fungi has now been subdivided into three kingdoms, Chromista, Fungi and Protozoa, (Carlile, 2001; Kirk, 2001) and all three kingdoms are Eukaryotic. The kingdom Chromista consists of three phyla, oomycota or water moulds, which include many important plant pathogens, notably *Phytophthora infestans* which causes potato blight (Carlile, 2001). The two remaining phyla, hyphochytriomycota and labyrinthulomycota, are aquatic with motile spores (Carlile, 2001; Kirk, 2001). The kingdom Protozoa comprise the plasmodial slime moulds myxomycota and the cellular slime moulds acrasiomycota, dictyosteliomycota and plasmodiophoromycota (Kirk, 2001).

The Kingdom Fungi is made up of four phyla, **chytridiomycota**, **zygomycota**, **ascomycota**, and **basidiomycota** (Kirk, 2001). The **basidiomycota** consist of three classes, basidiomycetes, urediniomycetes and ustilaginomycetes. The ustilaginomycetes have three subclasses, which are then further divided into orders. One of the orders, the ustilaginales, more commonly known as *smut* fungi includes the genus ustilago, an economically important plant pathogen of cereals. The urediniomycetes have five orders, one of which is the uredinales more commonly known as the rust fungi that are pathogens of cereals and grasses. The basidiomycetes are divided into two subclasses based on the distinction between primary and adventitious septa (Kirk, 2001). The subclass agaricomycetidae have adventitious septa and contain the order agaricales. The *Agaricus* genus is a member of the order agaricales and includes the cultivated mushroom *A. bisporus*.

1.3 LIFE CYCLE OF AGARICALES

In basidiomycetes sexual reproduction involves the production of haploid basidiospores which are produced from diploid basidia in which the nucleus undergoes a meiotic division (Carlile, 2001). Of the basidiomycetes species studied so far 90% are heterothallic and the remaining 10% homothallic (Raper, 1966). In homothallic species individual uninucleate spores germinate to produce mycelia that are capable of producing mushrooms, the spores are self-fertile. In heterothallic species basidiospores germinate to produce a primary mycelium which is non-self fertile and is a monokaryon, having uninucleate cells and only one type of nucleus. Fertility is under the genetic control of mating-type factors. About 72% have a bifactorial system involving two mating-type factors A and B and 28% a unifactorial or bipolar system with a single mating-type factor (Elliott, 1985a). An interaction between two monokaryons with different alleles of mating-type factors will lead to fertility, e.g., A1B1 interacting with A2B2 but not compatible with A1B1, A1B2 or A2B1 or A1 interacting with A2 (Carlile, 2001). This interaction between two strains that are fully compatible will lead to the nuclei from each strain migrating through the mycelium of the other resulting in each compartment containing paired nuclei with a nucleus of each mating-type factor. This secondary mycelium is termed dikaryon and the migration process is termed dikaryotization (Carlile, 2001). All subsequent growth will involve the synchronous division of the two nuclei in each compartment and their regular distribution as nuclear pairs throughout the mycelium. The maintenance of this dikaryotic state is maintained by the use of clamp connections (Figure 1.1). Although clamp connections are considered diagnostic of basidiomycetes, they are not found in any species of *Agaricus* (Elliott, 1985a; Carlile, 2001). In fungi that have clamp connections, one nucleus in the dikaryotic cell moves into a backward sidebranch and the other nucleus remains in the hypha but close to the sidebranch (Figure 1.1). The nuclei within the sidebranch and hypha then divide and move closer to the apical cell. A cross-wall is

produced at the base of the side branch and across the hypha, resulting in a new cell containing two nuclei of two different mating-type factors near the apical cell (Carlile, 2001).

Fruit bodies of *Agaricus* spp. are formed from the secondary mycelium and the gills lining the fruit body are covered with basidia and paraphyses which act as spacers keeping basidia separate (Carlile, 2001). In each basidium nuclear fusion and meiosis takes place during the final stages of button development producing four nuclei. Basidiospore production only takes place once the gills are exposed and typically in basidiomycetes four basidiospores are produced from a basidium each of which receives a nucleus and the basidiospores are actively discharged and are termed ballistospores (Carlile, 2001).

A major difference between the life cycle of *A. bisporus* and that of the typical basidiomycetes is the fertility of spores. The majority of spores produced by *A. bisporus* form a self-fertile mycelium that is capable of producing fruit bodies without the interaction of mycelium bearing a different mating-type. *A. bisporus* has a single mating-type factor e.g. A1 and A2, and the basidium produces four nuclei which are of different mating-types. The majority of the basidia, about 95%, are two spored, but three and four spored basidia also exist (Figure 1.2), and receive nuclei of different mating-type which after germination will produce a fertile mycelium (Elliott, 1985a). The life cycle of *A. bisporus* is termed secondarily homothallic. The occurrence of spores containing nuclei of different mating-types is twice as likely as spores containing nuclei of the same mating-type. Therefore, after germination the majority of these spores will produce a fertile mycelium capable of producing a fruit body.

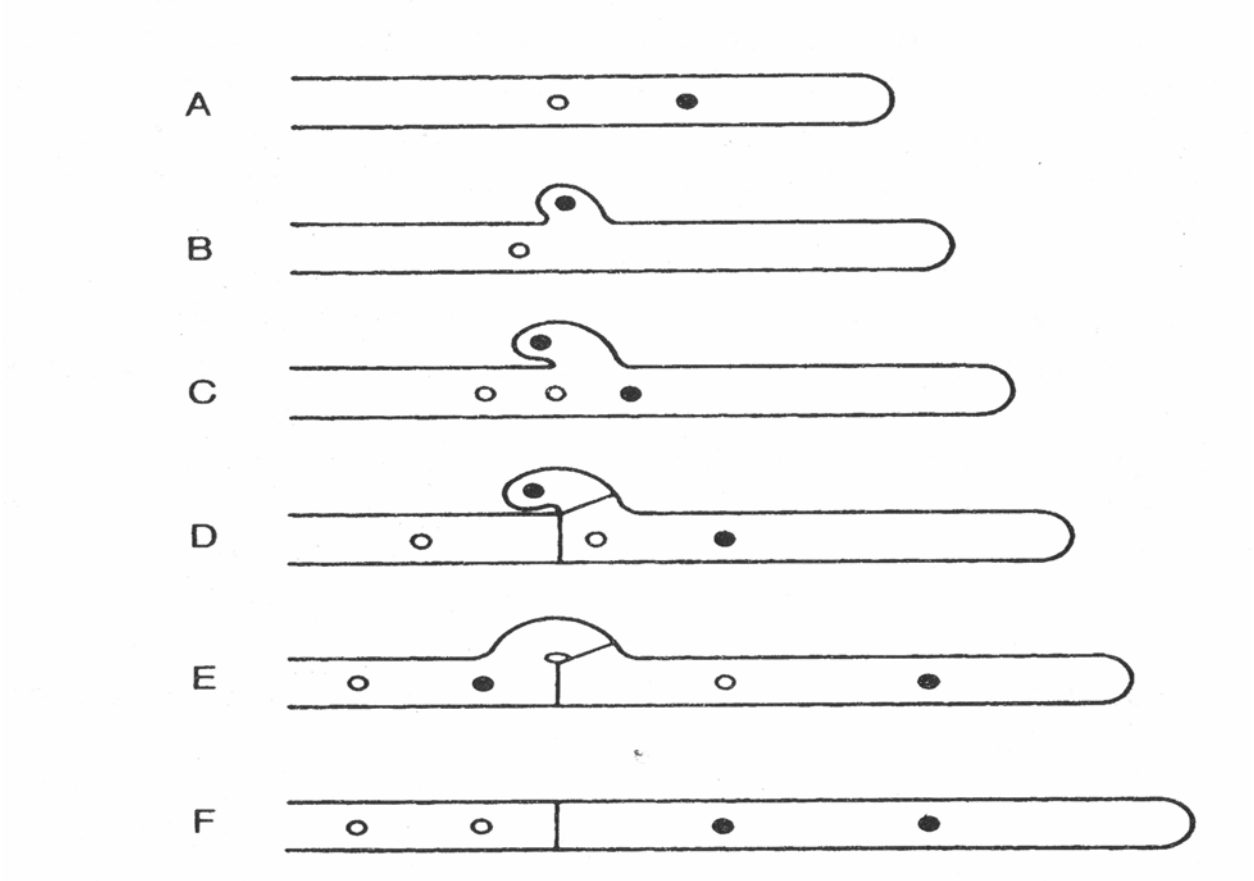


Figure 1.1: The formation of clamp connections in basidiomycetes demonstrating how the dikaryotic state of the mycelium is maintained (Carlile, 2001).

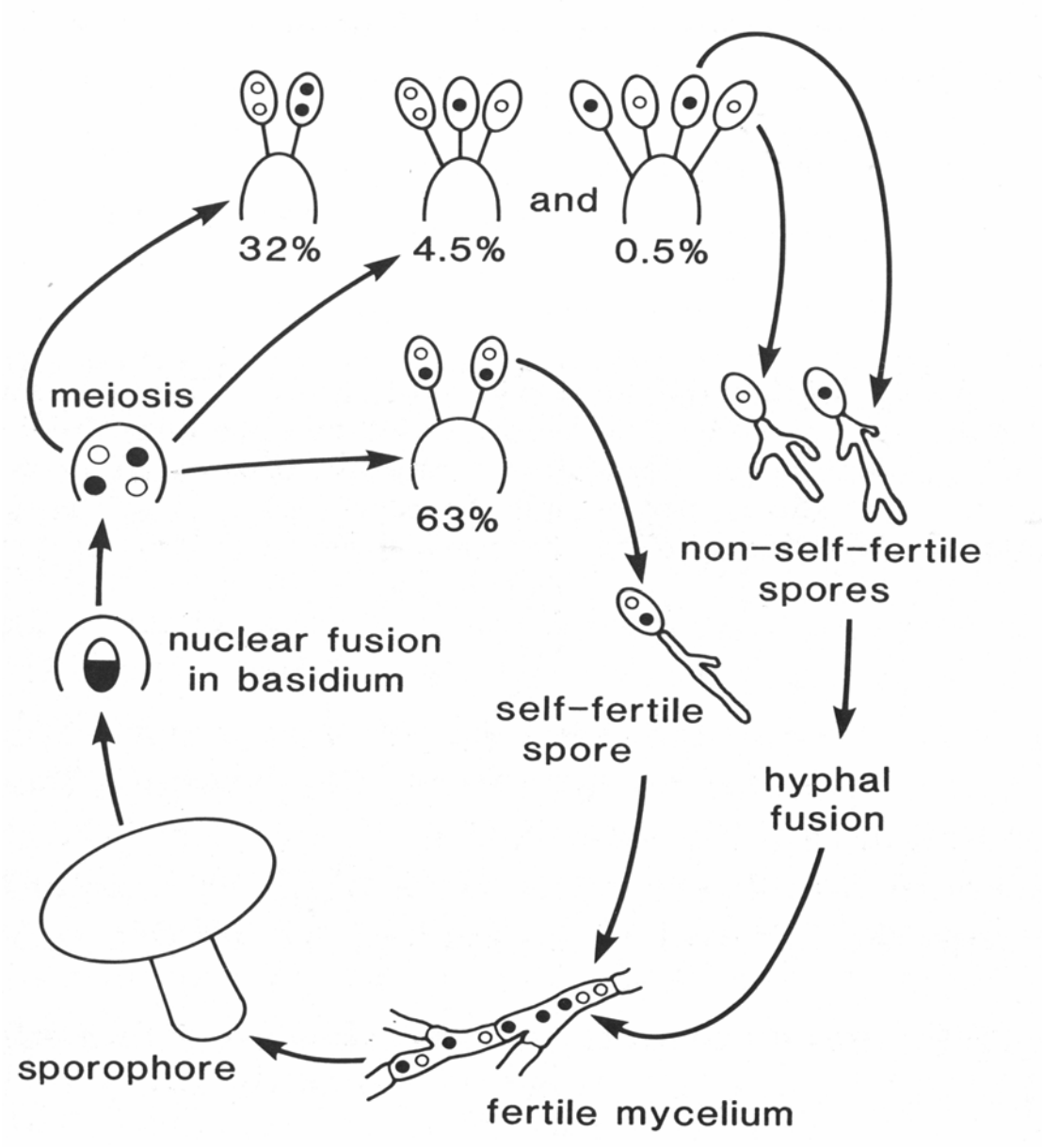


Figure 1.2: Life cycle of *Agaricus bisporus* showing the random migration of nuclei and the frequency of the aberrant basidia (Elliott, 1985a).

1.4 GROWTH AND DEVELOPMENT OF MUSHROOMS

1.4.1 Cultivation of *A. bisporus*

A. bisporus is grown on compost, which is derived from a mix of cereal straw (usually wheat), animal manure (horse or deep litter chicken) and water to which gypsum is added for structural stability and for stabilising the pH (Fermor *et al.*, 1985; Van Griensven, 1987). There are two phases involved in the composting procedure for *A. bisporus*, which is characterised by changing populations of micro-organisms. During phase I, which can be an outdoor or indoor composting procedure and usually takes around 10 days, the compost ingredients are mixed regularly with a composting machine to ensure uniform degradation of the compost and wetted to achieve 70-75% moisture content (Fermor *et al.*, 1985; Van Griensven, 1987). After the first three days of composting when a mixture of actinomycetes, microbial flora of bacteria and fungi have initiated the fermentation process, the temperature increases from 50–70°C and these organisms are displaced by thermophilic fungi like *Humicola insolens* var. *thermoidea* and *Torula thermophila* as well as thermophilic bacteria and actinomycetes proliferate (Fermor & Wood, 1981; Fermor *et al.*, 1985). Actinomycetes are present in large amounts at the end of phase I and give the compost the whitish grey appearance known as 'firefang'.

During the second phase of composting (phase II) thermophilic fungi proliferate especially *H.insolens* var. *thermoidea* together with thermophilic actinomycetes such as *Streptomyces thermovulgaris*, *Streptomyces* sp. B, *Thermoactinomyces vulgaris* and *Saccharomonospora viridis* (Fermor & Grant, 1985; Fermor *et al.*, 1985). This second phase of composting is carried out indoors where the compost is initially pasteurised (60°C) for 3-6 hours to rid the compost of mushroom pests and diseases after which the temperature is maintained at 45°C for 8-9 days (Fermor *et al.*, 1985). Straatsma & Samson (1993) examined isolates of the thermophilic fungi belonging to the *Torula-Humicola* complex found in mushroom compost and examined their growth promotion

ability of *A. bisporus* mycelium on sterilised phase II compost and their morphology. Straatsma & Samson (1993) found that *Scytalidium thermophilum* was the correct name for *H. insolens* var. *thermoidea* and *T. thermophila.* Straatsma *et al.* (1989) showed that mycelial growth of *A. bisporus* on sterilised phase II compost was greatly enhanced by pre-incubating it with the thermophilic fungus S. *thermophilum* and that the growth rate of *A. bisporus* was nearly as good as that with un-treated phase II compost. The thermophilic fungus *S*. *thermophilum* is important for compost selectivity, decreasing the ammonia concentration and enhancing the growth rate of mushroom mycelium (Straatsma *et al.*, 1989). Low yields of *A. bisporus* have been linked to low density of *S*. *thermophilum* and might be explained by the non-selective or toxic properties of the compost not counteracted by *S*. *thermophilum* (Straatsma *et al.*, 1989; Wiegant *et al.*, 1992). At temperatures greater than 55°C the dissipation of ammonia can be slow and make the compost unsuitable for *A. bisporus* by encouraging the growth of competitor fungi (Ross & Harris, 1983). Atkey & Wood (1983) showed that considerable breakdown of the cellular structure of cereal straw occurs during phase II and that some compost fungi and actinomycetes are capable of cellulose degradation. After composting, most of the carbon and nitrogen are in the form of polymers (polysaccharides, lignin and protein) embedded in a 'nitrogen-rich lignin-humus complex' (Gerrits *et al.*, 1967). Fermor & Grant (1985) found that at the end of composting the fungal and actinomycete mycelia represented 1.5% of the dry weight. These surface layers disappeared after colonisation of the compost by mushroom mycelium suggesting that this may be a nutrient source for *A. bisporus* and the microbial biomass can provide up to 10% of the nutritional requirement. However, the nitrogen content of the microbial biomass is high and since the micro-organisms will also concentrate minerals during composting, the biomass could act as a source of nitrogen and minerals (Fermor & Grant, 1985). The mycelium of the thermophilic fungus *H. insolens* var. *thermoidea*, a major component of the compost microbial biomass, contained glucose, glucosamine, galactose, mannose and traces of other

sugars (Fermor & Grant, 1985). During composting the indigenous microflora of the compost degraded lignin slightly but *A. bisporus* vegetative mycelium was found to degrade lignin by the activity of both laccase and manganese peroxidase while at the onset of fruiting a switch is made to cellulase activity (Wood & Goodenough, 1977; Wood & Leatham, 1983; Durrant *et al.*, 1991; Bonnen *et al.*, 1994; Ohga *et al.*, 1999).

Ross & Harris (1983) showed that there was an adverse effect on the selectivity of phase II compost caused by longer treatment of the compost at temperatures above 60°C which resulted in an increase in ammonia levels and by anti-microbial chemicals such as chloroform or ethanol which results in the death of microbial cells. After phase II the compost is ready to be inoculated with *A. bisporus*, in the form of millet or rye grain colonised with *A. bisporus* (spawning) (Elliott, 1985b). During the growth of the mycelium through the compost the temperature is maintained at 25°C and a relative humidity of 95% which is optimal for vegetative growth (Flegg & Wood, 1985). After 12-14 days when the compost has been completely colonised by the mycelium it is covered with the casing layer, which consists of moist peat and chalk or lime and one of its main functions is to act as a water barrier to protect the compost from desiccation and maintaining a sufficient humid climate around the sporophores (Flegg & Wood, 1985). It can also help to protect the compost from pests and diseases and to provide physical support for developing sporophores (Flegg & Wood, 1985; Van Griensven, 1987; Noble *et al.*, 1999). The temperature is then lowered to 20°C to ensure good growth into the casing layer. When the surface of the casing layer is completely colonised with the mycelium, after about 5-10 days, the mycelium is subject to environmental disturbance, a drop in temperature to 16-18°C and heavy watering and high humidity, which initiates sporophore production (Van Griensven, 1987). Following the production of the first mushrooms the crop appears in batches of fruit bodies known as flushes at intervals of 7-8 days (Flegg & Wood, 1985).

1.4.2 Sporophore tissue and developmental stages

The fruit body of basidiomycetes also known as a sporophore is the most complex differentiated structure formed by micro-organisms (Craig *et al.*, 1977). The fruit body of *A. bisporus* (Figure 1.3) produces and disperses large numbers of basidiospores and the macroscopic appearance of the fruit body is a reflection of the mechanism used to disperse the spores (Craig *et al.*, 1977). Fruit body formation begins as a knot of disorientated mass of hyphae which grows to a mycelial aggregate of cells (2 mm in diameter) (Craig *et al.*, 1977; Flegg & Wood, 1985). The aggregate develops to a fruit body primordium where differentiation into cap (pileus), gills and stipe occurs and this initial period of differentiation mainly involves cell division (Hagimoto, 1964; Craig *et al.*, 1977). The beginnings of the gill tissue can be discerned as an annulus developing within the primordium. Further development is characterised by gross expansion of the cap and gills and a rapid elongation of the stipe (Craig *et al.*, 1977).

The stipe is composed of vertically orientated hyphae and those of the cap are radially orientated. The function of the stipe is to support and raise the cap in a correct position for spore dispersal and to transport water and nutrients to the growing cap (Kamada, 1994). The gill tissue contains the basidia on which the spores are formed (Flegg & Wood, 1985). The mature gills consist of three regions; the trama, sub-hymenium and hymenium layer (Flegg & Wood, 1985). It is the hymenial surface, which produces the basidia onto which the basidiospores are formed and dispersed (Flegg & Wood, 1985). The gills only become visible at the veil-break stage of sporophore development and before then they are covered. The cap or pileus is shaped like an umbrella and the flesh of the pileus is indistinct and is known as the *pileus trama* and the outer surface of the pileus is the skin and is usually white or brown, has a smooth surface and is called the *pilei pellis* (Burton, 1988).

Hammond & Nichols (1976) divided sporophore development into 7 arbitrary stages (Figure 1.4). At stage 1 the sporophore emerges from the substrate and is referred to as a pinhead and is as small as 0.5 cm. Stage 2 sporophore is referred to as a closed button, where the cap is attached to the stipe by the velum that is intact but not visible. At stage 3 the velum is stretched and the gill tissue is not yet visible and the cap is about 2-3 cm in diameter. At stage 4 the velum has started to break revealing the gill tissue, which is covered by basidia. At this stage nuclear fusion and meiosis has taken place but not basidiospore production thus this is often referred to as the ‘veil break’ stage. At stage 5 the gills are fully exposed leaving a ring of tissue called the annulus where the veil was attached to the stipe. The region of the stipe above the annulus is termed the upper stipe and that below is termed the lower stipe. The mushroom cap is now cup shaped and the velum begins to curl underneath the rim of the cup. At stage 6 of sporophore development the cap is 4-6 cm in diameter. At the final stage of development ‘stage 7’ the gill surface is curving upwards.

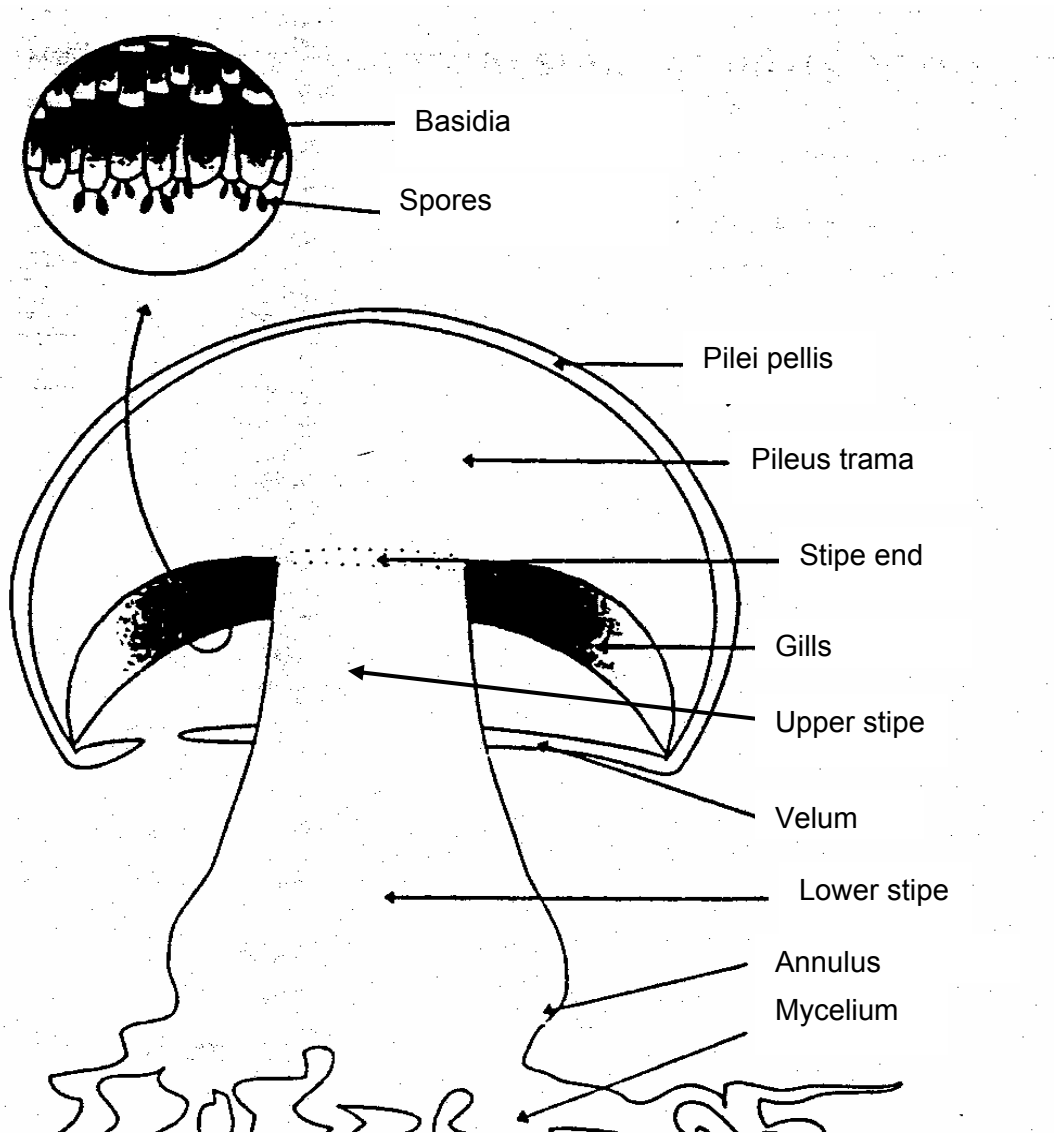


Figure 1.3: The fruit body of *Agaricus bisporus,* indicating the different structures/parts

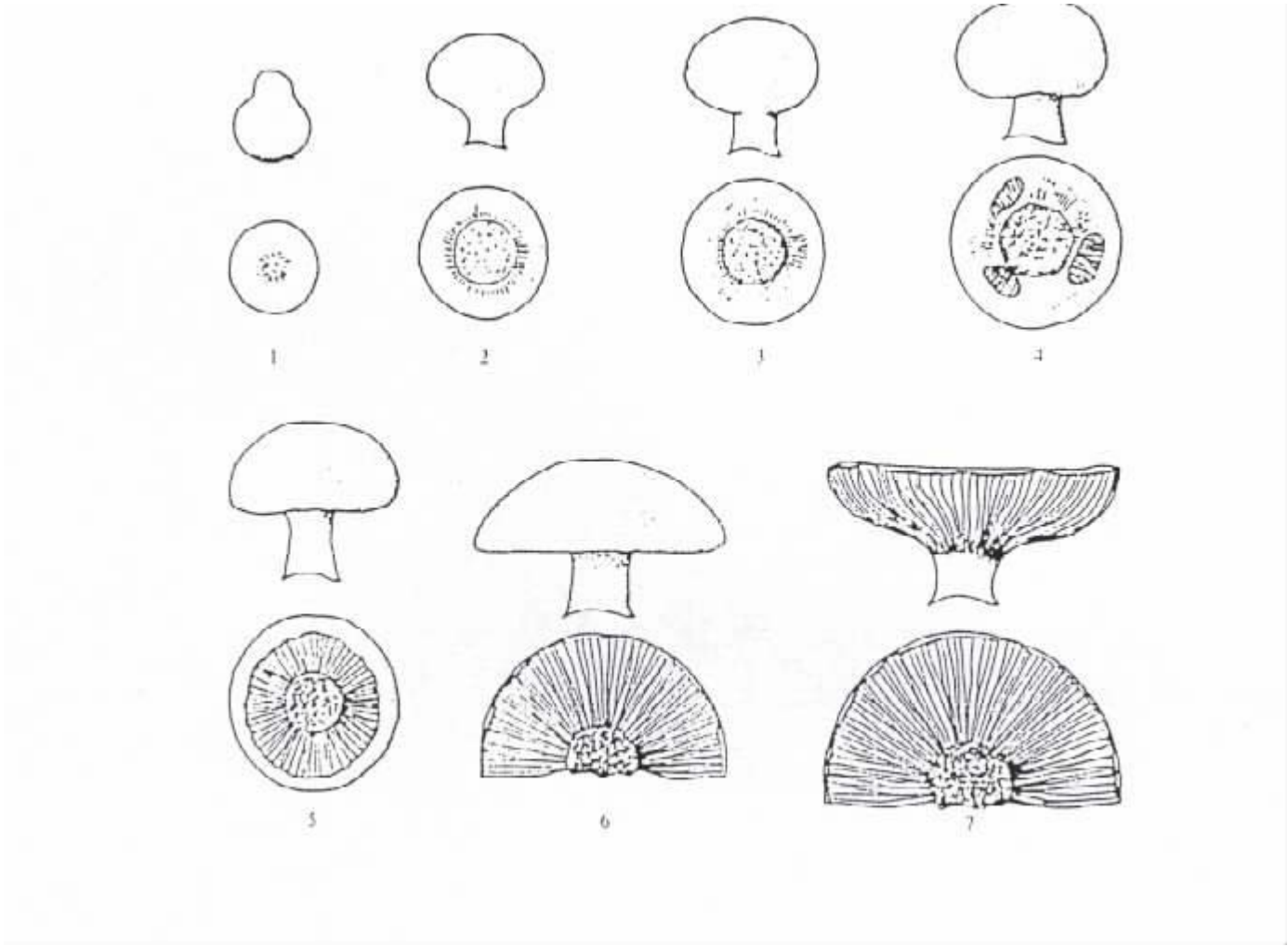


Figure 1.4: The appearance of the sporophore at stages 1-7 as described by Hammond & Nichols (1976)

1.4.3 Fungal hyphal structure

The hypha is the characteristic growth form of the fungal cells and allows the fungus to explore and exploit new environments (Gooday, 1995). The vegetative phase of the mushroom is the mycelium and this is made up of filaments called hyphae while the mushroom itself is composed of hyphae differentiated into tissue types (Elliott, 1985c). The hyphal cells of many basidiomycetes including *A. bisporus* are septate and have a complex septal structure called the dolipore septum (Moore & Marchant, 1972). Each separate cell contains all the organelles for independent growth (Elliott, 1985c). The mycelia of *A. bisporus* are multinucleate while most basidiomycetes are binucleate and this binucleate state is maintained by clamp-connections, which are not present in *Agaricus* species. The hyphal cells are highly vacuolated and the cytoplasmic strands between vacuoles contain organelles such as nuclei, mitochondria and endoplasmic reticulum (Kamada, 1994).

The hyphal cell walls of *A. bisporus* consist of 78% polysaccharides, 10% lipids, and 10.2% proteins and the major components of the polysaccharides are glucose, N-acetylglucosamine and glucosamine (Novaes-Ledieu & Mendoza, 1981). The hyphal polysaccharides consist mainly of glucan (44.8%) and glucosaminoglycan (chitin) (35.6%) (Mol & Wessels, 1990). Chitin is a polysaccharide of N-acetylglucosamine residues linked by β-1-4, glycosidic linkages, and also contained in the exoskeletons of insects and crustaceans. Craig *et al.* (1977) showed that new N-acetylglucosamine is incorporated into the cell walls and septa during elongation of the stipe and can be found all over the surface of the elongating cells. There is a rapid increase in stipe length during sporophore development and in the pileus greatest expansion takes place at its margin while little or no expansion takes place in the central region (Hagimoto, 1964). Chitin polymerisation is bought about by the enzyme chitin synthase and the activity of this enzyme increased during rapid elongation of the stipe and greater activity was found in the upper region of

the stipe where most elongation occurred (Craig *et al.*, 1981). A chitin synthase gene *chs1* has been cloned and characterised in *A. bisporus* and was shown to belong to the class III family of chitin synthase genes which are important in mycelial growth in other fungi (Sreenivasaprasad *et al.*, 2000a). In *A. bisporus* the transcript of this *chs1* gene was detected during fruit body development but could not be detected in vegetative mycelium indicating a developmental role. However, no difference in transcript levels could be detected between the button, veil break stage or the upper and lower stipe and cap tissue of the veil break stage (Sreenivasaprasad *et al.*, 2000a). The increase in stipe length during sporophore growth is largely accounted for by cell elongation (Hagimoto, 1964) but Craig *et al.* (1977) investigated elongation in the stipe by measuring the length of cells in pre-marked regions of the stipe from base to top of the same mushroom at two different stages of development (stage 2 and 6). Craig *et al.* (1977) found that elongation of the stipe in the lower and mid-stipe region was paralleled by an almost equivalent increase in cell length but the increase in upper stipe length was not paralleled by an increase in the length of the cells in that region and therefore implied that cell division also took place in the upper stipe region.

Filamentous fungi have cross-walls which subdivide their mycelia into compartments and these are known as septa (Markham, 1994). The septal structures differ according to the fungal species but nearly all species have septal perforations that permit some degree of cytoplasmic streaming and organelle migration between adjacent compartments (Markham, 1994). These perforations are called septal pores and can be plugged by electron dense materials in response to hyphal damage thus confining damage to a single compartment or permitting differentiation to occur and for compartments to develop. Ascomycete and deuteromycete fungi have specific organelles, which occlude the septal pores called Woronin bodies. These are electron dense, membrane-bound, proteinaceous spherical or ovoid organelles, which are located close to the septal pore. These organelles are capable of plugging the septal pore in both

damaged and undamaged hyphae. However, there is a low level of septal plugging observed in undamaged mycelia compared to damaged mycelia (Markham & Collinge, 1987). In *Penicillium chrysogenum* between 90 and 100% of pores were plugged in the vicinity of ruptured hyphae showing that pore plugging with Woronin bodies is a rapid and specific response to hyphal damage (Collinge & Markham, 1985). Although Woronin bodies are generally found as spherical structures they can also be found as crystalline hexagonal structures in a limited number of species including *Neurospora crassa* (Trinci & Collinge, 1974; Markham, 1994). In a small number of ascomycetes highly elongated membrane bound crystalline bodies have been identified close to the septal pore and these organelles share similarities with Woronin bodies although their function in plugging septal pores is unclear due to the fact that their diameter is smaller than the septal pore (Kimbrough, 1991; Turnau *et al.*, 1993). They have been found to occlude the septal pore in association with other electron dense material or to act as a cluster of 3-4 crystalloids above the septal pore (Kimbrough, 1991; Turnau *et al.*, 1993).

The septal structure of most basidiomycetes is a complex structure called the dolipore septum, which consists of two barrel shaped dolipores, with an orifice/pore (Moore & Marchant, 1972). On either side of the dolipores are bracket shaped membranous structures called parenthesomes which overarch the dolipore swellings and pores on both sides. The parenthesome consists of a differentiated membranous structure which is derived from the endoplasmic reticulum and often remains connected to it (Moore & Marchant, 1972). While basidiomycetes possess highly differentiated septal structures they lack pre-formed structures akin to Woronin bodies but they do produce electron dense material which plugs the septal pore in damaged hyphae (Casselton *et al.*, 1971; Moore & Marchant, 1972; Aylmore *et al.*, 1984). The plugging of septal pores in basidiomycetes was investigated by Aylmore *et al.* (1984) using *Coriolus versicolor* found that at either end of the dolipore channel the deposition of electron dense material in damaged hyphae occurred. The electron dense material was not membrane bound and

was approximately ovoid or spherical in shape and this material was deposited in order to occlude the pore and was progressively deposited until the dolipore channel was completely filled.

1.5 BIOCHEMICAL CHANGES DURING GROWTH

1.5.1 Extracellular degradation of carbohydrate polymers found in compost

After completion of the composting procedure the compost mainly consists of two principle nutritional components, lignocellulose and microbial biomass (De Groot *et al.*, 1998b; Whiteford *et al.*, 2000). Lignocellulose consists of lignin, cellulose and hemicellulose in a 3:3:1 ratio (Whiteford *et al.*, 2000). The lignin fraction encrusts cellulose and is chemically bound to hemicellulose making the compost a poor substrate for micro-organisms other than *A. bisporus* (De Groot *et al.*, 1998a). Utilisation of the compost requires that *A. bisporus* produces a range of enzymes capable of degrading insoluble polymers found in compost and it has been shown to produce a range of extracellular enzymes capable of breaking down these polymers. The degradation of the polysaccharide cellulose, which is an important potential carbon source for *A. bisporus*, has been shown to involve the degrading enzymes: endoglucanases, cellobiohydrolases and β-glucosidases (Wood & Goodenough, 1977; Raguz *et al.*, 1992; Chow *et al.*, 1994; Yague *et al.*, 1997). Endoglucanases cleave β-1-4 linkages of the cellulose chains at random. Exoglucanases mainly occur as cellobiohydrolases, removing cellobiose units from the non-reducing ends of cellulose chains, which are hydrolysed by β-glucosidases yielding glucose units.

Laccase and manganese peroxidase (MnP) are both known to participate in lignin degradation by *A. bisporus* (Bonnen *et al.*, 1994). The enzymes, laccase and cellulase which degrade lignin and cellulose respectively, have been extensively studied including their enzymatic properties, physiological role and protein structure and the gene

products controlling these enzymes have been identified. The other major constituent of compost is hemicellulose, which contains xylan a complex polymer of β-1-4 linked xylosyl residues (De Groot *et al.*, 1998a). Complete enzymatic conversion of xylan into monomeric sugars requires the concerted action of several enzymes (Whiteford *et al.*, 2000). The most important of these enzymes are endo-β-1,4-D-xylanase and β-xylosidase (exoxylanase) which have been found in *A. bisporus* compost extracts (De Groot *et al.*, 1998b; Whiteford *et al.*, 2000). While only limited information is available on the enzymatic activity of xylan degrading enzymes the activity of both endoxylanase and β-xylosidase has been detected in compost during fruit body formation of *A. bisporus* (Whiteford *et al.*, 2000).

Durrant *et al.* (1991) measured the lignin, cellulose and hemicellulose content of axenic mushroom compost and found that the lignin fraction was degraded primarily during the vegetative growth phase and the onset of fruiting markedly reduced this degradation. Cellulose and hemicellulose were degraded preferentially after the addition of the casing layer and at the onset of fruiting. Turner *et al.* (1975) and Wood & Goodenough (1977) showed that mycelium colonised compost had low levels of cellulase activity but that this increased with the onset of fruiting while laccase activity increased in the compost until just after the pinning stage when it sharply decreased. Claydon *et al.* (1988) also found low levels of endocellulase activity in compost samples up to the point of fruit body production but this increased sharply as fruiting commenced and endocellulase activity was closely related to fruit body biomass and peaked during each flush of mushrooms and fell sharply as harvesting of each flush was completed. This correlation between fruiting and cellulase activity was also reported by Ohga *et al*. (1999) who found that cellulase activity increased at the onset of fruiting up to the veil break stage of sporophore development. The relationship between fruit body biomass and endocellulase activity might influence the transfer of cellulose derived sugars such as glucose at a time of rapid growth of the fruit body. The precise role of laccases in lignin

degradation is unknown but it is thought to function through the oxidation of phenolics. The activities of two peroxidases, lignin peroxidase (LP) and manganese peroxidase (MnP), which are known to catalyse the depolymerisation of lignin in the white-rot fungus *Phanerochaete chrysosporium,* were investigated in compost colonised by *A. bisporus* (Bonnen *et al.*, 1994). The activity of MnP and laccase in mushroom colonised compost showed a similar trend, where they increased up to the pinning stage of fruit body development and declined at the onset of fruiting but no LP activity could be detected (Bonnen *et al.*, 1994). Both laccase and MnP seemed to be developmentally regulated and paralleled a loss of lignin prior to the onset of fruiting (Durrant *et al.*, 1991; Bonnen *et al.*, 1994). In compost culture endoxylanase activity increased slightly up to fruit body development and the largest increase in activity came after the veil break stage. However, there was very little exoxylanase activity up to the button stage of development but after this there was a large increase in activity of this enzyme (Whiteford *et al.*, 2000). This activity of endo- and exoxylanase enzymes at the onset of fruiting correlated with the decrease in hemicellulose during the cultivation of *A. bisporus* (Durrant *et al.*, 1991; Whiteford *et al.*, 2000).

The activity of both xylanase and cellulase enzymes was shown to be developmentally regulated with both enzymes increasing at the onset of fruit body development while laccase activity increased up to the pinning stage but then sharply declined (Claydon *et al.*, 1988; Bonnen *et al.*, 1994; Whiteford *et al.*, 2000). The understanding of these enzyme activities at the molecular level was undertaken with the isolation of genes encoding these enzymes and the investigation of their developmental regulation. An endo-glucanase antibody was used to isolate cellulase related genes by screening of a cDNA library made from 15 day old cellulose grown mycelium. This resulted in the identification of four cDNA clones designated *cel*1-4 and three were identified as cellulose induced genes (Chow *et al.*, 1994; Yague *et al.*, 1997; Tang *et al.*, 2001). Of these, *cel*2 and *cel*3 showed similarity to fungal cellobiohydrolase I and

cellobiohydrolase II, respectively and a *cel4* was found to be homologous to fungal mannanases, but *cel*1 showed no homology to cellulose degrading enzymes (Chow *et al.*, 1994; Yague *et al.*, 1997; Tang *et al.*, 2001;). Northern analysis showed that *cel*3 gene expression was induced by crystalline cellulose and repressed by glucose, fructose and lactose (Chow *et al.*, 1994) and this pattern of expression was similar for *cel*2 and *4* (Yague *et al.*, 1997). The transcription of *cel3* gene was about 13 times higher in cellulose grown cultures than in glucose grown cultures (Chow *et al.*, 1994). Ohga *et al.* (1999) found that the *cel3* gene from *A. bisporus* was developmentally regulated and was barely detectable in the mycelium prior to fruiting but increased as fruiting commenced reaching a maximum at the veil break.

In order to gain a better understanding of the hemicellulolytic activities associated with *A. bisporus* in the utilisation of compost a xylanase encoding gene *xlnA* was isolated (De Groot *et al.*, 1998b). The regulation of this gene was investigated in mycelium grown on phase II compost agar which was supplemented with glucose and no *xlnA* transcript could be detected (De Groot *et al.*, 1998b). However, after transfer of the mycelium to medium supplemented with phase II compost there was a constant and strong induction of *xlnA* but when transferred to medium supplemented with cellulose, xylan, xylose or glycerol there was a transient increase in the transcript levels of *xlnA*. This same pattern of *xlnA* expression and repression was observed with *cel*3 gene which codes for a cellobiohydrolase II (De Groot *et al.*, 1998b). This indicated that compost contains compounds that strongly induce expression of *xlnA* and *cel3*, which are not mimicked by other carbon sources. The identification of a laccase gene in *A. bisporus* resulted from the screening of a cDNA library from 11 day old mycelium with an anti-laccase antibody and resulted in the identification of two laccase genes *lcc1* and *lcc2* which differ in sequence only by 8.6% of the coding region (Smith *et al.*, 1998). Northern analysis showed that expression of the *lcc2* was greatest in colonised culture before fruiting but was undetectable after the onset of fruiting (Ohga *et al.*, 1999).

Examination of the genes coding for the cellulase, xylanase and laccase enzymes revealed that they are all developmentally regulated. Laccase accumulated during mycelial growth but declined at the onset of fruiting and both xylanase and cellulase increased at the onset of fruiting. It has been postulated that the role of cellulase is to supply carbohydrates to the mycelium where they can be translocated to the fruit body at a time of rapid growth.

1.5.2 Intracellular Carbohydrate Metabolism

Trehalose and mannitol are the principal carbohydrates produced by species within the kingdom Fungi (Jennings, 1984; Pfyffer *et al.*, 1990; Griffin, 1994; Hounsa *et al.*, 1998). Therefore studies on *A. bisporus* carbon metabolism have been mainly focused on the abundantly synthesised disaccharide trehalose and the sugar alcohol mannitol (polyol) because they are the main non-structural carbohydrates. Mannitol accumulates to high levels during sporophore growth (30-50% dry weight) but declines rapidly after sporulation while trehalose is present at lower levels and decreases during sporophore development from 5 to 1% dry weight (Hammond & Nichols, 1976). Mannitol is thought to act as an osmoticum during sporophore development where it accumulates and functions in creating a difference in osmotic pressure (or osmotic potential) between the sporophore and mycelium thus driving a net influx of water into the cell and increases the turgor pressure causing cell expansion and fruit body growth. Trehalose is thought to be the major sugar translocated from the mycelium to the growing sporophore where it acts as a carbon source (Hammond & Nichols, 1976).

1.5.2.1 Metabolism of mannitol

Mannitol, a six carbon sugar alcohol is produced in *A. bisporus* as the main storage carbon where it accumulates in both the pileus and stipe from 30 to 50% of the dry weight

between stages 2 and 7 of sporophore development (Hammond & Nichols, 1976). However, in the gill tissue it decreased from 23 to 14% between stages 2 and 7 while in the mycelium mannitol represented only 3-4% of the dry weight (Hammond & Nichols, 1976). Similar levels of mannitol were found during axenic fruiting of *A. bisporus* where it accumulated in aggregates 2-5 times higher than the surrounding mycelium (Wannet *et al.*, 1999) indicating that mannitol was not translocated from the mycelium to the sporophore as this would take place against a concentration gradient (Hammond & Nichols, 1976; Stoop & Mooibroek, 1998). Mannitol is produced in *A. bisporus* as a result of the reduction of fructose by mannitol dehydrogenase (MtDH) that uses NADPH as a coenzyme (Figure 1.5). NADPH production is regulated by the pentose phosphate pathway (PPP) via the enzyme glucose 6-phosphate (G6P) dehydrogenase which catalyses the dehydrogenation of glucose 6-phosphate (Edmundowicz & Wriston, 1963). The difference in mannitol level between the gill tissue and the rest of the sporophore might be related to the different function of the gills, which are concerned with spore production. High levels of biosynthesis are associated with spore production and it could utilise NADPH which might otherwise be required for mannitol synthesis (Hammond & Nichols, 1976). MtDH activity was found to be similar in both the mycelium and stage 2 sporophore (Hammond, 1977) but Wannet *et al.* (1999) found that the activity of this enzyme increased during aggregate development and was in fact 2-3 times higher than the supporting mycelium. MtDH activity increased during sporophore development and was in excess of the amount necessary to catalyse the reduction of fructose to mannitol. Thus a deficiency of this enzyme alone cannot be responsible for the low levels of mannitol found in the mycelium (Morton *et al.*, 1985b; Stoop & Mooibroek, 1998). The possible control points in the synthesis of mannitol are the supply of NADPH from the PPP, the supply of the enzyme MtDH which catalyses the reduction of fructose or as suggested by Morton *et al.* (1985a) the removal of the phosphate from fructose-6-

phosphate catalysed by the enzyme fructose-6-phosphatase which is a likely step as it is the control point for the supply of the substrate fructose.

The enzyme glucose-6-phosphate dehydrogenase is a key enzyme in the pentose phosphate pathway, which acts on glucose 6-phosphate and produces NADPH the coenzyme used in the conversion of fructose to mannitol, and is a possible control point in the production of mannitol. Hammond (1977) and Minamide & Hammond (1985) observed that G-6-P dehydrogenase activity was mainly confined to the fruit body and was in phase with the flushing cycle and the activity remained low in the mycelium supporting the sporophores. Wannet *et al.* (1999) found a similar trend in the activity of G-6-P dehydrogenase which increased in aggregates during axenic fruiting and was double that compared to the mycelium. This increase in G-6-P dehydrogenase the first enzyme in the PPP suggests an increase in activity of the PPP during sporophore development and a constant supply of NADPH for the reduction of fructose to mannitol by MtDH. The NADPH and NADP ratios were calculated using the anabolic reduction charge (ARC) which is calculated via the NADPH/(NADPH+$NADP^+$) ratio (Hammond, 1985). Hammond (1985) found that the ARC value showed a negative correlation with the mannitol content, thus the ARC is higher when the mannitol content is low. This indicates that the NADPH is unlikely to be the main controlling factor in the synthesis of mannitol.

The supply of free fructose as discussed earlier is a likely regulatory factor in the synthesis of mannitol. Fructose is produced from fructose 6-phosphate by the removal of a phosphate by an acid phosphatase and Hammond (1985) and Morton *et al.* (1985a) suggested that this dephosphorylating reaction could be a possible control point in the pathway to mannitol production as it is the first step. Hammond & Nichols (1977) observed the metabolism of ^{14}C hexoses when applied to the mycelium and sporophore of *A. bisporus*. When ^{14}C fructose was applied to both the mycelium and growing sporophore it was readily converted into mannitol. This is not surprising as Hammond (1977) found MtDH activity was similar in the sporophore and mycelium however low

levels of mannitol have been found in the mycelium compared to the growing sporophore suggesting that this enzyme is not a controlling factor in the production of mannitol. However when ^{14}C labelled fructose-6-phosphate was applied to the sporophore a lower proportion was incorporated into mannitol. This may be due to competition between the enzymes fructose-6-phosphatase and phosphofructokinase where a proportion of fructose-6-phosphate enters the glycolysis pathway compared to its use in the synthesis of mannitol but this would depend on the ATP status of the cell (Hammond & Nichols, 1977).

Stoop & Mooibroek (1998) cloned and characterised MtDH cDNA from *A. bisporus* and found the mannitol transcript level was higher in salt-stressed mushrooms (150 mM NaCl was added to casing layer) than in non-stressed mushrooms. Salt-stressed mushrooms would require larger amounts of mannitol to increase the osmotic potential and provide an influx of water into the cell and increase turgor and drive cell expansion than non salt-stressed mushrooms. The mannitol levels increased rapidly during early development and then remained relatively constant in non salt-stressed sporophores. Salt-stressed sporophores accumulated larger amounts of mannitol representing up to 60% of the dry weight. The specific activity of MtDH was greater in salt-stressed than non-stressed mushrooms along with an increased MtDH protein level. The MtDH transcript level was higher in salt-stressed fruit bodies than in non-stressed fruit bodies but it seemed to be under developmental control as the mRNA accumulation increased during fruit body development in both stressed and non-stressed mushrooms. Kalberer (1990) also found that adding salts to the casing layer of growing mushrooms resulted in an increase in osmotically active substances such as mannitol and a decrease in fruit body development and yield. He suggested that mushroom sporophores accumulated higher mannitol levels when salt was added thus acting as an osmoticum and enhancing the water potential gradient. Therefore it is likely that mannitol functions as an osmoregulatory compound and provides a continuous influx of water from the compost

to support turgor and fruit body development. Mannitol is unlikely to be transported from the mycelium but is synthesised in the sporophore as the MtDH activity is greater in the sporophore compared to the mycelium.

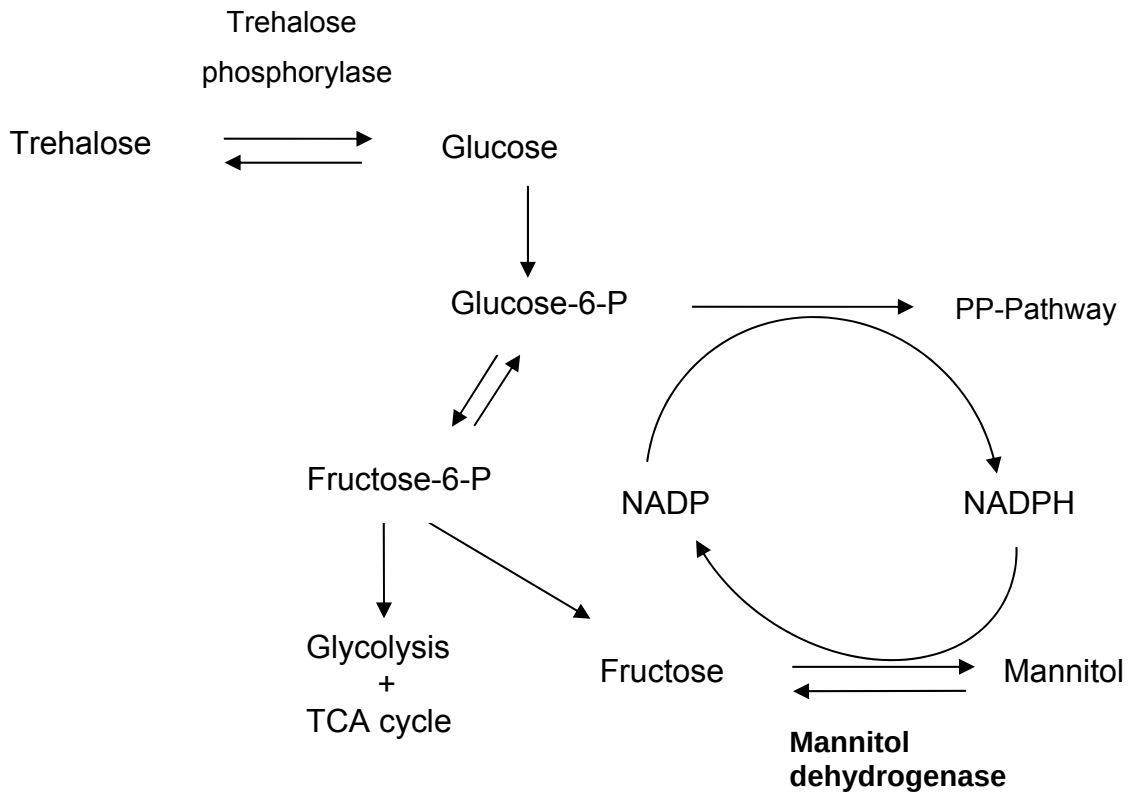


Figure1.5: Pathway of carbohydrate routing in *Agaricus bisporus* (Wannet, 1999)

1.5.2.2 Metabolism of trehalose

Trehalose is a non-reducing disaccharide (α-D-glucopyranosyl-α-D-glucopyranoside) and consists of two α-linked glucose sugars and in *A. bisporus* it is thought to serve as a reserve carbohydrate which is degraded under specific conditions, e.g. fructification, and has been suggested as a possible sugar translocated from the mycelium to the sporophore (Wells *et al.*, 1987; Wannet *et al.*, 1999). For a long time trehalose in fungi was thought to function as a storage compound but recently it has been found to function as a stress protectant. In S*accharomyces cerevisiae* a link was found between the induction of genes responsible for trehalose synthesis and stress conditions such as heat, dehydration and radiation (Parrou *et al.*, 1997). In *Escherichia coli* trehalose is synthesised under osmotic stress and is utilised by the enzyme trehalase yielding a carbon source, ultimately glucose (Horlacher *et al.*, 1996). Hammond & Nichols (1976) found that unlike mannitol, trehalose is found at similar levels in *A. bisporus* fruit bodies and mycelium but the level of trehalose in fruit body and mycelium fell during fruiting body development from 5% dry weight at stage 2 to 1-2% dry weight at stage 7. In *Flammulina velutipes* trehalose was found to disappear from the mycelium during fruit body development and was suggested to be the major translocation carbohydrate from mycelia to fruit bodies (Kitamoto & Gruen, 1976). This is a possible role for trehalose in *A. bisporus* also, where in the fruit body it can be used as a potential carbon source. Hammond & Nichols (1979) examined the levels of trehalose in *A. bisporus* fruit bodies during the flushing cycle and found that the amount of trehalose reached a maximum between flushes and decreased during flush growth. He hypothesised that the same trend might be seen in the mycelium as this pattern was in accordance with the trehalase enzyme activity observed by Wells *et al.* (1987). Wannet *et al.* (1999) observed similar patterns of trehalose levels during axenic periodic fruiting of *A. bisporus* where there were higher levels of trehalose in mycelium between fruiting and during fruiting the trehalose

levels dropped in the mycelium and aggregates. Synthesis of trehalose in bacteria and yeasts occurs predominantly via the trehalose synthase complex (trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase) and the hydrolytic cleavage of trehalose occurs via acid and neutral trehalases (Figure 1.6) (Wannet *et al.*, 1998). Wells *et al.* (1987) found activity of a presumed trehalase in *A. bisporus* sporophores which showed highest activity during the peak of each flushing cycle and minimum activity was found during the interflush period. This increase in trehalase activity coincided with a decrease in the corresponding substrate, trehalose, during the flushing cycle (Hammond & Nichols, 1979; Wells *et al.*, 1987).

An alternative for trehalose synthesis and degradation is provided by the enzyme trehalose phosphorylase which catalyses the degradation of trehalose and phosphate into one glucose molecule and one α-glucose1-phosphate molecule. The enzyme trehalose phosphorylase is a unique enzyme in trehalose metabolism from the following two standpoints (1) it can catalyse both the degradation (phosphorylation) and synthesis of trehalose and (2) it can synthesise trehalose without the formation of trehalose-6-phosphate (Aisaka & Masuda, 1995). So far trehalose phosphorylase activity has been found in a limited number of bacteria and fungi including the yeast, *Pichia fermentans*, the green alga *Euglena gracilis*, and the basidiomycete *F. velutipes* (Belocopitow & Marechal, 1970; Kitamoto *et al.*, 1988; Schick *et al.*, 1995). In *F. velutipes* trehalose phosphorylase activity was detected in both the sporophore and supporting mycelium together with trehalase activity (Kitamoto *et al.*, 1988), where the activity of both enzymes could be detected in the mycelium with higher activity of trehalose phosphorylase than trehalase. However, trehalose phosphorylase activity was 12 fold higher in the fruit body than trehalase indicating that trehalose phosphorylase acts in the degradation of trehalose in the fruit body (Kitamoto *et al.*, 1988).

Wannet *et al.* (1998) found no trehalose synthase or trehalase activity in *A. bisporus*, as had previously been reported by Wells *et al.* (1987), but purified and

characterised a trehalose phosphorylase enzyme. The synthetic activity of trehalose phosphorylase was found to increase in the mycelium during the interflush period of axenic fruiting of *A. bisporus* (Wannet *et al.*, 1999) which coincided with an increase in the amount of trehalose observed (Hammond & Nichols, 1979). During fruit body formation the degradative activity of trehalose phosphorylase was 2-3 times higher in fruit bodies than the mycelium and the level of trehalose decreased in the mycelium during this period (Wannet *et al.*, 1999). The rapid decline of trehalose during each flush coincided with the increasing degradative activity of trehalose phosphorylase and the release of hexoses during sporophore development for glycolysis and the pentose phosphate pathway which supplies NADPH as a coenzyme for mannitol synthesis.

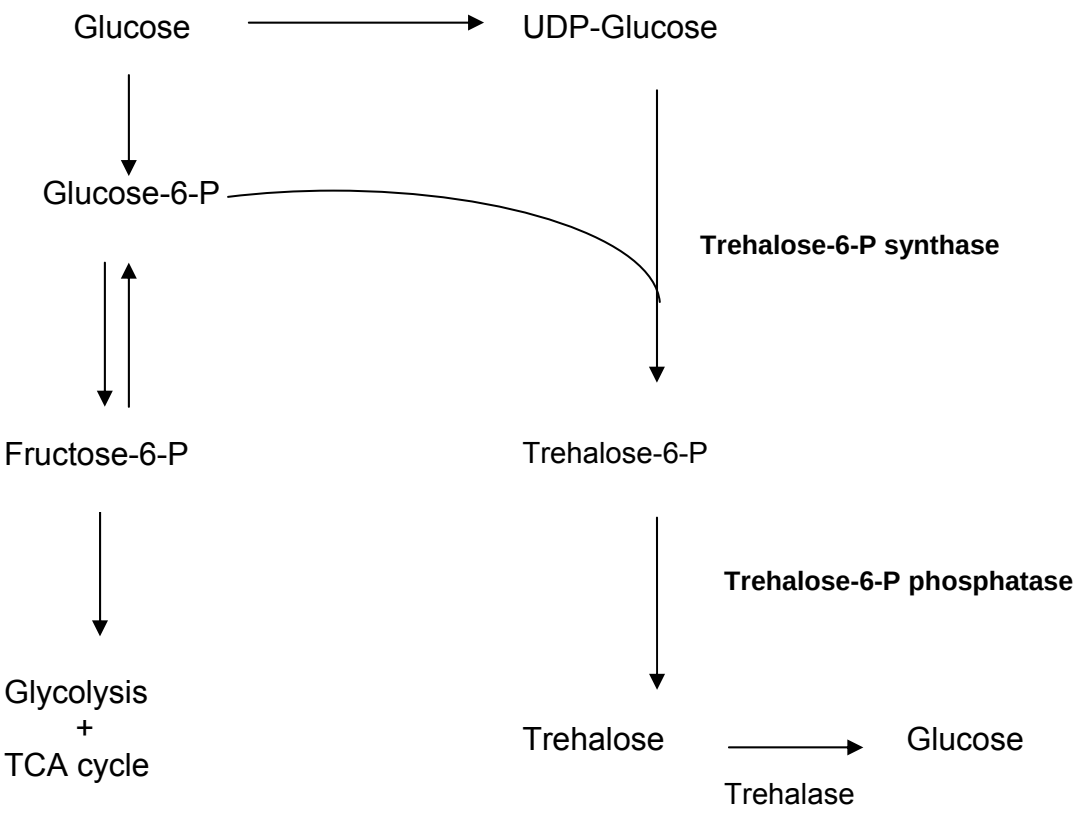


Figure 1.6: Scheme representing a typical trehalose synthesis and degradation in fungi (Wannet, 1999)

1.6 WATER MOVEMENT IN FUNGI (TRANSLOCATION)

The growth of the sporophore to a significant height above ground is an example of the ability of fungal mycelium to translocate nutrients over long distances (Jennings, 1987). However, the mechanism of transport within these organs has received little attention and not much is known about translocation in fungi while it has been extensively studied in plants. Translocation is the process by which nutrients are moved along the hyphae to other parts of the colony (Jennings, 1994). The developing fruit body, unlike the vegetative mycelium, is not in contact with the nutrient substratum and must therefore depend for its structural materials and energy source upon a translocating stream ascending from the stipe (Plunkett, 1958). All fungi translocate nutrients in their hyphae but some fungi produce conspicuous differentiated organs for bulk transport across nutrient-free environments. Depending on their structure and mode these are termed mycelial cords or rhizomorphs.

1.6.1 Water potential

Mushroom sporophores contain between 90 and 95% water (90% for the gill and 92-95% for the cap and stipe) and therefore water is a vital constituent of the growing sporophores (Kalberer, 1985; Donker & Van As, 1999). The growing sporophores obtain water from the casing and compost layers. Fruit bodies can obtain 54-83% of their water from the substrate and 17-46% from the casing layer (Kalberer, 1985). Only a percentage of the total water content of the substrate is available to the fruit bodies for growth and the rest is bound to the chemical structure of the substrate and is unavailable (Magan, 1997). Therefore water content is not a very good measure of water availability. Water activity (a_w) is a better measure of water availability for microbial activity (Scott, 1957). Thus a_w is the ratio between the vapour pressure of water in a substrate (P) and the vapour pressure of pure water(P_o) at the same temperature and pressure and is represented by the

equation: $a_w = P/P_o$ (Scott, 1957). A substrate containing little free water has a smaller vapour pressure than pure water and its a_w is consequently less. An alternative to measure a_w is to measure the water potential (Ψ_w) which is the sum of the osmotic (Ψ_π), matric (Ψ_m) and turgor (Ψ_p) potentials and is measured in pascals (Pa) and is related to the a_w by the following equation:

$$\text{Water potential } (\Psi) = RT/V \log_n a_w (+P)$$

R = the ideal gas constant

T= the absolute temperature (degrees Kelvin, or K)

P= the atmospheric pressure

V is the volume of 1mol of water

and provides an accurate measure of the available water (Magan, 1997).

In plant-water relations the following equation is used to describe the water potential (Passioura, 1980).

$$\Psi = P - \pi + \tau$$

However the components of the water potential can be represented by symbols where P is equal to Ψ_p the turgor pressure or hydrostatic pressure, - π is equal to Ψ_π the solute or osmotic potential and τ is equal to Ψ_m the matric potential.

Thus the water potential (Ψ_w) can be written as follows:

$$\Psi_w = \Psi_\pi + \Psi_m + \Psi_p$$

where Ψ_π= osmotic potential, Ψ_m= matric potential and Ψ_p = turgor potential (Griffin, 1981).

The osmotic potential Ψ_π is often referred to as the osmotic pressure but is equal in magnitude but opposite in sign and refers to the effect of dissolved solutes on Ψ_w and is given by the equation –RTC where R is the gas constant, T is the absolute temperature (degrees Kelvin, or K) and C_s is the osmolality of the solution. The osmolality is a measure of the concentration of dissolved solutes in a solution regardless of the

molecular species or mass. Therefore the osmotic potential –RTC is equal to - π and the negative value refers to the fact that the addition of solutes lowers the water potential thus having a negative effect (Money, 1994).

Osmolyte accumulation (dissolved osmotically–active substances) can create a differential osmotic pressure (osmotic potential) that drives a net influx of water into the cell and turgor pressure Ψ_p is generated when the plasma membrane is forced against the inner surface of the cell wall (Davis *et al.*, 2000). The difference in hydrostatic pressure between the cell and its surroundings is called turgor or pressure potential which is generally thought to be responsible for hyphal growth and for maintaining the volume, form and rigidity of fungal cells in sporophores by internal pressure (hydrostatic) on the cells walls (Money, 1994).

The matric potential Ψ_m is the reduction in water potential due to the interaction of water with the surface of a solid phase such as cell walls and soil particles. PEG of high molecular weight in solutions act like colloids and are referred to as a matricum rather then an osmoticum (Steuter *et al.*, 1981). However, the matric potential is often left out of the equation and only the osmotic and turgor potential is measured as components of the total water potential.

1.6.2 Water and sugar movement in fungi

The most coherent body of information about long-distance transport of solutes in basidiomycetes comes from studies on *Serpula lacrymans*, which can grow from a wood food source over nutritionally inert substances such as plaster or brick for long distances and must therefore possess the ability to translocate carbohydrate from the food source to the growing regions (Brownlee & Jennings, 1982a). Brownlee & Jennings (1981) using gas-liquid chromatography analysed *S. lacrymans* for soluble carbohydrates and found that the highest concentration of trehalose was detected in the mycelial strands and the

polyol arabitol was found in the highest amounts in the actively growing marginal and submarginal regions. Brownlee & Jennings (1981) investigated solute translocation by *S. lacrymans* using ^{14}C labelled glucose added to the food base and the bulk was incorporated into trehalose in all the mycelial regions after 6 hours and they postulated that this was the major carbohydrate translocated. A similar experiment was carried out in *F. velutipes* where ^{14}C glucose was added to media onto which *F. velutipes* was placed and the level of ^{14}C glucose incorporated into the carbohydrates was measured in the fruit bodies and trehalose was found to have the highest level of radioactive glucose (Kitamoto & Gruen, 1976). In *A. bisporus* ^{14}C labelled sugars were injected into the stipe of growing sporophores to study the mobility of trehalose and mannitol within the sporophore (Hammond & Nichols, 1977). When ^{14}C labelled glucose was injected into the stipe the majority of it was found to be incorporated into trehalose and ^{14}C labelled fructose was found to be incorporated into mannitol (Hammond & Nichols, 1977). Therefore it is likely that trehalose is translocated within the fruit body and is produced from glucose by the enzyme trehalose phosphorylase as indicated by Wannet *et al.* (1999). Trehalose seems to be the major translocation carbohydrate from the mycelia into the fruit bodies in *A. bisporus* and *F. velutipes* while in plants the disaccharide sucrose is the major sugar translocated (Kitamoto & Gruen, 1976; Wannet *et al.*, 1999; Williams *et al.*, 2000). However, the mechanism by which trehalose is translocated in *A. bisporus* is still unknown.

The pathway of translocation in *S. lacrymans* was investigated by feeding radioactive compounds to the feeding source and locating the radioisotope by autoradiography. ^{14}C labelled glucose was used for autoradiography on a large scale and [^{32}P] orthophosphate for micro-autoradiography (Brownlee & Jennings, 1982b). It was found that translocation took place in discrete pathways in young hyphal strands to the sub-marginal regions where there was an accumulation of carbohydrate. This is in agreement with previous results showing translocation of the carbohydrate trehalose in

strands and the accumulation and conversion to arabitol in the sub-marginal regions (Brownlee & Jennings, 1981; 1982b). Eamus & Jennings (1984) suggested that the mechanism of translocation in *S. lacrymans* is brought about by a pressure driven osmotically-derived mass flow which is the same mechanism that is now accepted for translocation in the phloem of higher plants. This mechanism of transport has been postulated for fungi whereby the presence of osmotically active solutes within the loading hyphae accumulate and lower the water potential below that of the external medium thus allowing a flux of water into the hyphae which increases the turgor and hydrostatic pressure thus driving water and solutes through the mycelium (Coggins *et al.*, 1980; Brownlee & Jennings, 1982a; Eamus & Jennings, 1984). For pressure driven mass flow to be an acceptable form of long distance transport there must be a turgor gradient generated in the direction of the proposed flow, namely from source to sink (Eamus & Jennings, 1984). Turgor gradients in basidiomycetes from source to sink were demonstrated in the hyphae of *Phallus impudicus, Phanerochaete velutina*, *S. lacrymans* and rhizomorphs of *Armillaria mellea* by Eamus & Jennings (1984). Clearly the demonstration of turgor gradients from the basal regions to the growing tips supports the theory that translocation in these basidiomycetes is by pressure driven mass flow. This gave the understanding that these turgor gradients are widespread amongst fungi and long distance transport in fungi is as a result of a pressure driven mass flow. Zimmermann (1971) suggested that three criteria must be fulfilled for pressure driven mass flow to be an acceptable means of translocation, these are (1) the conducting channel must be relatively impermeable to water in the lateral direction (2) it must be permeable to solutes and water in the longitudinal direction (3) turgor gradients must be demonstrated between source and sink.

In *A. bisporus* a pressure driven mass flow has been suggested by Kalberer (1987) as a mechanism that might explain water and solute transport from the mycelium to the growing sporophore. The driving force of translocation would be the higher turgor

potential in the substrate mycelium compared to that in the sporophore mycelium. Kalberer (1990) found that salt added to the casing layer reduced the yield of sporophores and slowed fruit body development and increased their average weight which is in agreement with the results observed by Awad & Nair (1989) who applied NaCl and PEG solutions over the range –0.07 to -0.62 MPa to the casing layer. The concentration of osmotically active substance mannitol was higher in fruit bodies with salt added to the casing soil (Kalberer, 1990). With the addition of NaCl to the casing soil translocation was delayed but the amount of solute translocated was not diminished. Kalberer (1990) hypothesised three mechanisms of translocation based on this increase in the osmolyte mannitol.

1.The mechanism of translocation is by osmotically derived pressure driven mass flow as described by Eamus & Jennings (1984) where a turgor gradient is generated from the substrate mycelium to the sporophore mycelium. However, in sporophores with salt added to the casing soil there is an increase in the amount of mannitol which can in turn increase the turgor pressure thereby reducing the turgor gradient from the mycelium to fruit body driving mass flow of solutes and slowing fruit body development.

2. According to Brownlee & Jennings (1982b) in *S. lacrymans* there are specific mycelial strands used for solute translocation and some strands used for water translocation. Salt added to the casing layer can reduce the water potential gradient between the casing soil and the fruit body therefore slowing water and solute uptake but the increase in osmolyte concentration in the fruit bodies can enhance the water potential gradient thus increasing water and solute uptake.

3. Water is taken up in the apoplast (intracellular capillaries of the cell walls) of the mycelial strands and fruit bodies. The growing fruit body cells take water from the apoplast of the fruit body. Water and solutes may move into the apoplast. A decrease in the water potential of the casing soil can decrease the water potential of the solution in

the apoplast and also the water potential difference between the fruit body cells and the solution in the apoplast. But with the increase in the concentration of mannitol in the cells the water potential difference can be restored.

1.6.3 Movement of water and solutes in plants

Water and solute transport has been well-characterised in plants and it can be considered as a possible model for water and solute transport from mycelium to sporophore. Water and mineral transport occurs in the xylem vessels, which move water from the roots to the shoots in response to water loss from the leaves (transpiration). Plants lose large quantities of water by transpiration and excessive transpiration retards their growth. Because the cuticle covering the leaf is so impervious to water, most of the leaf transpiration is restricted to loss of water vapour through the stomatal pores. The system of water transport in plants is by the cohesive tension theory, where water is pulled from the roots to the top of the trees. When water evaporates from the cell surface of leaves it is replaced by water from neighbouring cells and as a result the concentration of solutes within neighbouring cells increases and water potential decreases. A gradient of water potential becomes established which eventually exerts a tension on the water of the xylem. The tension is transferred down from the roots down to the stem and as a result water is distributed to the cells that are losing water. Jennings (1994) suggested this theory as possibly one of the mechanisms of translocation through the hypha.

Transport of sugars occurs in the phloem vessels by pressure flow hypothesis where photoassimilated sugars are transported from the source (leaves) to sink (growing points) along a gradient of turgor pressure developed osmotically. High sucrose concentrations in the phloem are established by active process of phloem loading. This decreases the water potential in the phloem and causes water to move into the phloem

by osmosis. With this movement of water into the phloem at the source, sucrose is carried passively to the sink.

1.7 MEMBRANE TRANSPORT PROTEINS

Transporters are membrane proteins and can be classified as peripheral or integral. Integral proteins interact with the hydrocarbon region of the lipid bilayer and nearly all of them span the lipid bilayer. Peripheral proteins bind to the surface of integral membrane proteins. While integral membrane proteins play important roles in solute transport and constitute approximately a third of all gene products sequenced (Wilson, 2001; Werten *et al.*, 2002) only 30 structures of membrane proteins have been solved at the atomic level compared to 3,000 crystal structures of soluble proteins. The 3-D structural elucidation of integral membrane proteins remains challenging due to difficulties with their stability, expression, purification and crystallisation (Tusnady & Simon, 1998; Wilson, 2001; Werten *et al.*, 2002), the functional characterisation of membrane proteins is more readily available through use of functional complementation of mutant yeast strains which are defective for the transport of a specific solute (Kaplan, 1993). However, in order to gain an insight into the structure of membrane proteins, topology predictions of the secondary structure of these membrane proteins based on their amino acid sequences are becoming increasingly popular and accurate (Tusnady & Simon, 1998). The secondary structure of transporters reveals the highly hydrophobic nature of these integral membrane proteins which are thought to consist of multiple α-helices serving as transmembrane domains (Sadee *et al.*, 1995) separated by hydrophilic loops. While the predicted secondary structures are useful and provide reasonable pictures of transport systems they give little knowledge of the arrangement of the helices in the membrane, which are most likely to be nearest each other or the likely interactions between them (Kaplan, 1993). However, it has been discovered that hydropathy profiles of membrane proteins are better conserved

than their primary structures (Henderson, 1991; Sadee *et al.*, 1995; Slotboom *et al.*, 1999). The deduced primary structure of transport membrane proteins serves to classify them together into gene families where there is high sequence similarity between individual genes in a family and sometimes very little sequence similarity between genes of different families (Sadee *et al.*, 1995). However, when the structural features of proteins are analysed there are often highly conserved shared sequence motifs, putative membrane domains and shared functions (transport) between gene families even when there is no primary structure similarity.

There are two primary modes of membrane transport: channel type and carrier type. The first channel proteins do not transduce energy but operate as selective pores that open in response to specific stimuli. There are two different types of channels: ligand gated and voltage gated. The acetylcholine receptor channel is a ligand gated channel and consists of five helical shaped subunits and uses electrical signalling for the purpose of transmitting nerve signals. If an expenditure of energy is coupled to transmembrane solute translocation then a system can be termed active transport (Saier, 2000a). Another subset of transport proteins are the ATP binding cassette (ABC) which are primary active transporters which use the energy released from ATP hydrolysis (e.g., Na^+/K^+ ATPase) to drive solute accumulation or efflux (Saier, 2000a). The third subset is the secondary active transporters known as major facilitator superfamily (MFS) that use a secondary source of energy (an ion electrochemical gradient, termed the proton motive force [PMF] in the case of protons or the sodium motive force [SMF] in the case of sodium ions), generated at the expense of a primary energy source, which is coupled to the process (Saier, 2000a). In animal cells the secondary active transport is dependant on the Na^+/K^+ ATPase transport system. For example, the Na^+ driven glucose transport protein depends on the sodium gradient across the membrane generated by the Na^+/K^+ ATPase to transport glucose against its concentration gradient across the membrane. In *E. coli* the lactose permease membrane transporter uses a free energy source called the proton

motive force generated from the flow of electrons from the respiratory chain. In this transport system the transport of lactose against its concentration gradient (active transport) is coupled to the downhill transport of one proton.

The MFS is one of the largest superfamilies of transporters and one of the most diverse (Saier, 2000b). The MFS was initially believed to function in the uptake of sugars (Maiden *et al.*, 1987) was then expanded to contain a five gene cluster (Marger & Saier, 1993) but it now contains 29 families of which 17 were recognised by Pao *et al.* (1998). Each of the members of the families of the superfamily share a common descent but are only distantly related but most of the proteins of the MFS share well characterised sequence motifs and their topology characteristically exhibits 12 transmembrane domains (TMD) (Saier, 2000b). With an increasing number of transport proteins being sequenced and topology predictions revealing predicted secondary structures of these proteins in the absence of their 3-D structures, a systematic classification system of all sequenced membrane proteins was devised by Saier (2000a). This classification system termed the transporter classification (TC) system places membrane proteins into families and sub-families based on sequence similarity and mode of transport. According to the TC system (Saier, 2000a; Busch & Saier, 2002) the MFS is assigned to subclass 2.A.1. The largest of the MFS families is the sugar porter (SP) family with over 200 currently sequenced members (Saier, 2000b).

1.8 HETEROLOGOUS EXPRESSION SYSTEMS

Several heterologous expression systems have been used for the identification and characterisation of membrane transport proteins (Frommer & Ninnemann, 1995). If appropriate expression systems are available, various forms of mutagenic analysis are performed with the aim of determining the structure–function of membrane proteins and their substrate specificity (Kaplan, 1993). Understanding the function of membrane

proteins has been greatly assisted by using yeast systems, which have been engineered with deficiencies in specific transport pathways. Cloning by functional complementation using a yeast system that has been engineered deficient for the transport of a specific solute can be used to demonstrate that the cloned transporter is capable of the transport of that specific solute (Dreyer *et al.*, 1999). Determining the function of membrane proteins is therefore more achievable than determining their structure.

Mutant strains of *S. cerevisiae* and *Schizosaccharomyces pombe* have been engineered with deficiencies in transport pathways and have been successfully used to determine the substrate specificity of membrane transporters (Dreyer *et al.*, 1999). Functional characterisation of the *Amanita muscaria* monosaccharide transporter was carried out by expressing the transporter in a *S. cerevisiae* mutant strain that was deficient in monosaccharide uptake and the transporter was revealed to have a preference for glucose (Wiese *et al.*, 2000). While mutant yeast strains can be used to identify the transport activity of a given system it can also provide additional information about transport kinetics. A sugar transporter gene HUP1, from *Chlorella kessleri* , belonging to the sugar transporter family of the MFS was successfully expressed in a *S. pombe* mutant unable to grow on glucose and was found to transport glucose (Will *et al.*, 1994). Two further genes HUP2 & 3 were isolated from *C. kessleri* and their substrate specificity was tested using *S. pombe* mutant strains and were found to transport galactose and glucose preferentially (Stadler *et al.*, 1995).

Other heterologous expression systems include the prokaryote *E. coli* which is a well established system for protein expression, oocytes of the South-African clawed toad *Xenopus laevis* and insect cells infected by recombinant baculoviruses (Dreyer *et al.*, 1999).

Although the prokaryote *E. coli* is a well established system for protein production, the heterologous expression of membrane proteins has been successful only in a few cases and many membrane proteins seem to be toxic when expressed in

bacteria (Dreyer *et al.*, 1999). The pET (plasmid for expression by T7 RNA polymerase) system (Novagen) has been widely used for the production of soluble proteins and has led to the production of both native membrane proteins and inclusion bodies (Ward *et al.*, 1999). To facilitate purification of membrane proteins, they are tagged to affinity fusions and the most widely used is hexahistidine, which allows for the rapid purification using nickel chelate chromatography. In the pET system transcription of the target gene is controlled by a T7 promoter and the production of T7 RNA polymerase.

Oocytes of the South African clawed toad *X. laevis* have been used as a powerful tool to study heterologous gene expression for more than 25 years (Dreyer *et al.*, 1999). Their popularity is due to the fact that they are quite robust and easy to handle due to their large size

While functional information of a membrane transporter can be obtained using mutant yeast strains, detailed structural analysis beyond the topology predictions of transport proteins is required especially due to the lack of membrane protein 3-D structures. Site-directed mutagenesis alters specific amino acids of a protein in order to determine their functional importance to the protein; it presupposes a concrete suspicion that a specific part of a protein determines a distinct feature. An example of what information can be gleaned from site-directed mutagenesis was demonstrated from the human glucose transporter Glut1 in which a conserved amino acid motif (RXGRR) was altered and expressed in *Xenopus* oocytes to determine its function (Sato & Mueckler, 1999). The conserved pentameric motif RXGRR, where R can be replaced by K and X is usually hydrophobic, duplicated in transmembrane helices 2/3 and 8/9 of many members of the MFS is thought to function in a structural rather than a functional role (Baldwin & Henderson, 1989; Sato & Mueckler, 1999). The positive charges in the pentameric motif are thought to function in the membrane topology of the protein, the elimination of these positive charges by the substitution of the arginines for glycine residues in the glucose

transporter resulted in a flipping of the loop to the exoplasmic compartment thus assigning a role as a cytoplasmic anchor to the conserved motif.

1.9 AIMS AND OBJECTIVES

The overall objective of this project was to understand the mechanism of translocation of water and solutes from the mycelium through the stipe and into the cap tissue of the developing sporophores and the factors which influence this translocation pathway. The mycelium and sporophores are more sensitive to matric than osmotic stresses and the effects of both were measured in relation to mycelial growth, the endogenous sugars and polyols and the water, osmotic and turgor potential on both defined and compost derived media. The extraction of water by the mycelium and sporophores has been reported to be linked to the components of the compost and casing soil and the matric potential. Therefore the effect of the two different media, compost extract and malt extract, were compared under osmotic and matric stress and the Ψ_w, Ψ_π, and Ψ_p were measured and sugars and sugar alcohols were quantified. The accumulation of polyols in sporophores and mycelium are required to create an osmotic gradient to allow for an influx of water into the cells to increase turgor and drive cell expansion. The sugars and polyols were measured in sporophore tissue during the stages of sporophore development to understand how they changed during the developmental stages. To understand how the carbohydrates are transported into the sporophore via the mycelium to provide a carbon source for the increasing mannitol production a previously identified SUT1 sugar transporter (Sreenivasaprasad *et al*., 2000b) was characterised during sporophore development (by Northern analysis) and attempts were made to determine the levels of SUT1 protein using Westerns.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Full descriptions of the buffers/reagents and growth media used are detailed in Appendix 1 and water used in media and buffer preparation was from the Elga Maxima ultra pure water system. All primer sequences are detailed in Appendix 2. All chemicals used were supplied by Merck BDH unless otherwise stated.

2.1 STRAINS AND GROWTH OF *A. BISPORUS*

The *A. bisporus* strains used in this study are shown in Table 2.1.

Table 2.1: Description of *Agaricus bisporus* strains used in this study.

A. bisporus strain	Description	Supplier
U3	Heterokaryotic	Horst (Fritsche, 1983)
A15	Heterokaryotic	Sylvan, UK
A12	Heterokaryotic	Sylvan, UK
PC1	Homokaryotic uracil auxotroph (=C28u) (Moore *et al.*, 2001)	HRI liquid nitrogen culture collection

2.1.1 Growth of *A. bisporus* fruit bodies on straw-based compost

Compost was prepared from a mixture of wheat straw, deep litter chicken manure and gypsum as described by Fermor *et al.* (1985). The composting procedure which is a two phase process is detailed in Section 1.4.1 after which the compost is inoculated with the appropriate *A. bisporus* strain in the form of colonised rye grain known as spawn. The conditions for mycelial growth and sporophore production were according to the standard procedure at the mushroom unit, HRI.

2.1.2 Harvest of fruit bodies and compost colonised mycelium

Fruit bodies were harvested from stage 1 through to stage 7 (Hammond & Nichols, 1976) from a second flush crop of mushrooms (strains A15 and U3) grown at the mushroom

unit, HRI and were immediately frozen in liquid nitrogen (N_2) and then stored at –70°C until required. Sporophore tissue (upper and lower stipe, cap, gills and cap skin) was removed from freshly picked stage 4 sporophores (A15) using a clean scalpel then wrapped in aluminium foil and frozen in liquid N_2 and stored as above. Also compost supporting a second flush crop of growing stage 2 and stage 4 sporophores (A15) was removed from the mushroom tray and wrapped in aluminium foil and frozen in liquid N_2 and stored at –70°C.

2.1.3 Growth of mycelium

A. bisporus (strains A12 and PC1) mycelial cultures were provided from the HRI liquid nitrogen culture collection (Challen & Elliott, 1986). These two strains were maintained on compost extract/complete yeast extract media (CE/CYM) agar, and incubated at 25°C unless otherwise stated.

A12 cultures were grown under water stress at two temperatures, 18°C and 25°C, and the following reagents: KCl and glycerol were added to CE/CYM agar and to malt extract agar (MEA, Lab M), and polyethylene glycol (PEG) 8000 was added to CE/CYM broth and malt extract broth (MEB, Oxoid) to lower the water potential of the media. The experimental details are described in Chapter 3.

2.2 MEDIA PREPARATION

All media were prepared using ultrapure water and autoclaved at 121°C and 15 psi for 15 minutes.

2.2.1 Media for fungal growth

Malt yeast peptone glucose (MYPG) broth was prepared by dissolving all the chemicals (composition as detailed in Appendix 1) in the appropriate amount of water, autoclaved and then kept at room temperature until required.

CE/CYM medium was prepared by thawing 400 ml of CE (Appendix 1) and 40 ml of 5X CYM (Appendix 1) in a waterbath at 55°C and mixing them in a jug with a magnetic stirrer and adjusting the volume to 2 litres with water. CE/CYM was then aliquoted into 500 ml Schott bottles and autoclaved as described above and kept at room temperature until required. CE/CYM agar was prepared as above except that 1.2% (w/v) technical agar No.3 (Oxoid) was added to the broth and after autoclaving it was placed in a 55°C waterbath to allow it to cool and then transferred (approx. 20 mls) into 9 cm Petri dishes.

The following media were prepared: 5% (w/v) MEA (Lab M) and 5% (w/v) MEB (Oxoid), by dissolving the appropriate amount of media in water and autoclaved. The MEA medium was then placed in a 55°C waterbath to allow it to cool and poured into 9 cm Petri dishes and MEB was kept at room temperature until required.

2.2.2 Bacterial media

Luria bertani (LB) broth was prepared by dissolving the chemicals (composition as detailed in Appendix 1) in the appropriate amount of water and then aliquoted into glass screw cap vials or conical flasks which were plugged with cotton wool, covered with aluminium foil and then sterilised by autoclaving. The broth was then kept at room temperature until required. LB agar was prepared as for LB broth except that 1.2% (w/v) agar was added to the medium, autoclaved and placed in a 55°C waterbath to allow it to cool. The necessary antibiotic (detailed in Section 2.2.3) was then added to the medium before it was poured into 9 cm Petri dishes.

SOC medium (1L) was prepared by dissolving 20 g tryptone (DIFCO), 5 g yeast extract (Oxoid), and 0.5 g NaCl in 950 ml of water to which 5 ml of 250 mM KCl was added. The pH was adjusted to 7 using 5 N NaOH and sterilised by autoclaving. The medium was then allowed to cool and 5 ml of sterile 2 M $MgCl_2$ and 20 ml of 1 M glucose

(filter sterilised using a 0.22 μm filter (25 mm diameter, Millipore), was added to the medium which was stored at 4°C.

2.2.3 Antibiotics

The antibiotics ampicillin and kanamycin (Sigma), were prepared as stock concentrations of 100 and 50 $mgml^{-1}$ respectively using ultrapure water and filter sterilised through a 0.22 μm filter (25 mm diameter) (Millipore). They were then aliquoted into sterile 1.5 ml microcentrifuge tubes (Treff Lab) and stored at -20°C.

2.3 BACTERIAL STRAINS AND VECTORS

The vectors used in this study are indicated in Table 2.2. The genomic library of the carboxin resistant mutant heterokaryon C54-carb8 was constructed in the 8153 bp cosmid vector Lawrist (Challen *et al.*, 1996). Lawrist was formed by fusing the 5386bp LoristX cosmid (Speek *et al*., 1988) with a 2767 bp modified pUC vector (Challen *et al.*, 1996). The following bacterial strains DH5α (Invitrogen) and BL21(DE3) (Novagen) contained the pET-43.1c(+) vector (Novagen) and XL1 blue contained the pBK-CMV Phagemid vector. These strains are detailed in Table 2.3.

Table 2.2: Vectors used in the characterisation of the sugar transporter gene, *SUT1*

Vector	Clones used	Antibiotic marker
pBK-CMV Phagemid (Stratagene)	STIIE2, STID4	kanamycin
pET-43.1c(+) (Novagen)	SUT1-C(C-terminal)	ampicillin
For the *A. bisporus* C54-*carb*.8 genomic library cosmid vector Lawrist was used (Challen *et al.*, 1996)	20A11, 32B10 ,25E6	kanamycin

Table 2.3: *Eschericha coli* strains used in the characterisation of the sugar transporter gene, *SUT1*

Bacterial Strain	Genotype
XL1 blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacI^q ZΔM15 Tn10 (tet^r)] (Stratagene)
DH5α	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZY* A-*argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(r_k^-, m_k^+) *phoA supE*44 *thi*-1 *gyrA*96 *relA*1 λ^- (Invitrogen)
BL21(DE3)	F$^-$ *omp*T *hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3) (Novagen)

2.4 RNA AND DNA ANALYSIS

2.4.1 Isolation of DNA

2.4.1.1 Isolation of phagemid and plasmid DNA

The *E. coli* strain containing plasmid or phagemid DNA was streaked from stock cultures stored at -70°C onto 9 cm Petri dishes containing LB agar supplemented with the appropriate antibiotic and incubated at 37°C overnight. A single colony was removed from the Petri dish and inoculated into 10 ml of LB broth supplemented with the appropriate antibiotic. Then 3 ml of the bacterial cells were harvested by centrifugation at 10,000 *g* for

1 minute and the DNA was extracted using Qiagen mini plasmid extraction kit (Qiagen). The DNA was dissolved in 30 μl of TE buffer and stored at -20°C.

2.4.1.2 Isolation of cosmid DNA

The *E. coli* strain containing the cosmid DNA was removed from stock culture stored at -70°C and streaked onto a 9 cm Petri dish containing LB agar supplemented with 50 $\mu g ml^{-1}$ of kanamycin and incubated at 37°C overnight. A single colony was removed and used to inoculate 10 ml of LB broth supplemented with 50 $\mu g ml^{-1}$ of kanamycin and the culture incubated at 37°C in an orbital shaker at 200 rpm overnight. Then the bacterial cells were harvested by centrifugation at 10,000 *g* for 1 minute. Cosmid DNA was extracted using Qiagen mini plasmid extraction kit (Qiagen) following the manufactures instructions for cosmid DNA extraction. DNA was dissolved in 30 μl of TE buffer and stored at -20°C.

2.4.1.3 Isolation of *A. bisporus* genomic DNA

A. bisporus genomic DNA was extracted from freeze dried mycelium according to the method described by Challen *et al.* (1995). The dried mycelium (approximately 100-200 mg per microcentrifuge tube) was ground using a sterile plastic inoculation loop. Extraction buffer (0.4 M KCl, 50 mM EDTA pH 8, 1% (v/v) Triton X-100) was freshly prepared and filter sterilised through a 0.2 μm sterile filter (Nalgene) before adding 5 $U ml^{-1}$ of Ribonuclease A (Sigma). To each 1.5 ml microcentrifuge tube (Treff Lab) containing 100-200 mg of ground mycelium 1 ml of the extraction buffer was added and mixed thoroughly. The samples were incubated at 70°C for 40 minutes and centrifuged at 13,000 rpm for 10 minutes after which the supernatants were removed from each tube and transferred to clean microcentrifuge tubes and centrifuged at 13,000 rpm for a further 5 minutes. The supernatant from each microcentrifuge tube was then individually applied to a Qiagen-tip 20 column which had been equilibrated with 1 ml of Qiagen buffer QBT,

after all the supernatants had been passed through the columns they were then washed with 4 X 1 ml of Qiagen buffer QC. The DNA was eluted from each column with 700 μl of Qiagen Buffer QF. The eluted DNA was precipitated with 0.7 volumes of isopropanol and centrifuged at 10,000 rpm for 20 minutes. The supernatant was decanted and the DNA washed with 1 ml of 70% ethanol (EtOH) and the pellet was air-dried and re-suspended in 50 μl of TE pH 8. The DNA was stored at -20°C until required.

2.4.2 DNA agarose gel electrophoresis

Agarose gels 0.8% to 2% (w/v) were prepared using 0.5X tris-borate EDTA (TBE) buffer to which ethidium bromide (stock 10 $mgml^{-1}$, Gibco BRL) was added to give a final concentration of 0.25 μgml^{-1}. DNA samples containing 1X DNA loading dye were loaded onto agarose gels and electrophoresis was carried out in 0.5X TBE buffer between 50 and 80 V in a Bio-Rad wide mini sub-cell (unless otherwise stated). The DNA was visualised under UV light at short wavelength (254 nm) on a transilluminator (Appligene). An image was captured using the Biogene imaging system.

2.4.3 Molecular size and quantification of DNA

DNA was diluted (1:10 or 1:20) with water and analysed by gel electrophoresis as described in Section 2.4.2 on the appropriate agarose gel along with DNA marker II or VI (Boehringer Mannheim) or Low DNA mass ladder (Invitrogen) which were used for comparative purposes to determine the size and quantity of the DNA.

2.4.4 PCR amplification of DNA

PCR amplification of DNA was carried out in a Hybaid multi block system (MBS) thermocycler. The conditions used for the PCR reaction are detailed in Table 2.4.

The annealing temperature varied between 50 and 64°C depending on the specific primers used in the PCR reaction. PCR (100 μl) reactions were performed using

either Red *Taq (Thermus aquaticus)* Readymix with $MgCl_2$ (Sigma-Aldrich) or *Pwo* (*Pyrococcus woesei)* DNA polymerase (Roche). PCR reactions performed with Red *Taq*, contained 1 μM of each forward and reverse primers, 50 μl Red *Taq* Readymix with $MgCl_2$ (Sigma-Aldrich), approx. 50 ng DNA, and the volume was adjusted to 100 μl using PCR grade water. PCR reactions carried out using *Pwo* DNA polymerase (to achieve high fidelity) contained 1 μM each of forward and reverse primers, 2.5 U *Pwo* DNA polymerase (5 $U\mu l^{-1}$), 1X PCR buffer with Mg^{2+} (Roche), 200 μM dNTP, approx. 50 ng DNA and the volume was adjusted to 100 μl using PCR grade water.

Table 2.4: Conditions used for PCR reaction

Temperature	Time	No. of cycles	
95°C	2 min	1 cycle	
94°C	45 sec	30 cycles	denaturation
50-64°C	45 sec		annealing
72°C	2 min		extension
72°C	5 min	1 cycle	

2.4.5 DNA Sequencing

Sequencing reactions were performed using ABI Prism® Big Dye™ terminator cycle sequencing ready reaction kit (PE Applied Biosystems). The sequencing reactions contained 3.2 pmol of primer (primer sequences are detailed in Appendix 2), 7 μl Big Dye sequencing mix, 10 ng-1 μg of DNA depending on the template and the volume was adjusted to 20 μl with sterile water. The sequencing cycle was as follows: 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes and 25 cycles were performed in a Hybaid MBS thermocycler. After the sequencing reaction was completed 2 μl of 3 M sodium acetate (NaAc) pH 5.2 and 50 μl 95% (v/v) EtOH were added to each tube and

they were placed on ice for 10 minutes. The tubes were then centrifuged at 14,000 *g* for 30 minutes after which the supernatant was removed and 250 μl of 70% (v/v) EtOH was added to wash the pellet and centrifuged for 2 minutes. The 70% EtOH was decanted and the pellet was allowed to air dry and electrophoresis of the reactions was performed either by HRI or Durham University sequencing service.

The sequence analysis was performed using the EditSeq package within DNASTAR (Dnastar Inc.). Nucelotide/amino acid sequence comparisons were carried out at the NCBI (http://www.ncbi.nlm.nih.gov) using the programme blastx. Multiple sequence alignments were generated using ClustalW (webserver, http://www.ch.embnet.org).

2.4.6 Restriction digestion of DNA

Restriction enzymes were supplied by Roche/Boehringer Mannheim. Restriction digestion reactions were carried out for 3 hours to overnight at 37°C in an incubator according to the manufacturers' instructions and contained: 1X of the appropriate buffer (supplied with the enzyme) and the appropriate amount of restriction enzyme added depending on the concentration of DNA whereby 1 unit of restriction enzyme digests 1 μg of DNA and adjusted to the final volume required using water.

2.4.7 Agarose gel extraction of DNA

PCR amplified DNA fragments and DNA restriction digests were electrophoresed as described in Section 2.4.2. The DNA band was visualised under UV as described in Section 2.4.2 and excised from agarose gel using a clean scalpel and placed into a 2 ml eppendorf tube (Eppendorf AG) of known weight and the gel fragment was then weighed. DNA was extracted from the agarose gel using the QIAquick gel extraction kit (Qiagen), following the manufacturers' instructions. The DNA was eluted from the QIAquick column using 30-50 μl of Buffer EB (provided with the kit), and stored at -20°C.

2.4.8 Transformation of vector DNA into *E. coli*

Chemically competent *E. coli* cells were removed from the -70°C freezer and allowed to thaw on ice and 100 μl of the cells were placed in pre-chilled 1.5 ml microcentrifuge tubes (Treff Lab) to which 1-10 ng of plasmid DNA was added. The cells were gently mixed and placed on ice for 30 minutes and subjected to heat shock by placing in a waterbath at 42°C for exactly 2 minutes and then held on ice for 2 minutes. SOC medium at room temperature was added to the transformation reaction adjusting the volume to 1 ml. The transformed cells were incubated at 37°C for 1 hour in an orbital shaker at 225 rpm and aliquots of the cells were spread on Petri dishes containing LB agar supplemented with the appropriate antibiotic. The Petri dishes were allowed to dry before being inverted and incubated overnight at 37°C.

2.4.9 Southern blotting and hybridisation

The agarose gel containing the DNA to be blotted was submerged in depurination solution (0.125 M HCl), which aids in the transfer of high molecular weight DNA fragments, and incubated for 10 minutes on a shaking platform (The Belly Dancer, Stovall Life Science Inc.) at 30 rpm and then rinsed in de-ionised water and covered with denaturation solution (1.5 M NaCl, 0.5 N NaOH, pH 9.9) and incubated shaking as described above for 30 minutes. This denaturation step produces single-stranded DNA that will hybridise to complementary DNA. The gel was then rinsed in de-ionised water and submerged in neutralisation solution pH 7 (1 M Tris base and 1.5 M NaCl) and placed on a shaking platform as described above for 30 minutes. The neutralisation solution was then removed, replaced with fresh neutralisation solution and the gel incubated shaking as described previously for a further 15 minutes. The DNA was then ready to be transferred onto Hybond-N nylon membrane (Amersham Pharmacia) and this was carried out by capillary action using 10X standard saline citrate (SSC) containing 1.5 M NaCl and 0.15 M tri-sodium citrate, pH 7 as the transfer buffer. Whatman 3MM filter paper was soaked in

10X SSC and then placed onto Saran wrap and the gel was inverted on top. The nylon membrane cut to the same size as the gel and wetted in 10X SSC was placed onto the gel. Any bubbles that might be trapped between the gel and membrane were removed by rolling a glass rod over the surface of the membrane. Onto the membrane 5 sheets of Whatman 3MM (cut to the same size as the membrane and soaked in 2X SSC) were placed and then absorbent paper towels were stacked on top to absorb the transfer buffer. A weight of approximately 500 g was placed on top to maintain contact between the layers and the blot was left overnight. The blot was dismantled and the positions of wells were marked onto the membrane. The membrane was then rinsed in 2X SSC for 5 minutes and allowed to dry at room temperature. The membrane was then placed between two sheets of 3MM filter paper and incubated in an oven at 80°C for two hours to immobilise the DNA to the membrane.

Southern blotting onto Hybond-N^{+} membrane (Amersham Pharmacia) was carried out as described above except that there was no neutralisation step and the denaturation solution was used as the transfer buffer. After overnight transfer was completed the membrane was rinsed in 2X SSC and then wrapped in Saran wrap (since the DNA was covalently attached to the membrane during the transfer process with NaOH, the membrane did not need to be baked as for nylon membrane).

The probe DNA for hybridisation was radiolabelled by incorporating ^{32}P α-dCTP (Amersham Pharmacia) into it. The DNA to be labelled was diluted to a final concentration of 25 ng in 45 μl of TE buffer and denatured by incubating at 100°C for 5 minutes and snap cooling on ice for 5 minutes. The denatured DNA was then added to a Rediprime II random primer reaction tube (Amersham Pharmacia), and to this 5 μl of 370 μCiμl^{-1} of ^{32}P labelled α-dCTP was added to introduce a radioactively labelled nucleotide. The labelling reaction was allowed to proceed at room temperature for 1 hour. To stop the reaction, 5 μl of 0.2 M EDTA was added to the reaction. The labelled DNA was denatured

by incubating at 100°C for 5 minutes and snap cooling on ice for 5 minutes, just prior to adding it to the pre-hybridisation solution.

The membrane containing the immobilised DNA was placed in a Hybaid hybridisation cylinder containing 25 ml of pre-hybridisation solution (6X SSC, 0.5% SDS, 5X Denhardts and 100 $\mu g ml^{-1}$ herring sperm DNA (which was denatured for 5 minutes at 100°C and snap cooled on ice for 5 minutes) and placed in a Hybaid rotating hybridisation oven at 65°C and left overnight. After overnight incubation of the membrane, 50 µl of the ^{32}P labelled and denatured DNA probe was added to the pre-hybridisation solution. The hybridisation reaction was allowed to proceed overnight at 65°C after which the hybridisation solution was removed. All non-specifically bound probe was removed by washing the membrane first with post-hybridisation wash solution (PHW) I (2X SSC and 0.1% SDS) three times, each time for 30 minutes and then with PHW II (0.1X SSC and 0.1% SDS) three times, each time for 30 minutes and both solutions were pre-heated to 65°C. The membrane was removed from the Hybaid cylinder and wrapped in Saran wrap and placed in light proof Kodak cassette and exposed to X-ray film (Kodak, blue sensitive) under safe light illumination and placed at -70°C. After 1-2 days the film was developed using a Hyperprocessor (Amersham).

2.4.10 Extraction of total RNA from fungal tissue

In order to prevent degradation of RNA by ribonucleases, all reagents used in RNA extractions were prepared with diethylpyrocarbonate (DEPC, Sigma) treated water, which was prepared as detailed in Appendix 1. Pestles and mortars used for RNA extractions were autoclaved at 121°C and 15 psi for 15 minutes and centrifuge tubes were soaked overnight in water containing 0.1% (v/v) DEPC and then removed and autoclaved.

RNA was extracted from fungal tissue and compost according to the method described previously by Sreenivasaprasad (2000). Both whole sporophores and

sporophore tissue were ground to a fine powder in a pestle and mortar under liquid N_2. VM and compost was ground to a fine powder in a Krups 75 coffee grinder using dry ice and 5.5 g of the ground fungal material was transferred to a 50 ml Nalgene round bottomed centrifuge tube containing: 15 ml of extraction buffer (0.2 M NaAc, 10 mM EDTA, 1% (v/v) SDS and 0.5% (v/v) β-mercaptoethanol) and 15 ml of phenol:chloroform:isoamyl alcohol 25:24:1, pH 5.2 (Sigma) pre-heated to 65°C. The sample was incubated in a waterbath at 65°C for 15 minutes and centrifuged at 15,000 *g* for 30 minutes at 4°C. The aqueous layer was transferred to a 35 ml graduated Nalgene centrifuge tube and an equal volume of chloroform:isoamyl alcohol 24:1 was added to remove any residual phenol and the mixture was centrifuged at 15,000 *g* for 30 minutes. The aqueous phase was removed and 12 M Lithium Chloride (LiCl, Sigma) was added to give a final concentration of 2 M LiCl and mixed thoroughly and incubated overnight at 4°C to precipitate the RNA. The RNA was pelleted by centrifugation at 15,000 *g* for 30 minutes at 4°C and the supernatant was removed and the pellet washed with 5 ml of 3 M NaAc pH 5.2 by centrifugation at 15,000 *g* for 15 minutes at 4°C. The supernatant was decanted and the RNA pellet was washed with 20 ml of 70% (v/v) EtOH by centrifugation as above. The 70% (v/v) EtOH wash was repeated to remove salts and trace amounts of organic solvents and the pellet was dried in a desiccator. The RNA pellet was re-suspended in 250-1000 μl of DEPC treated water and transferred to a 2 ml siliconised eppendorf tube and quantified and analysed by agarose gel electrophoresis as described in Section 2.4.11 and 2.4.12 and stored at -70°C.

2.4.11 Quantification of RNA

RNA quantification was carried out by preparing a 1:50 dilution of the RNA samples using DEPC treated water and placing it in a quartz cuvette and the absorbance was measured

at 260 nm using a Philips PU8720 UV/Vis scanning spectrophotometer. The RNA concentration (μgml^{-1}) was calculated as follows: 40 x A_{260} x dilution factor.

2.4.12 RNA agarose gel electrophoresis

Agarose gels 1% (w/v) were prepared using DEPC treated water and contained 1.8% (v/v) formaldehyde and 1X MOPS. The electrophoresis buffer used contained 1X MOPS and 1.8% (v/v) formaldehyde. Prior to electrophoresis 11 μl RNA (approx.15 μg) samples and RNA marker I (Boehringer Mannheim) were denatured by adding: 3 μl 10X MOPS, 3.5 μl formaldehyde, 10 μl formamide and incubated for 15 minutes at 55°C and then placed on ice for 5 minutes. To each sample and RNA marker I, 2.5 μl of ethidium bromide (400 μgml^{-1}, Gibco BRL) was added to give a final concentration of 35 μgml^{-1} and 4 μl of 5X RNA loading buffer were added. The RNA was separated by electrophoresis using a Bio-Rad wide mini sub-cell, at between 50 and 80 V for 2-3 hours and the RNA was visualised as described in Section 2.4.2. An image was captured using Biogene imaging system.

2.4.13 Northern blotting and hybridisation

RNA transfer onto Hybond-N nylon membrane (Amersham Pharmacia) was carried out by capillary action as for Southern blot (Section 2.4.9) except that 20X SSPE was used as the transfer buffer and the gel was not pre-treated prior to transfer due to the single stranded nature of RNA. The membrane was removed after overnight transfer was completed and the position of the wells was marked onto the membrane. The membrane was then rinsed in 6X SSPE to remove any traces of the agarose gel on the membrane and then allowed to dry at room temperature. The membrane was then placed between two sheets of 3MM filter paper and incubated in an oven at 80°C for 2 hours to immobilise the RNA onto the membrane. The membrane was stained in 0.5% Methylene Blue dissolved in 0.5 M NaAc at pH 5.2 and excess stain was removed by rinsing the

membrane in DEPC treated water. The stained membrane was photographed using a white light source.

Membrane blots containing immobilised RNA were placed in a Hybaid hybridisation cylinder, containing 25 ml of pre-hybridisation solution (5X SSPE, 50% formamide, Denhardts 5X, 0.5% SDS and 100 μgml^{-1} herring sperm DNA (denatured for 5 minutes at 100°C and snap cooled on ice for 5 minutes) and placed in a Hybaid rotating hybridisation oven at 42°C and left overnight. After overnight incubation of the membrane, the ^{32}P labelled and denatured DNA probe (prepared as described in Section 2.4.9) was added to the pre-hybridisation solution and hybridisation was carried out overnight at 42°C and then the hybridisation solution was removed. All non-specifically bound probe was removed by washing the membrane with PHW I (4X SSC, 25 mM NaP pH 7, 0.1% SDS) and PHW II (1X SSPE, 0.1% SDS) for 30 minutes each and the final wash was carried out using PHW solution III (0.1X SSPE, 0.1% SDS) for 15 minutes, all PHW solutions were pre-heated to 42°C. The membrane was removed and wrapped in Saran wrap, placed in a Kodak cassette and exposed to X-ray film (Kodak, blue sensitive) and the cassette placed at -70°C. After 1 or 2 days the film was developed using a Hyperprocessor.

2.5 PROTEIN ANALYSIS

2.5.1 SDS-polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels were prepared using Bio-Rad Mini Protean II assembly apparatus (as per the manufacturers' instructions). Polyacrylamide gels (12% Tris-HCl) comprised of 12% separating gels and 4% stacking gels (composition in Appendix 1) and were 0.75 mm in thickness. Protein samples were mixed 1:2 ratio with Laemmli sample buffer (Bio-Rad) to which 5% (v/v) β-mercaptoethanol was added just prior to use. Protein samples and molecular mass standards, SDS VII-L, SDS VII-B (Sigma) or pre-stained SDS-PAGE

low range marker (Bio-Rad), were incubated in a boiling waterbath for 5 minutes and then allowed to cool to room temperature. The protein samples (15-20 μl) and the molecular mass standard (5 μl) were loaded onto the polyacrylamide gel. Electrophoresis of the protein samples was performed in 1X Tris-glycine buffer containing 0.1% SDS at 100 V for 5 minutes and then the voltage was increased to 200 V until the dye migrated to the bottom of the gel. The polyacrylamide gels were carefully removed from the glass plates and the protein was either stained with 0.1% (w/v) Coomassie Blue R-250 dissolved in 40% (v/v) methanol (MeOH) and 10% (v/v) acetic acid (HoAc) or transferred onto nitrocellulose membrane (Western blotting) as described in Section 2.5.2. To stain the protein the polyacrylamide gels were placed in square Petri dishes and covered with 0.1% (w/v) Coomassie Blue R-250 solution. The polyacrylamide gels were incubated shaking gently for one hour at 15 rpm on Belly Dancer and then the stain was removed and the gels were covered with de-staining solution (40% (v/v) MeOH, 10% (v/v) HoAc) and shaken for 1 hour as described previously to remove background staining.

2.5.2 Western blotting

Proteins were transferred from the polyacrylamide gel to nitrocellulose membrane using a Trans-blot Semi-dry blotter (Bio-Rad) and Tris-glycine transfer buffer (48 mM Tris, 39 mM glycine, 20% MeOH, 1.3 mM SDS). Two pieces of Bio-Rad extra thick filter paper were cut to the same size as the gel and soaked in pre-chilled Tris-glycine transfer buffer (with SDS). Nitrocellulose membrane (Schleicher & Schuell) was cut to the size of the polyacrylamide gel and was wetted in transfer buffer. The polyacrylamide gel containing the protein to be transferred was equilibrated in transfer buffer for 15 minutes with one change of buffer to allow the removal of electrophoresis buffer and salts. One pre-soaked filter paper was placed onto the platinum anode of the Semi-dry blotter over which a glass rod was rolled over to remove any air bubbles. On top of the filter paper the pre-wetted nitrocellulose membrane was placed and again any air bubbles were removed by rolling

with a glass rod. The equilibrated gel was carefully placed on top of the nitrocellulose membrane and any air bubbles removed. Another sheet of pre-soaked filter paper was then placed onto the gel and the cathode was carefully placed onto the stack. Transfer was carried out at 15 V for 45 minutes after which the blot was dismantled and the membrane was placed in a square Petri dish and wrapped with Saran wrap and left overnight.

2.5.3 Staining for the detection of proteins

All reagents were prepared in TBS-Tween (TBS containing 0.5% (v/v) Tween 20) unless otherwise stated. The membrane was covered with 3% (w/v) skimmed milk powder (Marvel) blocking reagent prepared in TBS and incubated for 1 hour at room temperature on a shaking platform (The Belly Dancer) at 15 rpm. The membrane was then washed thrice for 5 minutes each time in TBS-Tween. The membrane was then covered with a second blocking reagent which comprised of 1% (w/v) bovine albumin (Sigma) and 1% (v/v) bovine serum and incubated for 1 hour at room temperature shaking as described above. The membrane was washed thrice for 5 minutes each time in TBS-Tween. The membrane was then covered with 1:250 dilution of antibody prepared in 1% (w/v) bovine albumin and incubated for 2 hours at room temperature shaking as described above after which it was washed thrice for 5 minutes each time in TBS-Tween. The membrane was then covered with either of the two following conjugates: goat anti-rabbit labelled horseradish peroxidase (HRP, Sigma) or alkaline phosphatase (AP, Sigma) each of which were prepared as a 1:2000 dilution in 1% (w/v) bovine albumin and incubated for 2 hours at room temperature shaking at 15 rpm. After this incubation the membrane was washed twice for 5 minutes with TBS-Tween and once for 5 minutes in TBS and the corresponding substrate was added. The substrate 4-chloro-1-napthol (CN, Sigma) was used to detect the conjugate enzyme HRP and gives an intense blue-black colour when it reacts with HRP. The CN substrate was prepared as follows: a 30 mg tablet (Sigma) was

dissolved in 1 ml of EtOH and just prior to developing the blot it was added to 100 ml of 50 mM Tris/HCl pH 7.6 and 100 μl of 30% hydrogen peroxide (H_2O_2, Sigma). The blot was covered with the substrate and agitated until bands were detected. To stop the reaction the membrane was rinsed with PBS. The substrate bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Sigma) was used to detect the presence of the conjugated enzyme AP and gives an intense black-purple colour. A BCIP/NBT tablet containing all the reagents required was dissolved in 10 ml of distilled water and the blot was covered with the substrate and agitated until bands were detected and the reaction was stopped by rinsing the membrane with PBS containing 20 mM EDTA.

2.6 ANALYSIS OF FUNGAL SUGARS AND POLYOLS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

To each fungal sample of known weight, 1 ml of HPLC grade water (Fischer Scientific) was added and samples sonicated for 2 minutes using a Soniprep 150 (Fisons) at 26 μm amplitude. The sample was then incubated for 5.5 minutes in a boiling waterbath followed by the addition of 0.67 ml of acetonitrile and then centrifuged at 13 000 rpm for 15 minutes (Hallsworth & Magan, 1997). The resulting supernatant was removed into a sterile 1 ml syringe (Terumo) and filtered through a 0.2 μm, (13 mm diam.) nylon syringe filter (Whatman) into HPLC vials.

After sealing the vials the sugar/polyol contents were analysed using a Gilson modular HPLC system, a Hamilton HC-75 CA^{2+} column and a refractive index (RI) detector using a mobile phase of acetonitrile:water (40:60) and a flow rate of 1 $mlmin^{-1}$. The solutes analysed were the sugars trehalose and glucose and the polyols glycerol, erythritol, arabitol and mannitol. Standards of 100, 200, 400 and 800 ppm of the sugars and polyols prepared in the mobile phase acetonitrile:water (40:60) were used for interpretation of the actual concentrations of the unknown samples. These solutes were quantified with Gilson software adapted to HPLC equipment and the peak areas were

integrated by the software and the amounts were obtained for the calibration curves (100-800 ppm). The amount of solutes were obtained in ppm from the calibration curve for the fungal tissue and converted into mmolar concentration.

CHAPTER 3

IN VITRO EFFECT OF DIFFERENT WATER STRESSES ON THE GROWTH, WATER POTENTIAL AND SUGAR/POLYOL CONTENT OF *A. BISPORUS*

3.1 INTRODUCTION

Mushroom sporophores contain 90-95% water and the growing sporophores obtain water from both the casing (17-46%) and the compost (54-83%) layer (Kalberer, 1985; Donker & Van As, 1999). However, only a percentage of the total water content of the substrate is available for growth and the rest is held by the chemical and physical structure of the compost and casing layer (Magan, 1997). The total water content is often known but does not describe the amount of water actually available to the growing mushrooms (Lee *et al.*, 2000). The water availability is defined by the water potential of the substrate and the water potential (Ψ_w) is the sum of the osmotic (Ψ_π), matric (Ψ_m) and turgor (Ψ_p) potentials and is measured in pascals (Pa) (Griffin, 1981) and these parameters were discussed in more detail in Section 1.6.1. The difference in water potential between the substrate and the mycelial cells strongly influences water uptake by the mycelium (Kalberer, 1990). Thus the water potential of the substrate depends on the concentration of substrate water-soluble solutes (Kalberer, 1990). Translocation in *A. bisporus* from the mycelium to the sporophore has been suggested to occur by pressure driven mass flow as a mechanism that might explain solute and water transport and is discussed in Section 1.6.2.

The total Ψ_w and its osmotic and matric components and their interactions with temperature affect the efficient colonisation of the substrate and are important for effective fruiting (Lee *et al.*, 2000). The mycelium colonising the compost and casing layer and the sporophore yield are influenced by the matric potential of the casing layer and relates to the physical and chemical properties of the casing soil (Noble *et al*., 1999). The mushroom is more sensitive to the matric than osmotic potential of the casing soil in terms of yield and water status of the sporophores and mycelial growth (Noble *et al*., 1999). Therefore it is necessary to measure both the effect of water stress by matric and osmotic forces on the growth response of mycelium and *A. bisporus* sporophores and the

accumulation of both polyols and solutes. Growth under water stress due to reduced osmotic potential causes a reduction in the water potential gradient between the substrate and the sporophore and affects the nutrient and sugar uptake from the substrate to the sporophore. Therefore to maintain the cell turgor for hyphal growth the fungal cells accumulate osmolytes (dissolved osmotically-active substances), in response to osmotic stress, that create a difference in osmotic potential thus driving an influx of water into the cells (Davis *et al.*, 2000).

Previous work has examined the effect on sporophore yield and solute production under osmotic and matric stresses applied to the casing layer and will be further discussed. Awad & Nair (1989) applied solutions of NaCl and polyethylene glycol (PEG) (unspecified molecular weight) to alter the water potential of the casing layer osmotically and matrically respectively in the range -0.07 to -0.62 MPa. They found that with both NaCl and PEG at osmotic potentials between –0.7 and -0.37 MPa the yield of sporophores were significantly reduced and both treatments had similar effects. Sporophore production was completely suppressed at osmotic potentials below -0.37 MPa with NaCl but only decreased to 56% of the control when PEG was used. This could be due to the toxic effects of the Na ions on the sporophore which has been suggested to occur at lowered water potentials (ionically using NaCl) in wood rotting basidiomycete fungi (Boddy, 1983). Because Awad & Nair (1989) did not specify the molecular weight of the PEG that they used it is not known whether they exerted a matric effect with the PEG solution as PEG with molecular weight <6000 exerts predominantly osmotic effects and only exerts a matric effect at a molecular size >6000 (Michel & Kaufmann, 1973; Steuter *et al*., 1981). Kalberer (1990) examined the effect of reducing the water potential of the casing soil of an *A. bisporus* crop osmotically using NaCl on the yield of sporophores and the accumulation of solutes and found with increasing salt concentration added to the casing layer fruit body development decreased and fruit body yield was reduced along with an increase in fruit body mannitol levels. When salt was added to the casing layer

this reduced the water potential difference/gradient between the casing and fruit body thus reducing water and solute translocation and slowing fruit body development. However Kalberer (1990) found the formation of the osmotically-active substance mannitol can enhance the water potential gradient by reducing the water potential of the cells. The possible mechanisms of translocation in *A. bisporus* were discussed in Section 1.6.2.

Magan *et al.* (1995) examined the effect of reduced osmotic and matric potential between -0.2 and -4.0 MPa using KCl and PEG 6000 respectively on mycelial growth on agar of commercial and hybrid strains of *A. bisporus* and *Agaricus bitorquis* at 20 and 25°C. They found that both were more sensitive to changes in matric than osmotic potential, similar to the results which Noble *et al*. (1999) obtained for mushroom production. Magan *et al.* (1995) also found that the optimum growth of *A. bisporus* mycelium was 20°C although a temperature of 16-18°C is required for sporophore production.

Previous work has examined the effect of reduced osmotic and matric potentials on the growth rate of *A. bisporus* mycelium on agar medium (Magan *et al*., 1995) and on sporophore yield through the addition of salts, by ionically reduced osmotic potential, to the casing layer (Kalberer, 1990). No work has been carried out to examine how *A. bisporus* mycelium extension responds to reduced matric and osmotic potentials and their effect on the endogenous sugars and polyols and the osmotic, water and matric potential of the mycelium. Previous work carried out by Kalberer, (1990); Magan *et al.* (1995) and Noble (1998) have shown that *A. bisporus* mycelium and fruit bodies were more sensitive to changes in matric than osmotic potential. Noble *et al*. (1999) reported that mycelium growth and sporophore production are linked to the chemical and physical structure of the compost and casing soil and can withstand decreases in the casing soil matric potential below -0.1 MPa without any adverse effect on sporophore yield. What is not known is how the sporophore can withstand changes in the matric potential of the

compost and casing soil and if it creates an osmotic potential gradient in response to the decrease in the matric potential by accumulating mannitol or if it has adjusted over time to changes in compost and casing soil matric potential without creating an osmotic gradient? This chapter examines the differences in *A. bisporus* mycelial growth, osmotic, matric and water potential and the accumulation of endogenous sugars and polyols on two different media: malt extract media which is a nutrient rich defined media and media prepared from phase II compost supplemented with yeast extract under both osmotic and matric stresses.

3.2 MATERIALS AND METHODS

The *A. bisporus* strain A12 was used to study the effects of osmotic and matric potential on the growth rate and endogenous sugars and polyols. The media used in this study were compost extract/complete yeast extract media (CE/CYM) and malt extract agar medium (MEA) (Oxoid). The ionic solute KCl and the non-ionic solute glycerol were used to lower the water potential osmotically and PEG 8000 to lower the water potential matrically. The effect of the lowered water potentials was measured at two temperatures 18 and 25°C.

3.2.1 Osmotic and matric adjustment of media

The amount of each of the following solutes: KCl (Merck BDH), glycerol (Sigma) and PEG 8000 (Sigma) added to lower the water potential of malt extract media to -0.98, -1.48 and -2.48 MPa are indicated in Table 3.1 (Dallyn & Fox, 1980; Whiting & Rizo, 1999). Because agar does not solidify in the presence of PEG 8000 it was directly added to 5% (w/v) malt extract broth (MEB, Lab M) while KCl and glycerol were added to 5% (w/v) malt extract agar (MEA, Oxoid). The media was autoclaved as described in Section 2.2.

The water potential of CE/CYM (prepared as detailed in Section 2.2.1) with 2% (w/v) technical agar no.3 (Oxoid) and CE/CYM broth was determined by constructing calibration curves of the media when modified with different concentrations of the following solutes: KCl, glycerol and PEG 8000. The following amounts: 0.5, 1, 1.5, 2, 2.5 and 3 g of KCl and glycerol were individually added to 50 ml of CE/CYM with 2% (w/v) technical agar no.3 (Oxoid) and the media autoclaved as described above. To individual 50 ml aliquots of CE/CYM broth 5, 10, 15, 20, 25 and 30 g of PEG 8000 were added and the media autoclaved as described above. From these calibration curves the amount of KCl, glycerol and PEG 8000 solute required to lower the water potential of CE/CYM media to –0.48, -0.98, -1.48 and -2.48 MPa was calculated and is shown in Table 3.2. The solute-adjusted media was autoclaved at 121°C and 15 psi for 20 minutes and then placed in a 55°C waterbath to allow it to cool.

Solidified media (approx. 20 ml) were dispensed into 9 cm Petri dishes using a sterile graduated 50 ml centrifuge tube (TRP, Switzerland). The media were then covered with sterile cellophane discs (9 cm in diameter, P400, Cannings Ltd, Bristol) which had been placed in kilner jars and rinsed with distilled water for 5 minutes and then covered with distilled water and autoclaved at 121°C and 15 psi for 15 minutes. For liquid media modified matrically with PEG 8000 and the un-modified liquid media, approx. 20 ml were dispensed onto 9 cm circular discs of sterile capillary matting which were then covered with sterile discs of black polyester and cellophane which provided a support for fungal growth (Mswaka & Magan, 1999).

The MEA and MEB media treatments were centrally inoculated with 5 mm plugs removed (using a no.2 cork borer) from the growing margin of *A. bisporus* (A12) cultures grown on 5% (w/v) MEA. The CE/CYM agar and CE/CYM broth media were also centrally inoculated with 5 mm plugs which were removed from the growing margin of A12 cultures grown on CE/CYM with 2% (w/v) agar.

Each modified and un-modified media treatment was replicated five times and numbered 1-5, a single replicate from each of the treatments was randomly stacked together and placed in polyethylene bags to prevent desiccation. Therefore for MEA/MEB media treatments there were two sets of fives stacks (each stack had 11 plates and a single replicate from each solute treatment) and for CE/CYM media treatments there were also two sets of fives stacks (each stack had 14 plates and a single replicate from each solute treatment). For each media treatment one set of replicated plates were incubated at 18°C and another set at 25°C.

The colony radial growth was measured by measuring the colony diameter in cms. Two measurements were taken from the centre of the inoculum: one was taken to the growing edge of the largest diameter of the colony and the other was at right angles to this measurement and the mean of these measurements was then calculated. Measurements were taken for up to four weeks or until the mycelial colonies had fully colonised the media treatments and then a sample of the mycelium was removed from the cellophane of each of the treatments and placed in a 2 ml Eppendorf tube (Ependorf AG), weighed and then frozen in liquid N_2 and kept at -70°C until required for sugar and polyol analysis as described in Section 2.6. A second fungal mycelial sample was also removed at the same time from the cellophane and the total water (Ψ_w), osmotic (Ψ_π) and matric (Ψ_m) potential were measured as described in Section. 3.2.3.

3.2.3 Mycelial water, osmotic and matric potential measurements

The water potential of fungal samples was measured using a Wescor C-52 sample chamber which contains a thermocouple transducer which was used as a psychrometer (wet bulb depression method) and connected to the Wescor HR-33T microvoltmeter which gave measurements in microvolts (μV) which were converted to MPa.

Fungal mycelium samples were placed in the sample holder ensuring that it was only 50-75% full to avoid coming into contact with the thermocouple as this would lead to incorrect readings. Total water potential (Ψ_w) measurements were made by placing the sample holder in the sample chamber and taking readings from the microvoltmeter. The osmotic potential (Ψ_π) was measured by freeze-thawing the sample where it was wrapped in aluminium foil and frozen in liquid N_2 and then removed and allowed to thaw at room temperature before the sample osmotic potential was measured in the sample chamber. This freeze-thawing of the sample ruptures the cells thereby reducing the turgor potential to zero. The turgor potential was then calculated on the basis of the following equation: $\Psi_w = \Psi_\pi + \Psi_p$ i.e. the difference between the water and osmotic potential (Eamus & Jennings, 1984).

Table 3.1: Solute concentrations of PEG 8000, KCl and glycerol required to give specified water potentials in 5% (w/v) malt extract agar (MEA) and malt extract broth (MEB) media.

	Solute g/300 ml of media		
Water Potential MPa	PEG 8000 +MEB	KCl + MEA	Glycerol + MEA
-0.48	0 + 15	0 +15	0 +15
-0.98	60 + 15	2.24 + 15	5.52 + 15
-1.48	90 + 15	4.47 + 15	11.04 + 15
-2.48	127.5 + 15	11.2 + 15	22.08 + 15

Table 3.2: Solute concentrations of PEG 8000, KCl and glycerol required to give specified water potentials in compost extract/complete yeast extract media (CE/CYM).

	Solute g/200 ml of CE/CYM broth	Solute:g/300 ml of CE/CYM agar	
Water potential MPa	PEG 8000	KCl	Glycerol
-0.28	0	0	0
-0.48	16.24	1.41	3.84
-0.98	36.2	4.08	5.62
-1.48	56.1	6.84	13.01
-2.48	96.06	12.27	22.18

3.3 RESULTS

3.3.1 In vitro growth of *A. bisporus* colonies in response to reduced osmotic and matric potential on two treatments MEA and CE/CYM

The growth of *A. bisporus* (A12) mycelia, measured as the diameter of the mycelial colony, at 18 and 25°C on two media treatments: CE/CYM and MEA/MEB which were modified ionically with KCl, non-ionically with glycerol and matrically using PEG 8000 and the un-modified CE/CYM broth and agar media and MEA/MEB media was measured over a period of 26 days. For MEA/MEB treatments the water potential of the media was –0.48 MPa and was modified with the above solutes to achieve water potentials of –0.98, -1.48 and –2.48 MPa. For CE/CYM treatment the water potential of the media was measured and found to be –0.28 MPa and adjusted with the above solutes to achieve water potentials of –0.48, -0.98, -1.48 and –2.48 MPa.

The *A. bisporus* growth data was analysed using Genstat v.6 for Windows unless otherwise stated. There were five replica plates for each modified and un-modified media treatment and the mean (of five replicates) change in colony diameter over time was taken and plotted for each modified and un-modified treatment. The growth rate (change in colony diameter with time) $cmday^{-1}$ was measured for each of the 5 replicates per treatment and was log transformed using the following equation:

$$\log_{10}(100 \text{ X growth rate} + \alpha)$$

A value of alpha (α) was chosen, using the statistical package R Foundation for Statistical Computing Version 1.8.1, for all the growth rate data based on the log transformation model which shows the best value for α for the transformation of the data (Venables & Ripley, 2002) and this plot is shown in Appendix 3 and from this plot a value of 1 was chosen for α. Statistical analysis was carried out on the mean log transformation of the growth rate of the 5 replicates for each modified and un-modified media treatment using Genstat v.6 for Windows.

3.3.1.1. *A. bisporus* hyphal growth on MEA and MEB stressed media

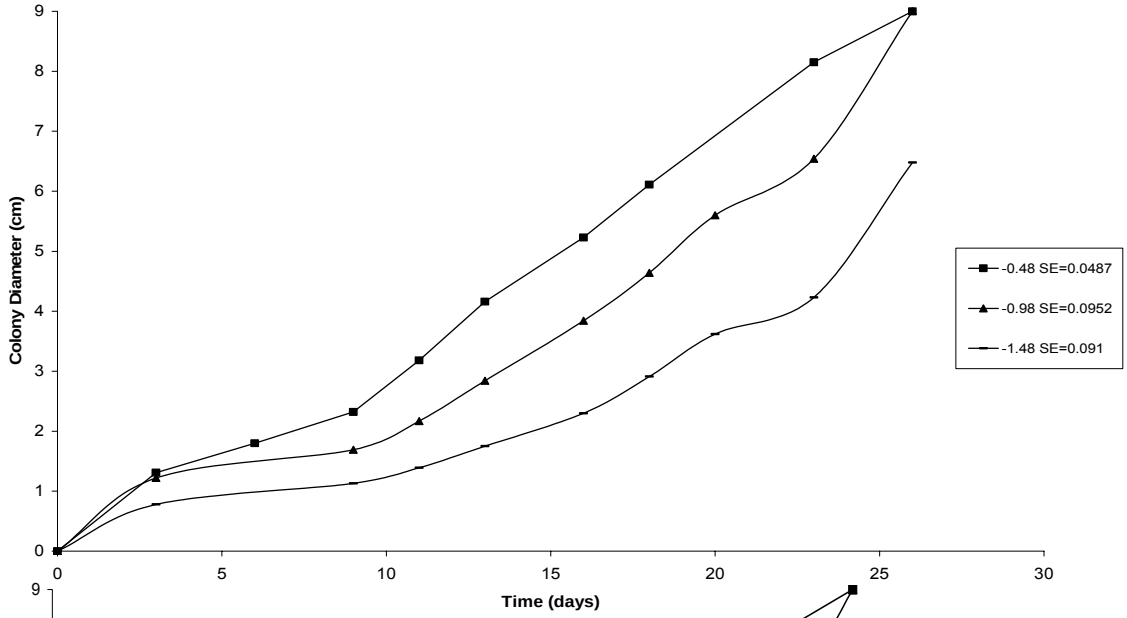
The mean (of five replicates) change in *A. bisporus* hyphal growth measured as colony diameter (cm) with time (days) for each of the un-modified and ionically, non-ionically and matrically modified malt extract media treatments at 18°C is shown in Figure 3.1 and at 25°C in Figure 3.2. At both 18 and 25°C the mycelial growth rate was reduced on all the solute adjusted media compared to the non-adjusted media and with increasing water stress the mycelial growth decreased on all three solute adjusted malt extract media treatments.

At 18°C there was mycelial growth on all the osmotically (non-ionically) stressed MEA media at –0.98, -1.48 and –2.48 MPa. There was no hyphal growth on the driest treatment (–2.48 MPa) on matrically and ionically modified MEB and MEA respectively and there was also no growth on matrically modified MEB at –1.48 MPa. There was no growth of *A. bisporus* mycelia on PEG 8000 adjusted media at –0.98 MPa during the first five days and after this time period the mycelial growth was slow indicating that the mycelia must undergo a period of adjustment before any growth can take place. *A. bisporus* mycelia was more sensitive to matric than osmotic stresses and mycelia was least sensitive to non-ionic than ionic water stresses which was evident from the observed mycelial growth pattern whereby the mycelia grew on all ionically induced water stresses but not on all non-ionically induced water stresses.

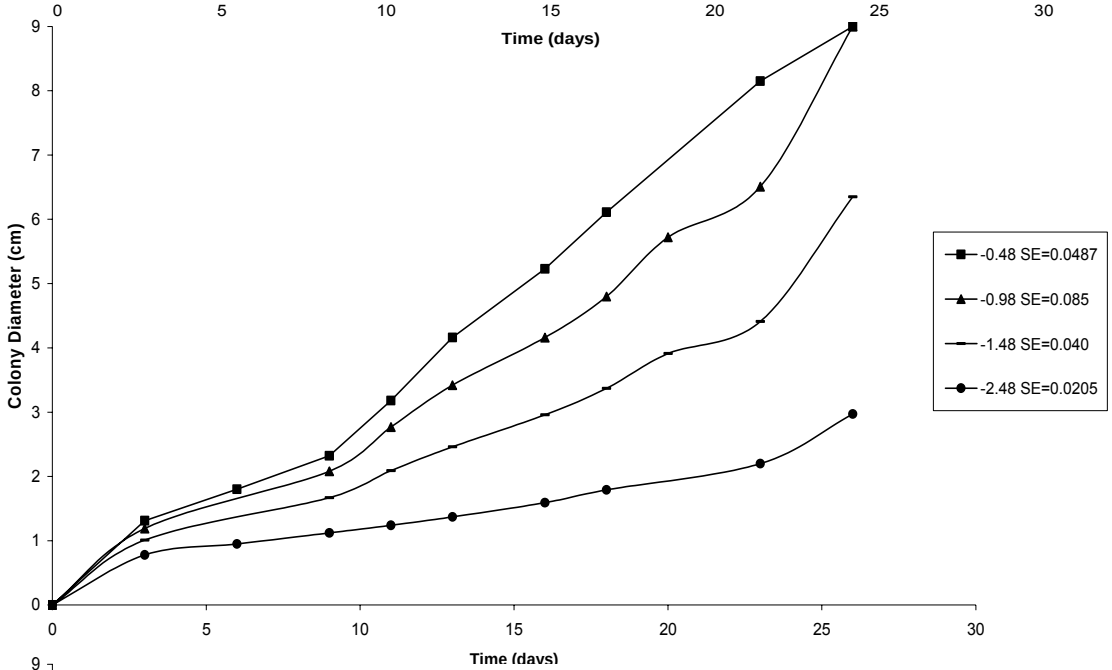
At 25°C there was mycelial growth on all the non-ionically stressed media at –0.98, -1.48 and –2.48 MPa. There was no hyphal growth at –2.48 MPa on matrically and ionically modified MEB and MEA media respectively. There was no growth of *A. bisporus* mycelia on PEG 8000 adjusted media at –0.98 MPa during the first 3 days and at -1.48 MPa there was no growth for the first 9 days after which there was slow growth of the mycelia indicating that the mycelia had an adjustment period to the matrically stressed media.

Figure: 3.1: The mean change in colony diameter (cm) with time (days) of *Agaricus bisporus* (A12) mycelia grown on un-modified and matrically and osmotically modified malt extract media was measured at 18°C. *A. bisporus* hyphal growth was measured on malt extract agar (MEA) (water potential of -0.48 MPa) and osmotically modified MEA with KCl (A) and glycerol (B) to produce the following water potentials -0.98, -1.48 and -2.48 MPa. The effect on *A. bisporus* hyphal growth was also measured on malt extract broth (MEB) (-0.48 MPa) and MEB modified matrically with PEG 8000 (C) to reduce the water potentials as described above. There was no mycelial growth on matrically modified media at -1.48 and -2.48 MPa and on ionically (KCl) modified media at -2.48 MPa.

(A)


Colony Diameter (cm)
Time (days)
-0.48 SE=0.0487
-0.98 SE=0.0952
-1.48 SE=0.091

(B)


Colony Diameter (cm)
Time (days)
-0.48 SE=0.0487
-0.98 SE=0.085
-1.48 SE=0.040
-2.48 SE=0.0205

(C)

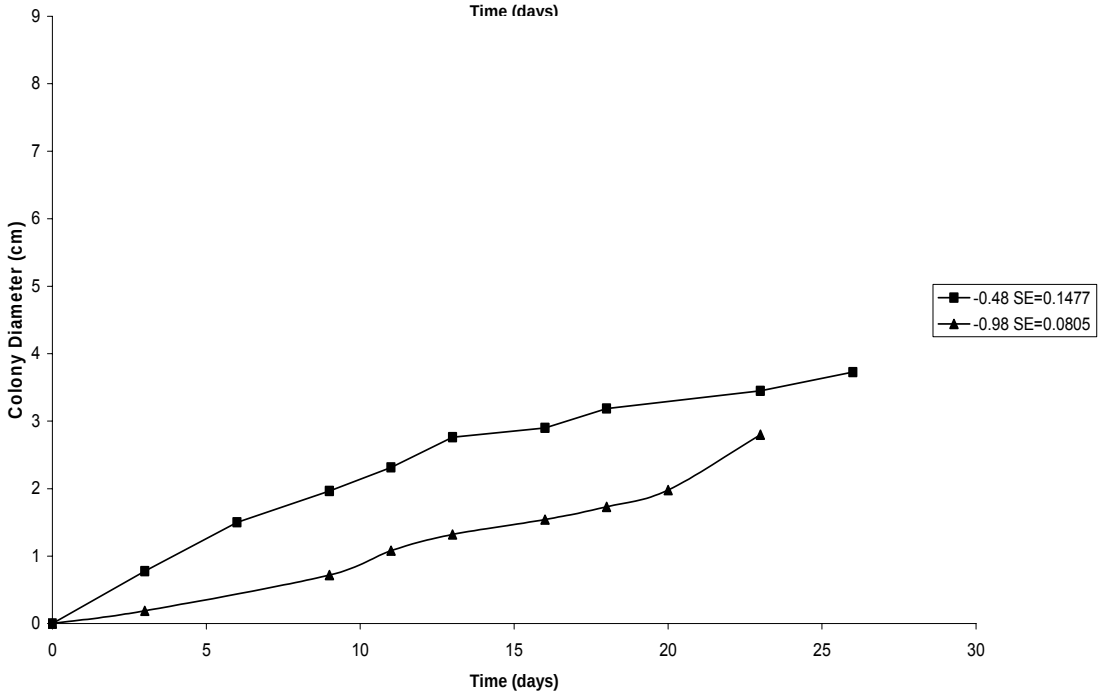
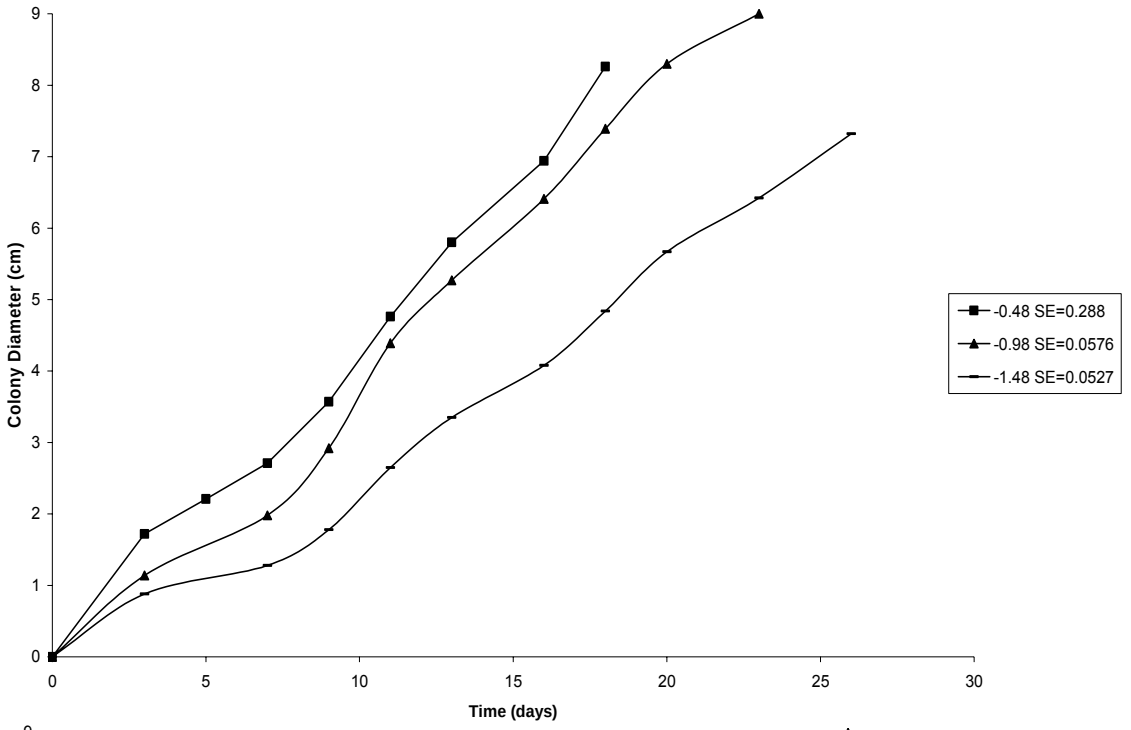
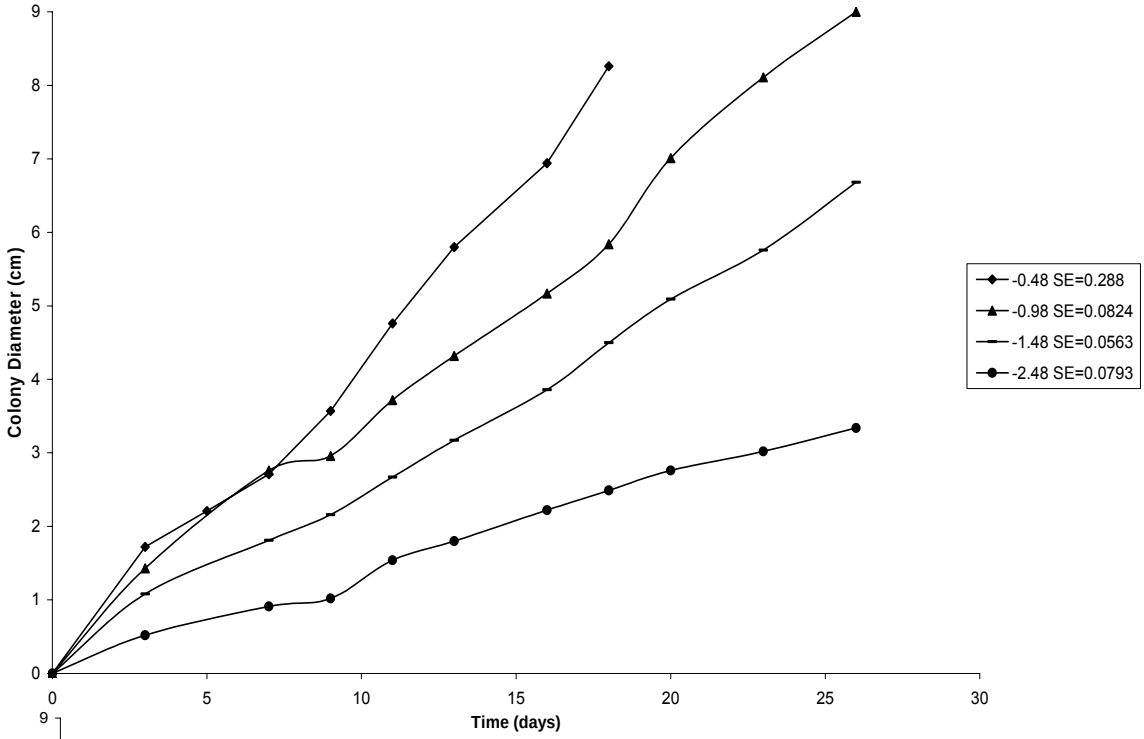

Colony Diameter (cm)
Time (days)
-0.48 SE=0.1477
-0.98 SE=0.0805

Figure 3.2: The mean change in colony diameter (cm) with time (days) of *Agaricus bisporus* (A12) mycelia grown on un-modified and matrically and osmotically modified malt extract media was measured at 25° C. *A. bisporus* hyphal growth was measured on malt extract agar (MEA) (water potential of -0.48 MPa) and osmotically modified MEA with KCl (A) and glycerol (B) to produce the following water potentials -0.98, -1.48 and -2.48 MPa. The effect on *A. bisporus* hyphal growth was also measured on malt extract broth (MEB) (-0.48 MPa) and MEB modified matrically with PEG 8000 (C) to reduce the water potentials as described above. There was no mycelial growth on matrically modified media at -1.48 and -2.48 MPa and on ionically (KCl) modified media at -2.48 MPa.

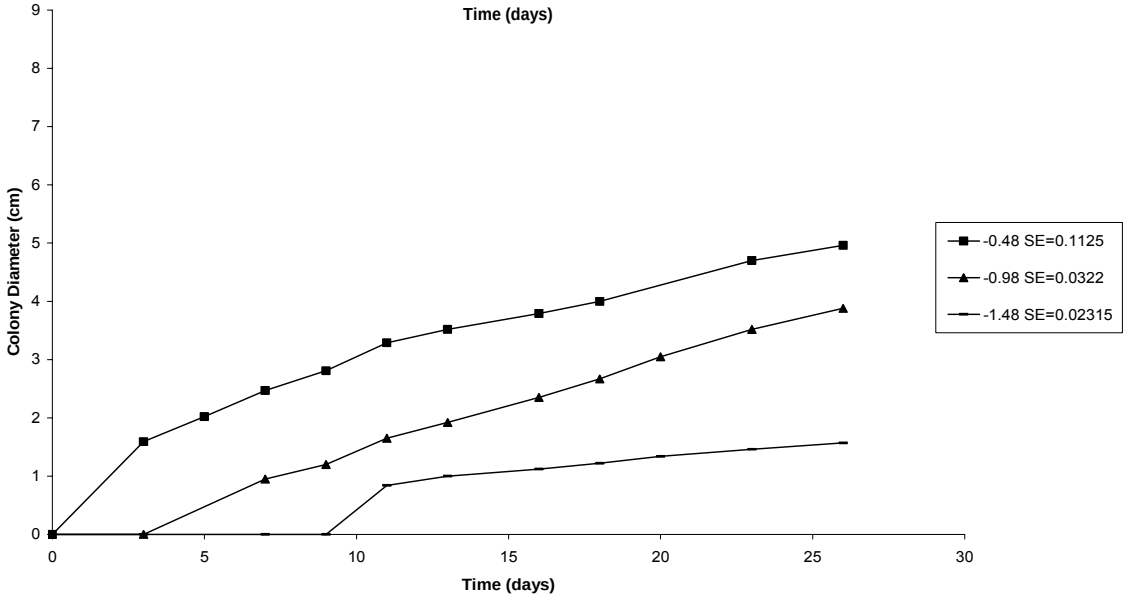
(A)


Colony Diameter (cm)
Time (days)
-0.48 SE=0.288
-0.98 SE=0.0576
-1.48 SE=0.0527

(B)


Colony Diameter (cm)
Time (days)
-0.48 SE=0.288
-0.98 SE=0.0824
-1.48 SE=0.0563
-2.48 SE=0.0793

(C)


Colony Diameter (cm)
Time (days)
-0.48 SE=0.1125
-0.98 SE=0.0322
-1.48 SE=0.02315

3.3.1.2 *A. bisporus* hyphal growth on CE/CYM stressed media

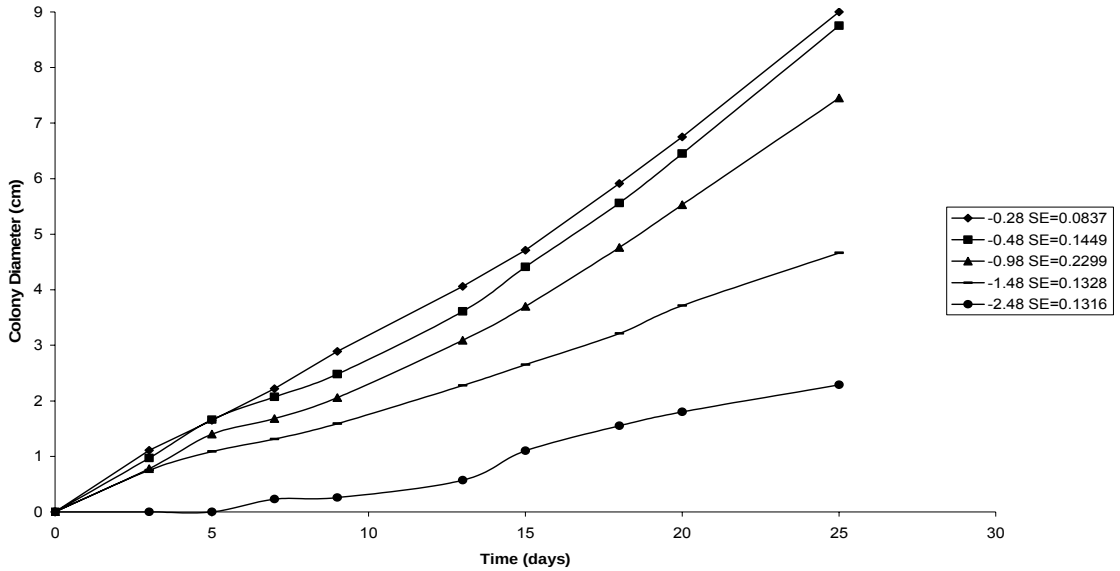
The mean (of five replicates) change in *A. bisporus* hyphal growth measured as colony diameter (cm) with time (days) on each of the un-modified and ionically, non-ionically and matrically modified CE/CYM media at 18 and 25°C are shown in Figure 3.3 and 3.4 respectively. At both 18 and 25°C the mycelial growth rate was reduced on all the solute adjusted media compared to the non-adjusted media and with increasing water potential stress the mycelial growth decreased for all three solute adjusted CE/CYM media. However the mycelial growth was higher for PEG 8000 adjusted CE/CYM broth at 18°C than on un-adjusted CE/CYM broth but in this instance only there was one replicate for mycelial growth on un-adjusted CE/CYM broth due to contamination of the other 4 replica plates (unlike all the other adjusted and un-adjusted media treatments where there were five replicates). Therefore the mycelial growth pattern for the un-adjusted CE/CYM broth cannot be taken as a reliable data set.

At 18°C there was mycelial growth on ionically and non-ionically stressed media at –0.48, -0.98, -1.48 and –2.48 MPa and there was no mycelial growth at –2.48 MPa on CE/CYM broth modified matrically. At –1.48 MPa there was no growth during the first five days on PEG 8000 adjusted CE/CYM broth after which there was mycelial growth and this pattern of an initial lag phase in growth was also seen on ionically adjusted CE/CYM agar at –2.48 MPa.

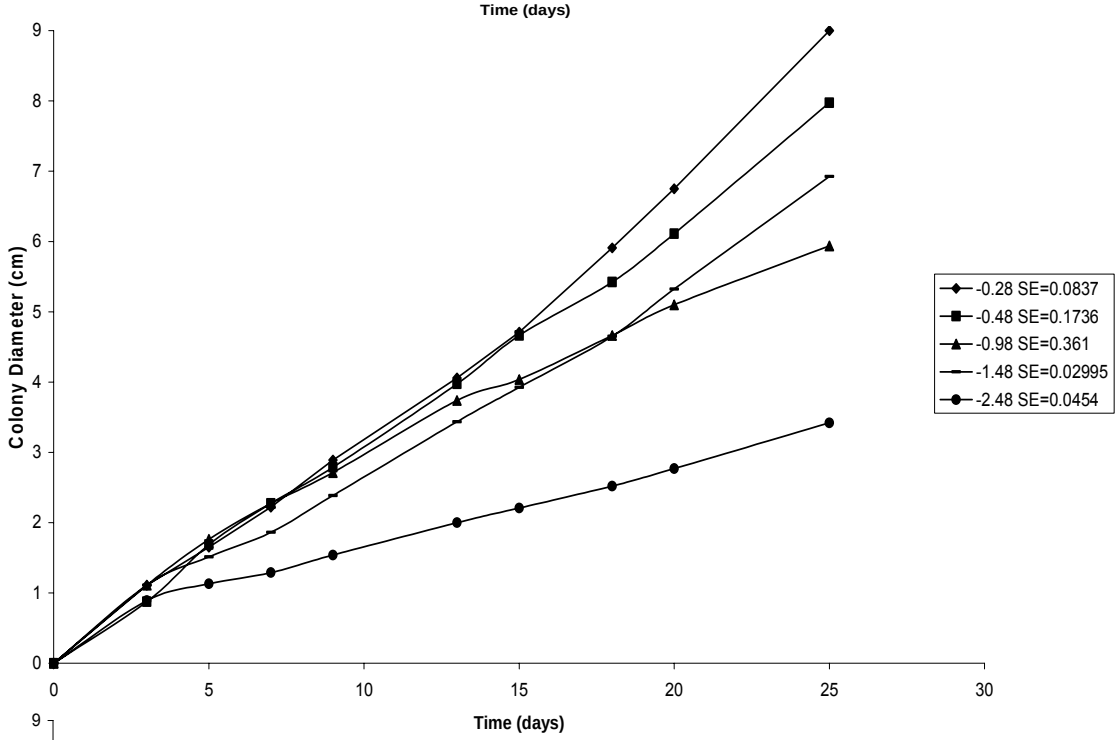
At 25°C *A. bisporus* mycelia grew on the CE/CYM media modified matrically and osmotically (non-ionically and ionically) at all lowered water potentials (–0.48, -0.98, -1.48 and –2.48 MPa). At a water stress of –2.48 MPa there was no growth during the first five days on PEG 8000 adjusted CE/CYM broth after which there was mycelial growth and this pattern of an initial lag phase in mycelial growth was also observed on KCl adjusted CE/CYM agar at –2.48 MPa.

Figure 3.3: The mean change in colony diameter (cm) with time (days) of *Agaricus bisporus* (A12) mycelia grown on un-modified and matrically and osmotically modified compost extract/complete yeast extract media (CE/CYM) was measured at 18° C. *A. bisporus* hyphal growth was measured on CE/CYM solid media (water potential of -0.28 MPa) and osmotically modified CE/CYM with KCl (A) and glycerol (B) to produce the following water potentials -0.48, -0.98, -1.48 and -2.48 MPa. The effect on *A. bisporus* hyphal growth was also measured on CE/CYM broth (-0.28 MPa) and matrically modified CE/CYM broth with PEG 8000 (C) to the reduced water potentials as described above. There was no mycelial growth on matrically modified media at a water stress of -2.48 MPa.

(A)


Colony Diameter (cm)
Time (days)
-0.28 SE=0.0837
-0.48 SE=0.1449
-0.98 SE=0.2299
-1.48 SE=0.1328
-2.48 SE=0.1316

(B)


Colony Diameter (cm)
Time (days)
-0.28 SE=0.0837
-0.48 SE=0.1736
-0.98 SE=0.361
-1.48 SE=0.02995
-2.48 SE=0.0454

(C)

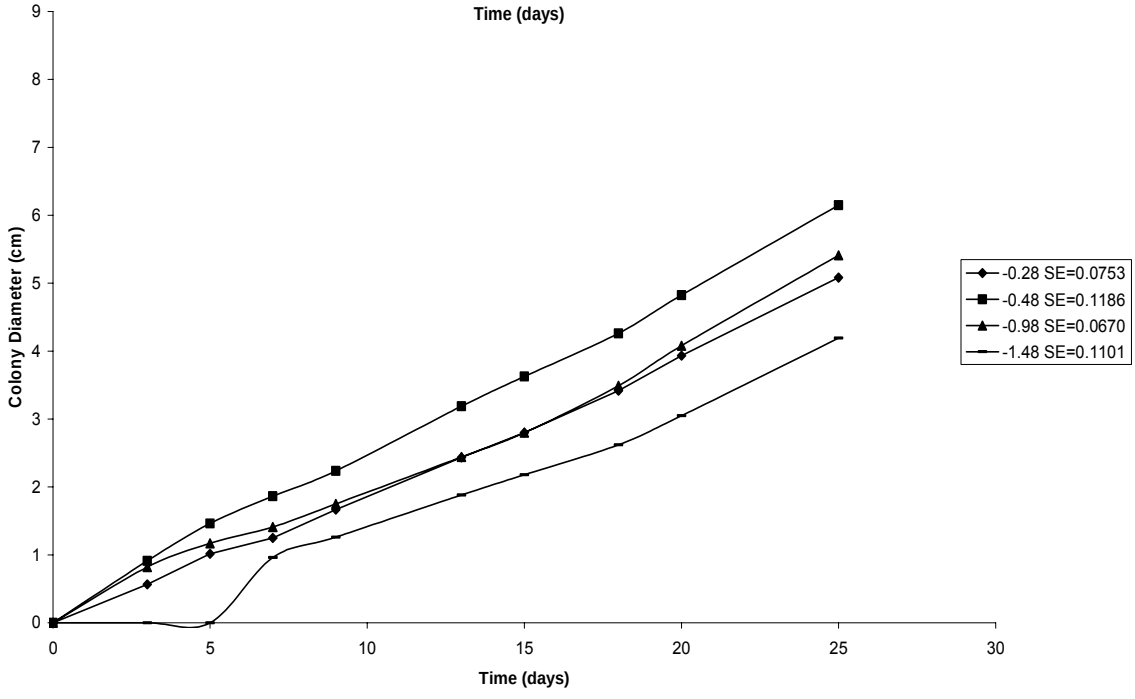
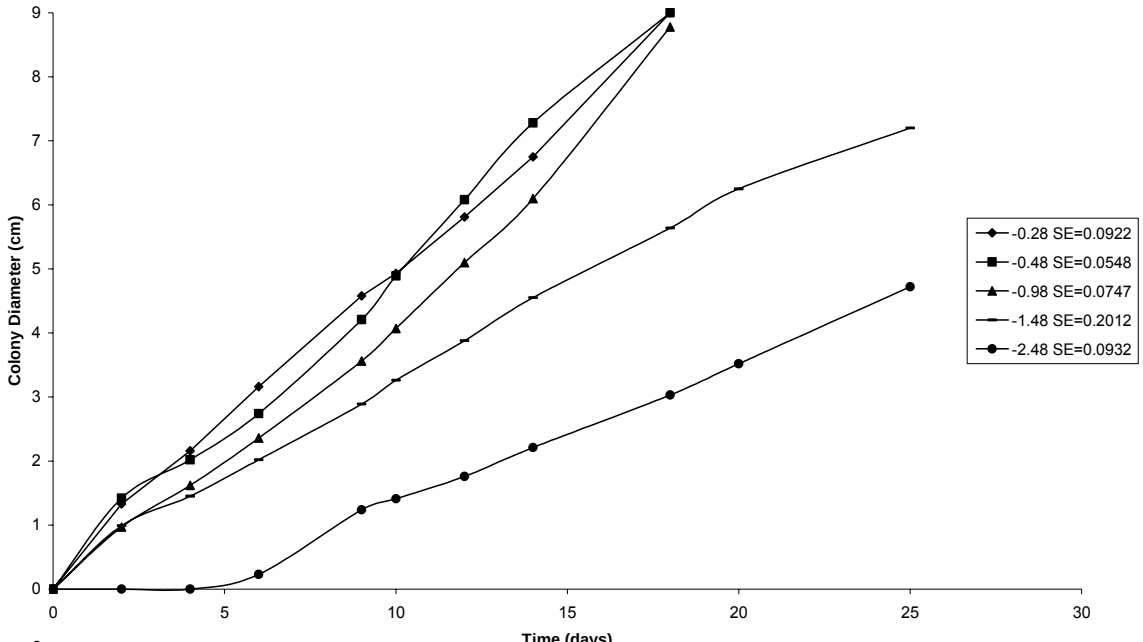
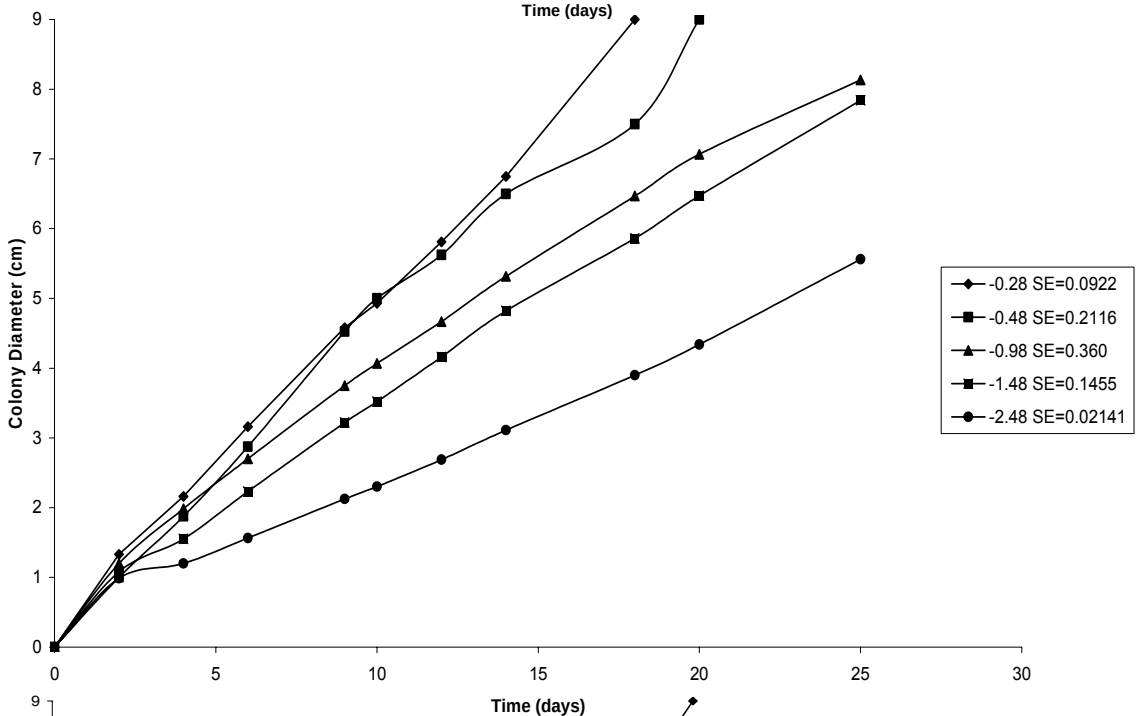

Colony Diameter (cm)
Time (days)
-0.28 SE=0.0753
-0.48 SE=0.1186
-0.98 SE=0.0670
-1.48 SE=0.1101

Figure 3.4: The mean change in colony diameter (cm) with time (days) of *Agaricus bisporus* (A12) mycelia grown on un-modified and matrically and osmotically modified compost extract/complete yeast extract media (CE/CYM) was measured at 25° C. *A. bisporus* hyphal growth was measured on CE/CYM solidified media (water potential of -0.28 MPa) and osmotically modified CE/CYM with KCl (A) and glycerol (B) to produce the following water potentials -0.48, -0.98, -1.48 and -2.48 MPa. The effect on *A. bisporus* hyphal growth was also measured on CE/CYM broth (-0.28 MPa) and matrically modified CE/CYM broth with PEG 8000 (C) to reduce the water potentials as described above. There was mycelial growth on all solute adjusted media treatments.

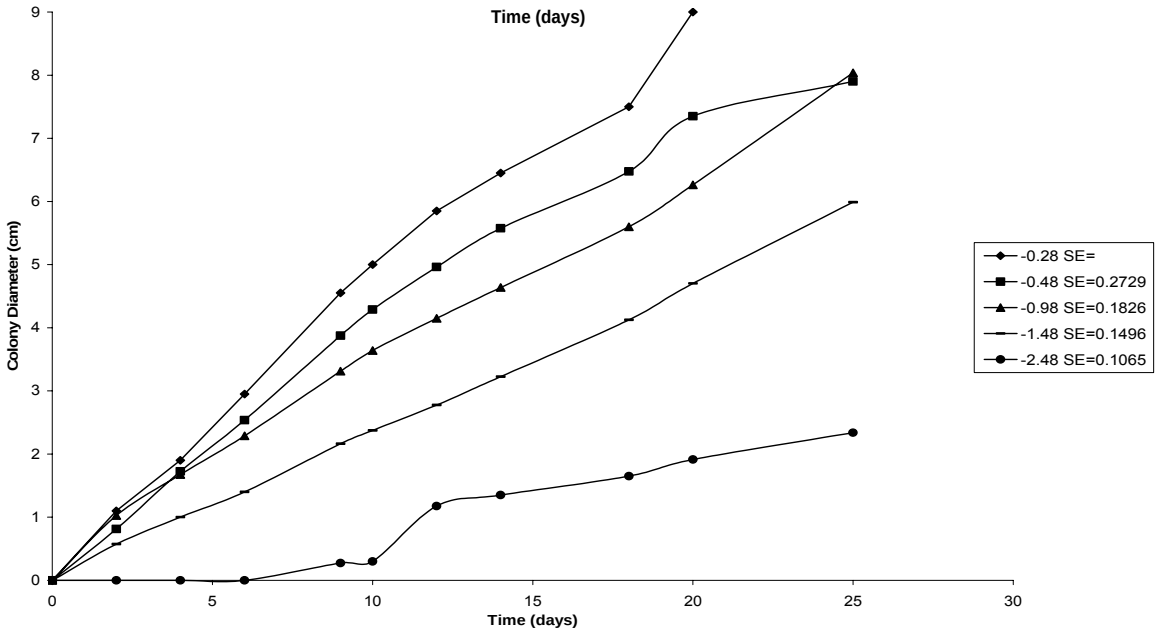
(A)


Colony Diameter (cm)
Time (days)
-0.28 SE=0.0922
-0.48 SE=0.0548
-0.98 SE=0.0747
-1.48 SE=0.2012
-2.48 SE=0.0932

(B)


Colony Diameter (cm)
Time (days)
-0.28 SE=0.0922
-0.48 SE=0.2116
-0.98 SE=0.360
-1.48 SE=0.1455
-2.48 SE=0.02141

(C)


Colony Diameter (cm)
Time (days)
-0.28 SE=
-0.48 SE=0.2729
-0.98 SE=0.1826
-1.48 SE=0.1496
-2.48 SE=0.1065

The log transformed growth rates of *A. bisporus* mycelia grown on un-adjusted and solute adjusted malt extract media at 18 and 25°C are shown in Figure 3.5. There was a significant ($P<0.001$) water potential effect on the growth rate, which decreased with decreasing water potential. There was a significant ($P<0.001$) combined solute and water potential effect, whereby the increase in solute concentration lowered the water potential which slowed the growth rate of the mycelia. The mycelia grown on matrically modified media had slower growth than that on non-ionically and ionically modified media and there was no mycelial growth on ionically and matrically adjusted media at -2.48 MPa. The mycelia was most sensitive to water stresses induced matrically than osmotically and was least sensitive to non-ionically and this was reflected in the higher mycelial growth rates measured on non-ionically adjusted media compared with those on ionically and matrically modified media. Although at -0.98 and -1.48 MPa the mycelial growth rate was slightly higher on ionically than non-ionically modified media at 25°C. There was also a significant temperature effect on the mycelial growth rate which was consistently lower for all three solute adjusted media treatments at 18°C than at 25°C.

The log transformed growth rates of *A. bisporus* mycelia grown on un-adjusted and solute adjusted CE/CYM media at 18 and 25°C are shown in Figure 3.6. There was a significant ($P<0.001$) water potential effect on the growth rate at both temperatures. There was also a significant ($P<0.001$) solute effect on the growth rate whereby at 18°C there was no mycelial growth on matrically modified media on the driest treatment (-2.48 MPa). The mycelial growth rate at both 18 and 25°C was least sensitive to non-ionically modified media and most sensitive to matrically modified media and this is reflected in the mycelial growth rates measured on these solute treatments. There was also a significant temperature effect on the growth rate which was slower at 18°C than at 25°C.

The mycelial growth rate at 18 and 25°C was higher on CE/CYM media compared to malt extract media for all three solute treatments. The mycelium was most

sensitive to changes in matric than osmotic potential and this is reflected in the lower mycelial growth rate measured on matrically modified media (for both CE/CYM and MEA/MEB media) at both temperatures compared to the ionically and non-ionically modified media.

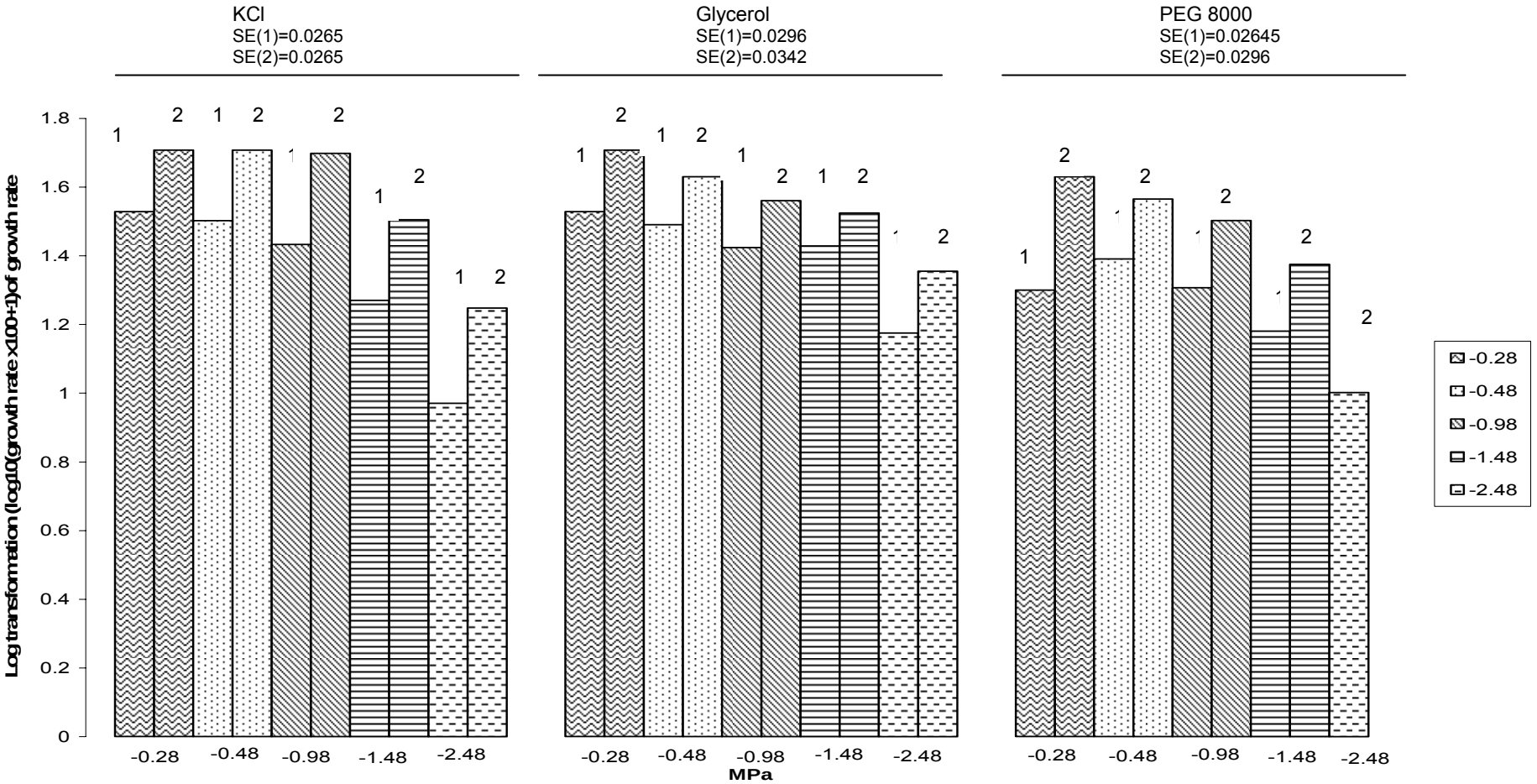


Figure 3.6: The log transformation of the growth rates (cmday^{-1}) of *Agaricus bisporus* mycelia in response to matric and osmotic stresses at 18°C (1) and 25°C (2) on compost extract/complete yeast extract media (CE/CYM). The solutes KCl and glycerol were added to the media to lower the water potential osmotically and PEG 8000 was added to lower it matrically. There was no mycelial growth on matrically modified media at -2.48 MPa at 18°C.

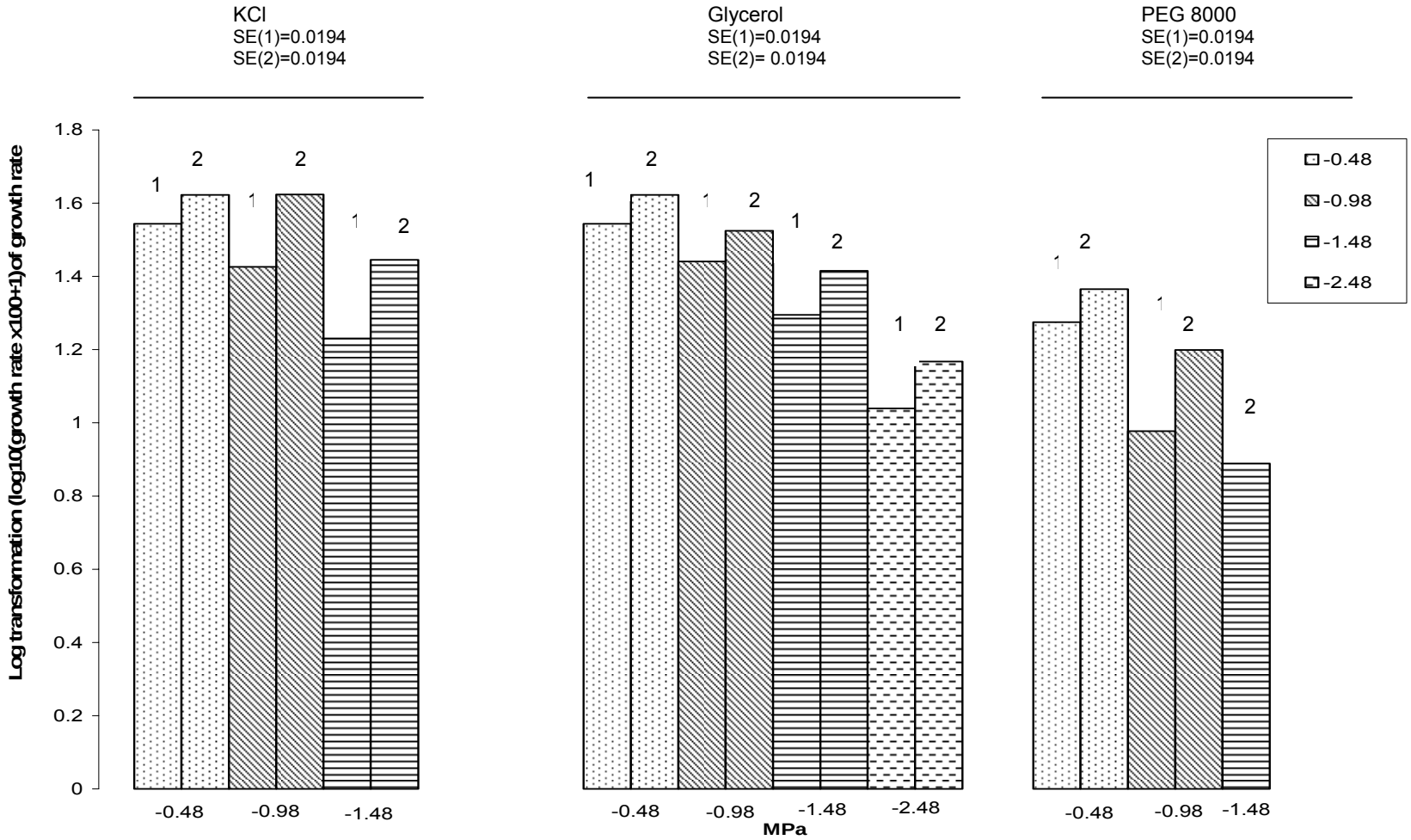


Figure 3.5: The log transformation of the growth rates (cmday^{-1}) of *Agaricus bisporus* mycelia in response to matric and osmotic stresses at 18°C (1) and 25°C (2) on malt extract media. The solutes KCl and glycerol were added to the media to lower the water potential osmotically and PEG 8000 was added to lower it matrically. There was no mycelial growth on matrically modified media at -1.48 MPa/18°C and at water potential of -2.48 MPa there was no mycelial growth on osmotically (KCl) and matrically modified media at both 18 and 25°C.

3.3.2 Water, osmotic and turgor potential measurements of mycelial samples grown on stressed MEA/MEB and CE/CYM media

When the mycelium had fully colonised the media in the 9 cm Petri plates or after 4 weeks growth for slower growing mycelium, it was removed from the surface of the cellophane overlays on the two different media treatments: CE/CYM and MEA/MEB, which were subjected to water stresses using the following solutes: KCl, glycerol and PEG 8000. The water, osmotic and turgor potentials of the mycelium were measured (Section 3.2.3) and converted to MPa for each of the five replicates for all of the un-modified and solute modified media and the mean (of five replicates) water, osmotic and matric potential was calculated. Statistical analysis of the data was carried out using Genstat v.6 for Windows.

3.3.2.1 MEA/MEB media

The mean hyphal water, osmotic and turgor potential of *A. bisporus* mycelia grown on un-modified MEA and MEB media and on ionically (KCl) and non-ionically (glycerol) modified MEA and matrically (PEG 8000) modified MEB media at 18 and 25°C are shown in Figure 3.7 and 3.8 respectively. At both temperatures and for all solute adjusted media treatments the mycelia had lower water potentials than that of the media on which they were grown.

At 18°C there was a significant ($P<0.001$) water potential effect on the mycelial osmotic and water potential and with increasing water potential stress the mean osmotic and water potential of the mycelia decreased for all the 3 solute adjusted media. The solute added also had a significant effect ($P<0.001$) on the water and osmotic potential of the mycelia, where the lowest osmotic and water potential were found on glycerol modified on the most stressed treatment (-2.48 MPa). However at -2.48 MPa there was no mycelial growth on ionically and matrically modified media and also at -1.48 MPa on matrically modified media, therefore there is no data for these treatments. At -0.98 MPa

the mycelia grown on PEG 8000 modified media had the lowest water and osmotic potential compared to the other solute treatments. The highest osmotic and water potential were found in mycelia grown on un-modified media (-0.48 MPa) as this media would have the greatest quantity of freely available water.

There were no significant solute or water potential effects on the mycelial turgor potential. There was no similar trend in the mycelial turgor potential as was observed for the osmotic and water potential for the solute treatments. The lowest turgor potential was found on unmodified MEA media (-0.48 MPa) and the highest mean turgor potential was found in mycelium grown on osmotically (non-ionically with glycerol) modified media at –2.48 MPa which also had the lowest osmotic and water potential.

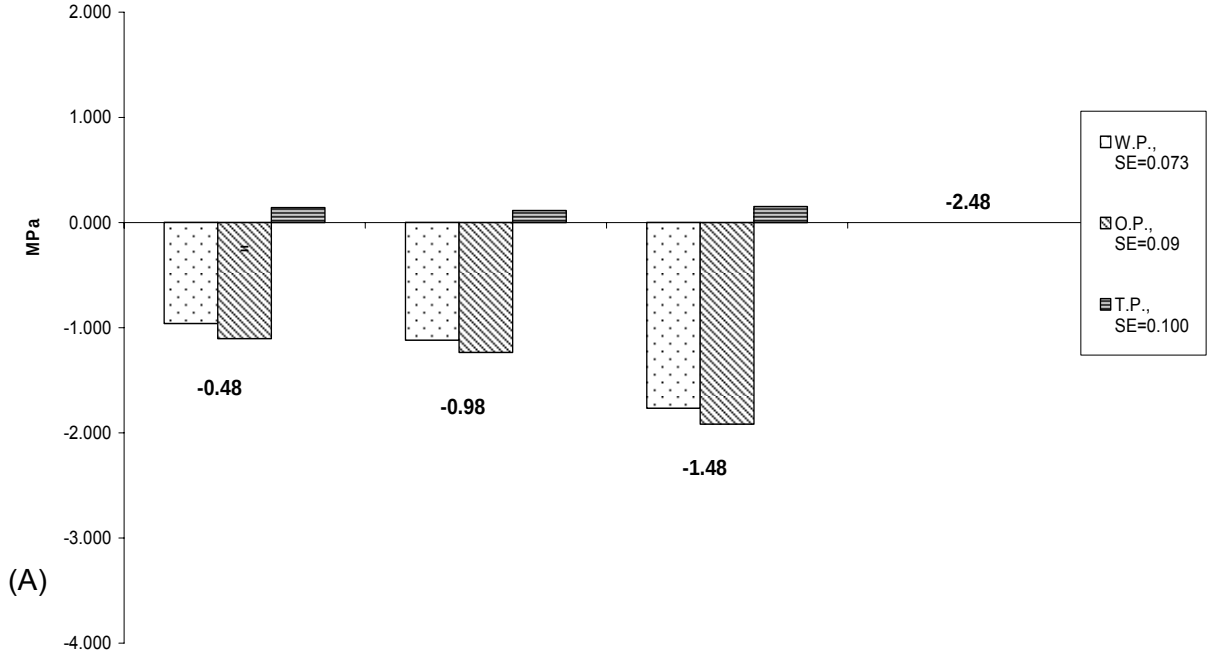
At 25°C there was a significant ($P<0.001$) water potential effect on the water and osmotic potential of the mycelia grown on all 3 solute adjusted media whereby the osmotic and water potential decreased with increasing water stress. There was also a significant ($P<0.001$) solute effect on the water and osmotic potential of the mycelia. The lowest osmotic and water potential were found on glycerol modified media at -2.48 MPa but there was no mycelial growth on KCl and PEG 8000 modified media at this water potential therefore we only have data for these treatments at -0.98 and -1.48 MPa. The lowest water and osmotic potential was consistently found on matrically modified media as the mycelia was most sensitive to changes in matric potential and the mycelia grew slowest on all the matrically modified treatments compared with the growth rates on KCl and glycerol modified media. However the media to which no solutes were added (-0.48 MPa) had the highest water and osmotic potential as this media would have the greatest quantity of freely available water.

There was no significant water potential or solute effect on the turgor potential of the mycelia at 25°C. The lowest mean turgor potential (+0.098 MPa) was found on KCl

modified media at –1.48 MPa compared to +0.364 MPa and +0.228MPa on glycerol and PEG 8000 modified media respectively.

There was no significant temperature effect on the water, osmotic or turgor potential of the mycelia. Overall, at both temperatures the mycelial water and osmotic potential tended to be higher on non-ionically modified media as was the mycelial growth rate compared with ionically and matrically modified media. At any imposed water stress media treatment the lowest water and osmotic potential was on matrically modified media which had the greatest effect on the water and osmotic potential.

Figure 3.7: The mean, of 5 replicates, total water (WP), osmotic (OP) and turgor potentials (TP) measured in mega pascals (MPa) of *Agaricus bisporus* (A12) mycelia grown on (A) KCl and (B) glycerol amended malt extract agar and (C) PEG 8000 amended malt extract broth, at 18°C. The mycelia were grown on un-modified malt extract media (-0.48 MPa) and malt extract media to which the above solutes were added to lower the water potential to -0.98, -1.48 and -2.48 MPa and the WP, OP, and TP of the mycelia were measured after growing on the media for 26 days or before this time if the mycelium had colonised the media. There was no mycelial growth on ionically (KCl) or matrically (PEG 8000) modified media at the lowest water stress -2.48 MPa or on matrically modified media at -1.48 MPa therefore there is no data for these treatments.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MPa
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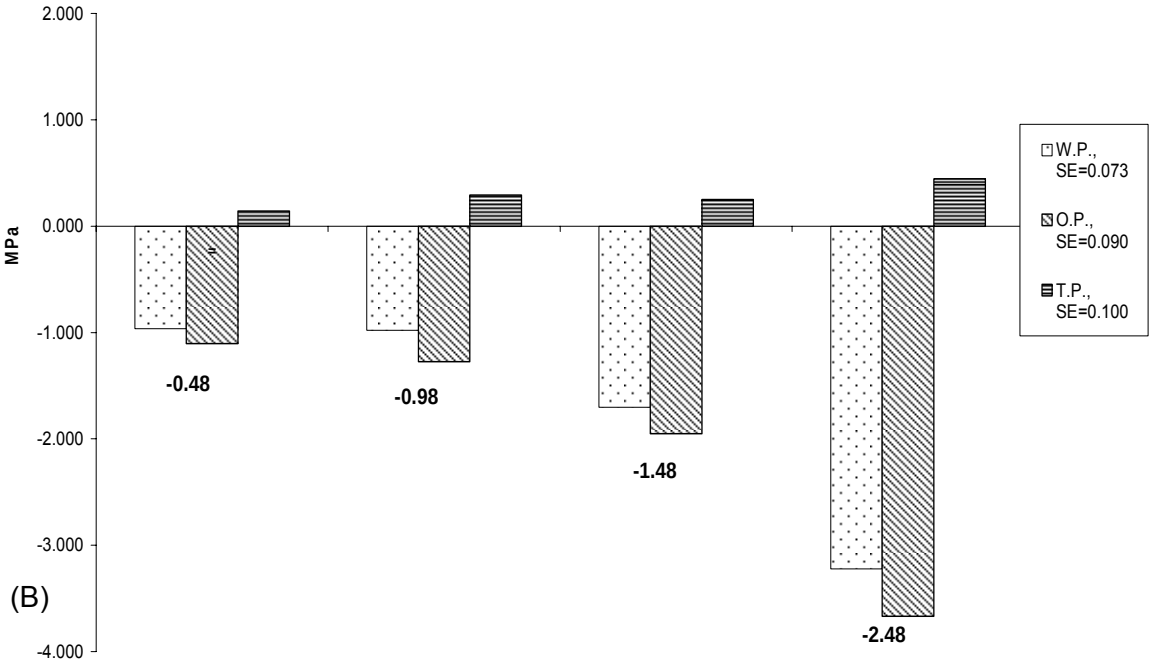
-2.48
W.P., SE=0.073
O.P., SE=0.09
T.P., SE=0.100

(A)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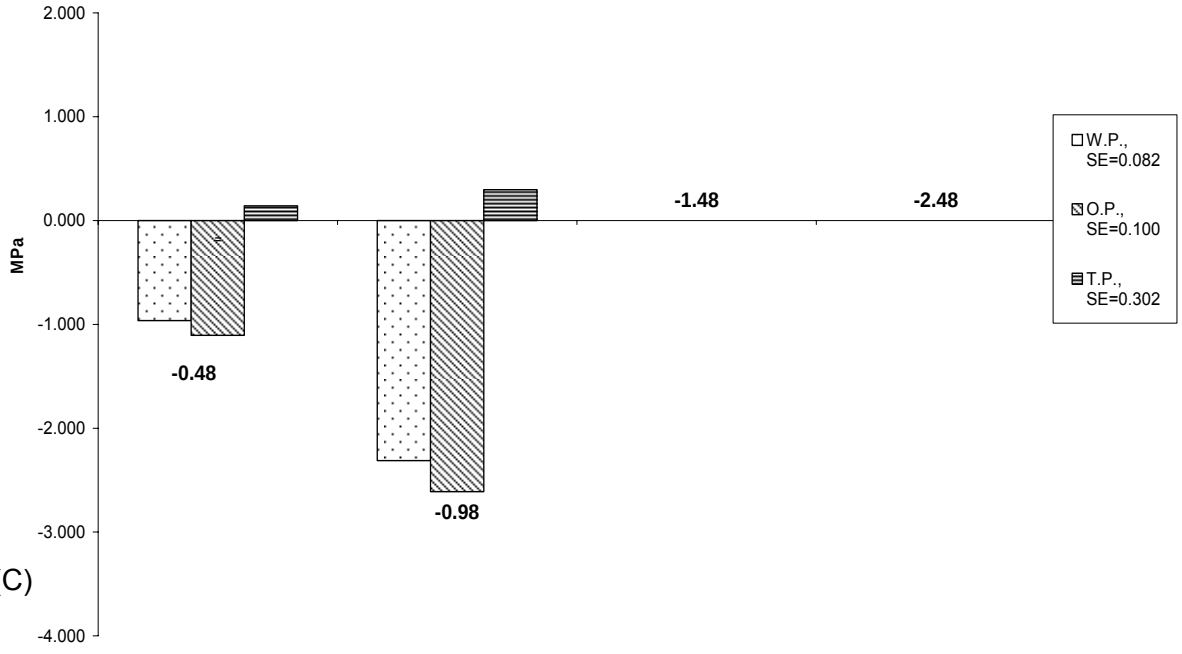
W.P., SE=0.073
O.P., SE=0.090
T.P., SE=0.100

(B)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-2.48
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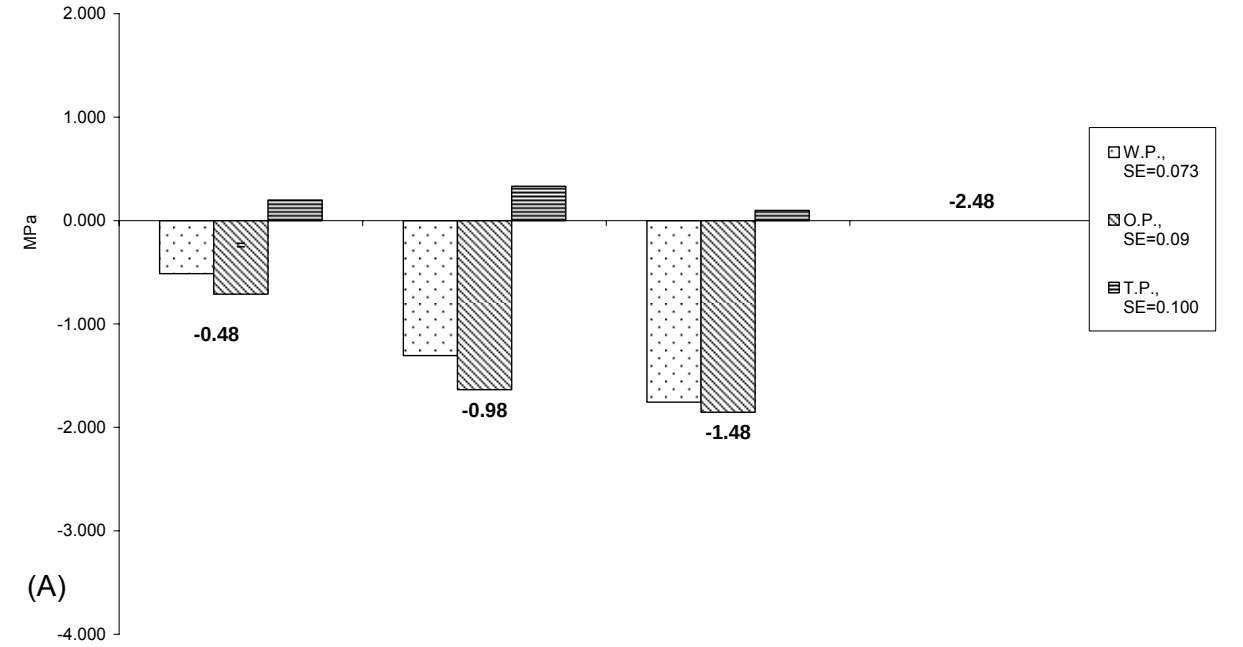
O.P., SE=0.100
T.P., SE=0.302

(C)

Figure 3.8: The mean, of 5 replicates, total water (WP), osmotic (OP) and turgor potentials (TP) measured in mega pascals (MPa) of *Agaricus bisporus* (A12) mycelia grown on (A) KCl and (B) glycerol amended malt extract agar and (C) PEG 8000 amended malt extract broth, at 25°C. The mycelia were grown on un-modified malt extract media (-0.48 MPa) and malt extract media to which the above solutes were added to lower the water potential to -0.98, -1.48 and -2.48 MPa and the WP, OP, and TP of the mycelia were measured after growing on the media for 26 days or before this time if the mycelium had colonised the media. There was no mycelial growth on ionically (KCl) or matrically (PEG 8000) modified media at the lowest water stress -2.48 MPa therefore there is no data for these treatments.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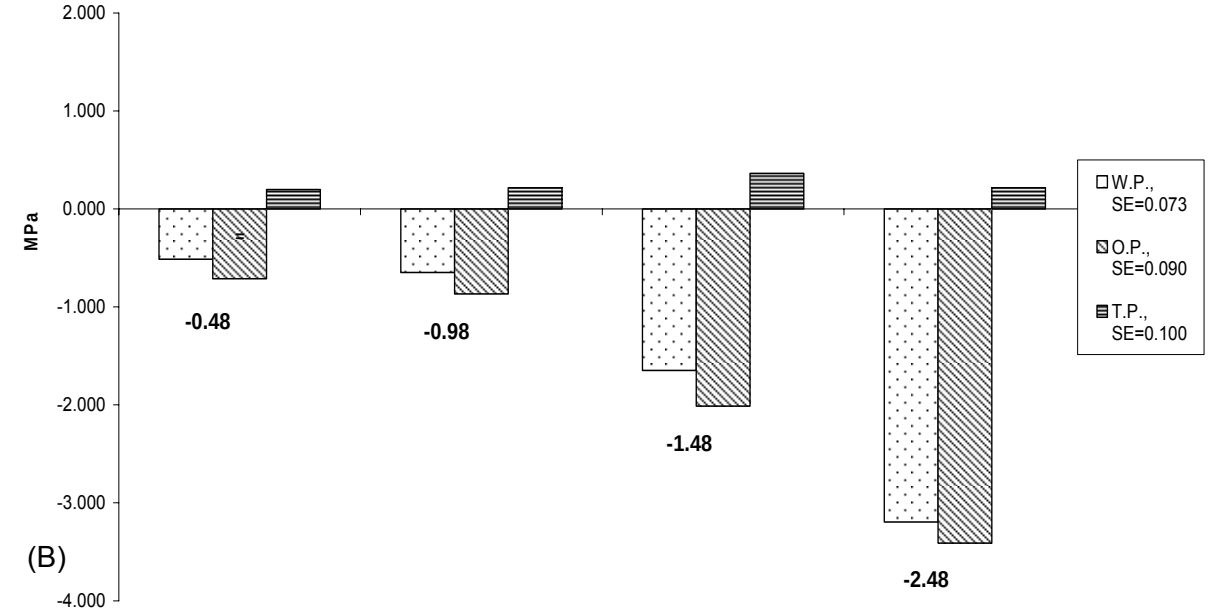
W.P., SE=0.073
O.P., SE=0.09
T.P., SE=0.100

(A)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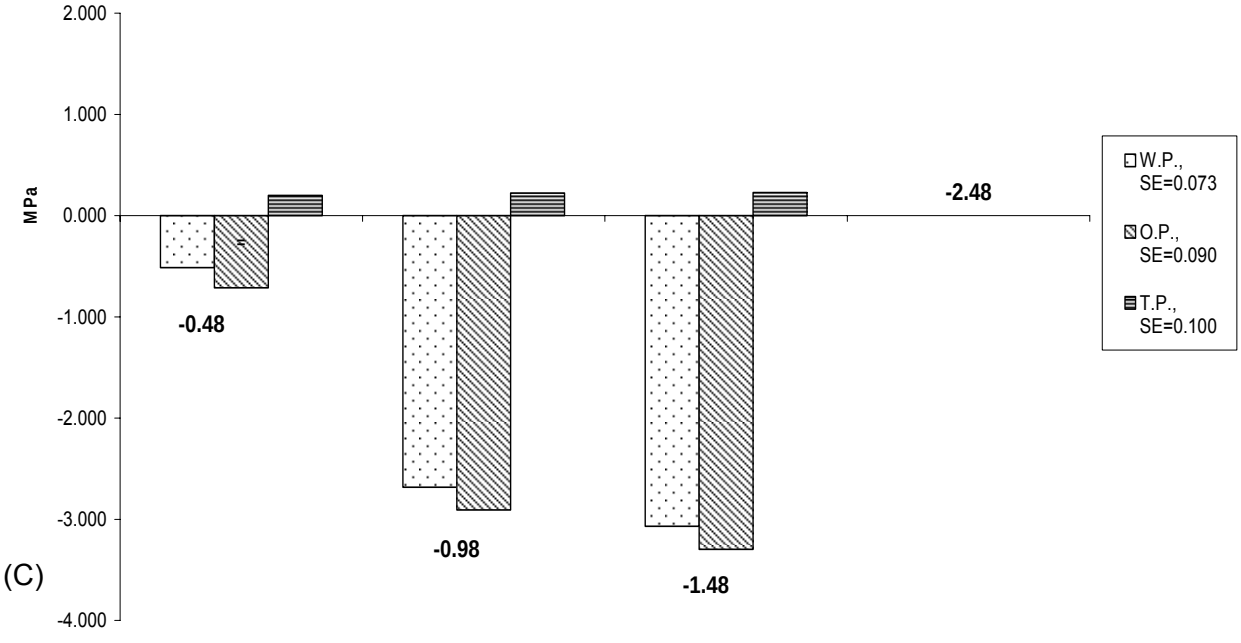
W.P., SE=0.073
O.P., SE=0.090
T.P., SE=0.100

(B)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-2.48
W.P., SE=0.073
O.P., SE=0.090
T.P., SE=0.100

(C)

3.3.2.2 CE/CYM media

The hyphal water, osmotic and turgor potential of *A. bisporus* mycelia grown on un-modified CE/CYM media and on ionically and non-ionically modified CE/CYM media and matrically modified CE/CYM broth media at 18 and 25°C are shown in Figure 3.9 and 3.10 respectively. At both 18 and 25°C the mycelial water and osmotic potential decreased with increasing water stress.

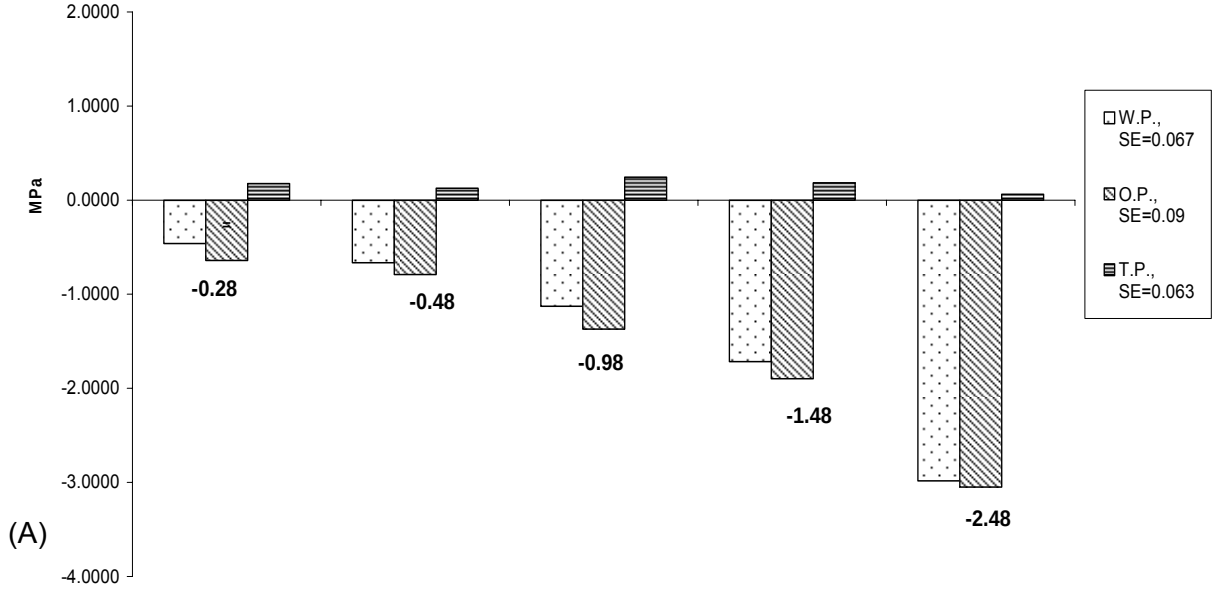
At 18°C there was no mycelial growth on matrically modified media at the highest water stress (-2.48 MPa) therefore there is no data for this treatment. There was a significant ($P<0.001$) water potential effect on the mycelial water and osmotic potential which decreased with increasing water stress and this trend was generally found for all solute treatments. However the highest mycelial osmotic and water potential was not found on the un-modified media (-0.28 MPa) which would have the most freely available water but on the least stressed (-0.48 MPa) matrically modified media. Between the three solute adjusted treatments the highest water potential was found at all water stresses (-0.48, -0.98 and -1.48 MPa) on matrically modified media although the mycelia had the slowest growth rate on this media. The lowest water and osmotic potential were generated on ionically modified media on the driest treatment (-2.48 MPa). There was a significant ($P<0.001$) water potential effect on the turgor potential whereby it generally increased with decreasing water potential and this trend was generally found on all solute treatments. The highest mean turgor potential (+0.495 MPa) was found on the most stressed media (non-ionically) at -2.48 MPa compared with +0.065 MPa on ionically modified media and this was the lowest turgor potential generated.

At 25°C there was a significant ($P<0.001$) water potential effect on the mycelial osmotic and water potential which decreased with increasing water stress and the highest osmotic and water potential were found on un-modified media which would have the most freely available water. There was a significant solute effect on the mycelial osmotic and

water potential which were highest at each water stress treatment on matrically modified media, although it had the slowest growth rate, except at -0.98 MPa where the highest osmotic and water potential was found on non-ionically modified media. There was a significant water potential effect on the turgor potential where it generally increased for all solute treatments with decreasing water potential and the highest turgor potential (+0.375 MPa) was in mycelia grown on ionically modified media at a water stress of -1.48 MPa compared with turgor potentials of +0.304 and +0.367 MPa generated on non-ionically and matrically modified media respectively.

For both MEA and CE/CYM the mycelial osmotic and water potential decreased with increasing water stress. For MEA media treatments the mycelia grown on non-ionically modified media had higher water and osmotic potentials, compared with mycelia grown on ionically and matrically modified media, and this was in parallel with the higher growth rates observed on non-ionically modified media. However this was not the case for CE/CYM media treatments whereby the highest osmotic and water potentials were found on matrically modified media although the mycelia grown on this treatment had the lowest growth rates. There was no significant temperature effect on the osmotic, water and turgor potential

Figure 3.9: The mean, of 5 replicates, total water (WP), osmotic (OP) and turgor potentials (TP) measured in mega pascals (MPa) of *Agaricus bisporus* (A12) mycelia grown on (A) KCl and (B) glycerol amended compost extract/complete yeast extract media (CE/CYM) and (C) PEG 8000 amended CE/CYM broth, at 18°C. The mycelia were grown on un-modified CE/CYM media (-0.28 MPa) and CE/CYM media to which the above solutes were added to lower the water potential to -0.48, -0.98, -1.48 and -2.48 MPa and the WP, OP, and TP of the mycelia were measured after growing on the media for 26 days or before this time if the mycelium had colonised the media. There was no mycelial growth on matrically (PEG 8000) modified media at -2.48 MPa therefore there is no data for this treatment.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MPa
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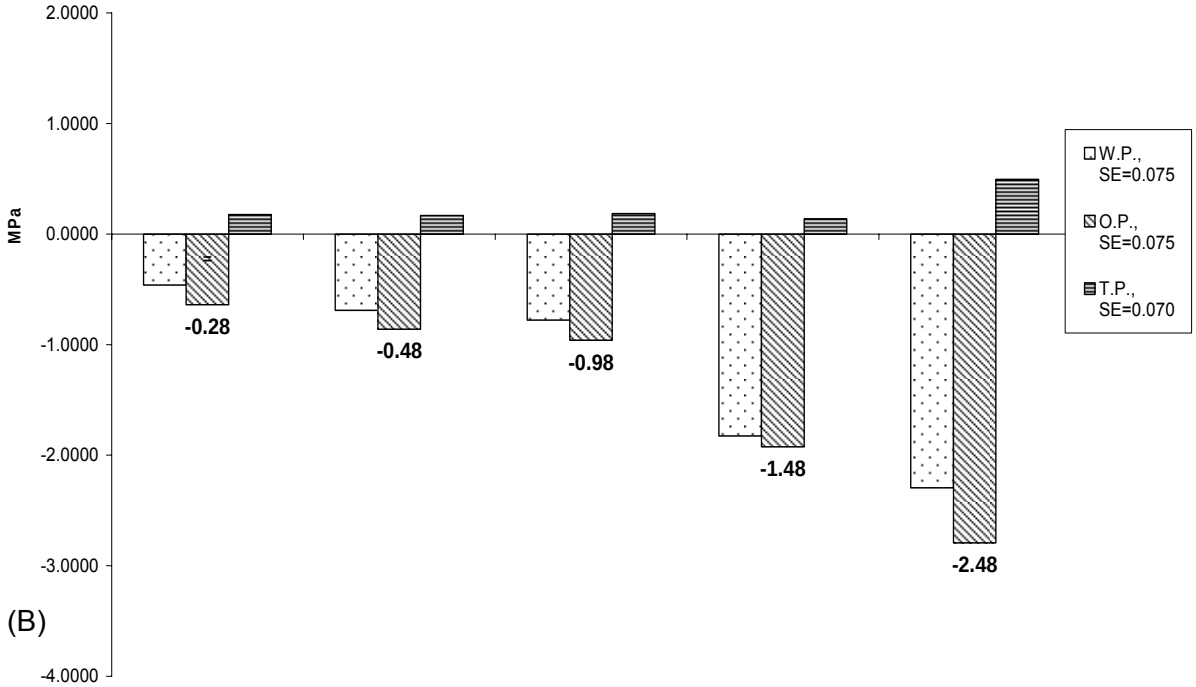
W.P., SE=0.067
O.P., SE=0.09
T.P., SE=0.063

(A)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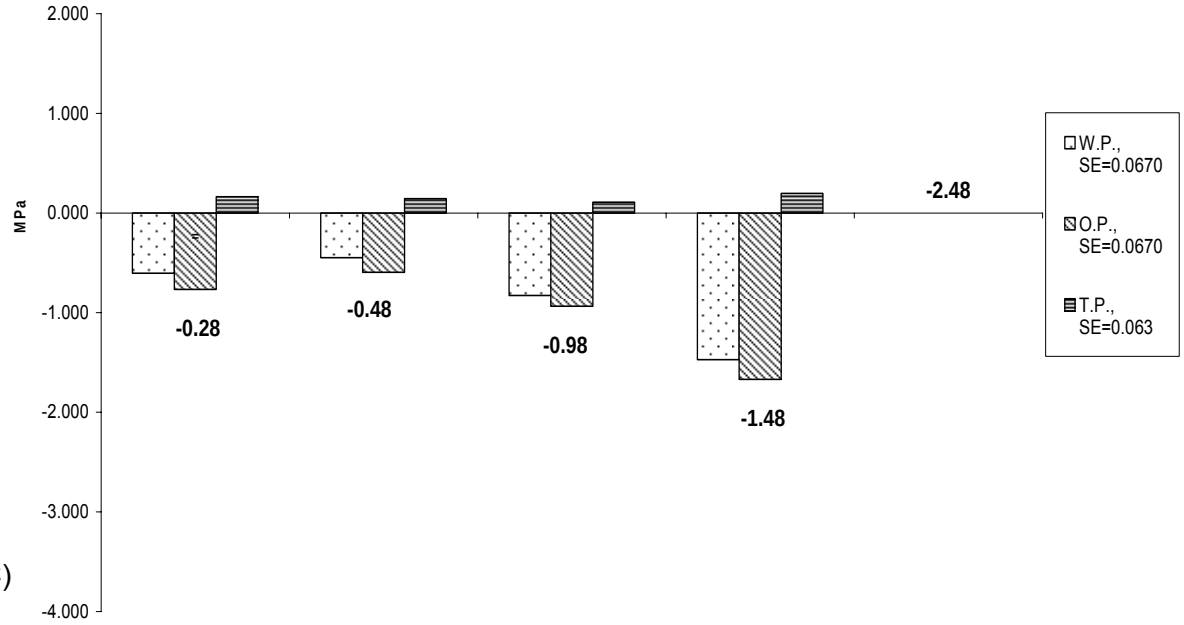
(B)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W.P., SE=0.0670
O.P., SE=0.0670
T.P., SE=0.063

(C)

Figure 3.10: The mean, of 5 replicates, total water (WP), osmotic (OP) and turgor potentials (TP) measured in mega pascals (MPa) of *Agaricus bisporus* (A12) mycelia grown on (A) KCl and (B) glycerol amended compost extract/complete yeast extract media (CE/CYM) and (C) PEG 8000 amended CE/CYM broth, at 25°C. The mycelia were grown on un-modified CE/CYM media (-0.28 MPa) and CE/CYM media to which the above solutes were added to lower the water potential to -0.48, -0.98, -1.48 and -2.48 MPa and the WP, OP, and TP of the mycelia were measured after growing on the media for 26 days or before this time if the mycelium had colonised the media.

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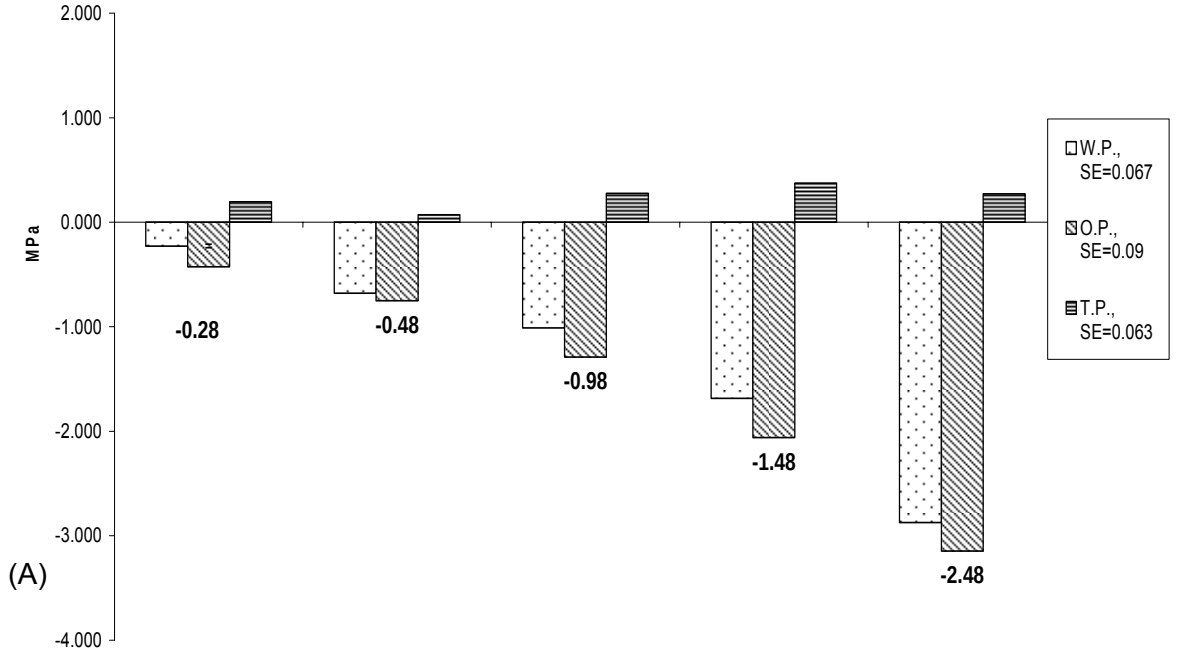
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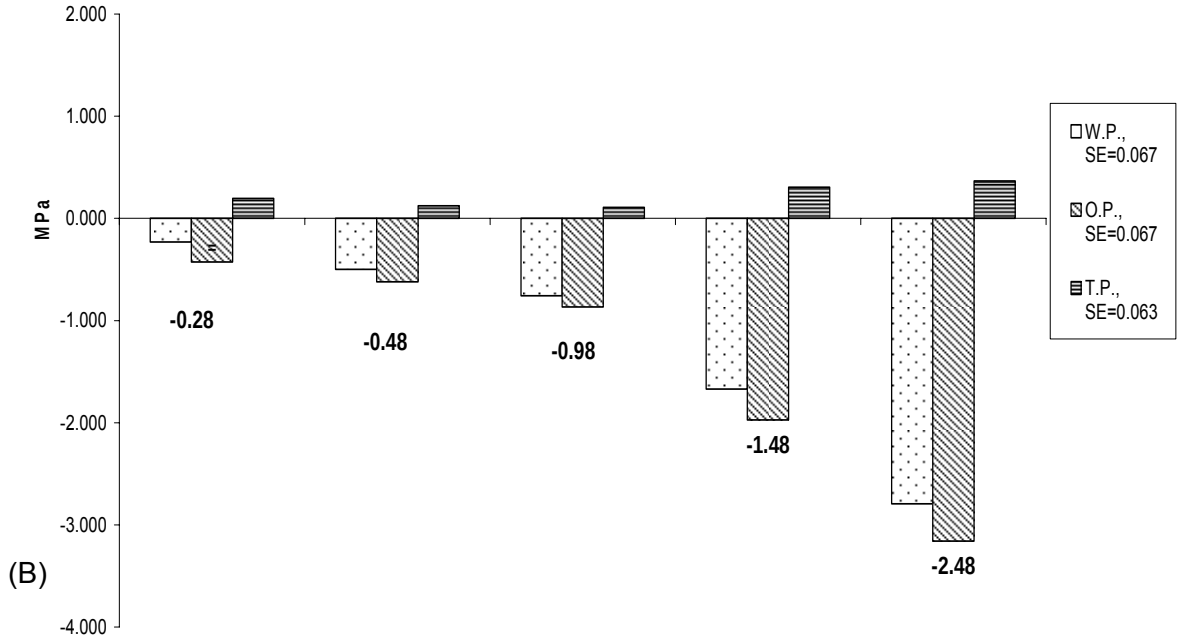
W.P., SE=0.067
O.P., SE=0.09
T.P., SE=0.063

(A)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W.P., SE=0.067
O.P., SE=0.067
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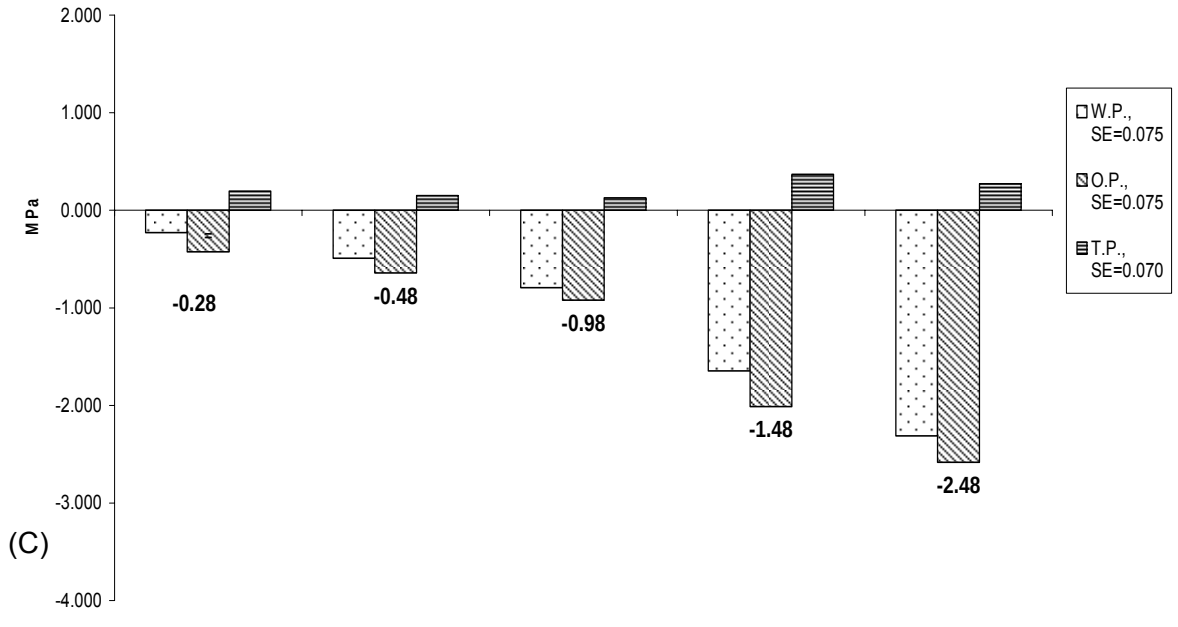
(B)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-0.98
-1.48
-2.48
W.P., SE=0.075
O.P., SE=0.075
T.P., SE=0.070

(C)

3.3.3 The endogenous sugar and polyol concentrations of the mycelium grown on different water stress treatments

The *A. bisporus* mycelia were removed from the surface of the cellophane overlays on two different media treatments: CE/CYM and MEA/MEB, which were subjected to water stresses using the following solutes: KCl, glycerol and PEG 8000. The endogenous sugars and polyols of the mycelia grown on the un-adjusted media (MEA/MEB) and on all solute adjusted media (MEA/MEB) at –0.98 and –2.48 MPa were measured at both 18 and 25°C. The endogenous sugars and polyols of the mycelia grown on the un-adjusted media (CE/CYM) and on all solute adjusted media (CE/CYM) at –0.48, -0.98 and –2.48 MPa were measured at both 18 and 25°C. The following sugars: glucose and trehalose and the polyols: mannitol, glycerol, erythritol and arabitol were measured (mM) in the mycelium. The concentration of the sugars and polyols were transformed using a log transformation equation:

$$\log_{10}(y+\alpha)$$

y=sugar or polyol concentrations in mM

α is a value based on the log transformation of the sugar/polyols

α=4 for glucose, trehalose, erythritol, and arabitol

α=70 for mannitol and glycerol

A value of alpha (α) was chosen using the package R Foundation for Statistical Computing v1.8.1 for each of the sugars and polyols measured based on the log transformation model which shows the best value of α for the transformation of the data. Statistical analysis of the log transformed data was carried out using Genstat v.6 for Windows.

3.3.3.1. Sugars and polyols in MEA/ MEB media

At 18 and 25°C the mean (of five replicates) concentration of the accumulated mycelial sugars and polyols grown on MEA/MEB media are shown in Table 3.3 and 3.4 respectively. There is no sugar or polyol data for mycelia grown on ionically or matrically modified media at –2.48 MPa because there was no growth at this water potential. No arabitol could be detected in the mycelia grown on MEA/MEB media at -0.48, -0.98 and -2.48 MPa water stress treatments.

At 18°C there was a significant water potential effect on the endogenous glucose levels which increased with increasing water stress and the highest concentration of glucose was found on non-ionically modified media on the driest treatment (-2.48 MPa). There was a significant water potential effect on the trehalose concentration and the lowest concentration of trehalose (7.69 mM) was detected on non-ionically modified media at -2.48 MPa. There were similar levels of trehalose detected on un-modified (74.66 mM) and ionically (77.26 mM) modified media at -0.48 and -0.98 MPa respectively. The lowest concentration of trehalose (7.68 mM) was detected in mycelia grown on the most stressed media (-2.48 MPa) modified non-ionically and this could be could be accounted for by the fact that trehalose is a known storage compound in *A. bisporus* and could be metabolised by the enzyme trehalose phosphorylase to produce glucose and glucose-1-P (Wannet *et al*., 1999). Glucose could then be re-routed into the carbohydrate biosynthesis pathway (see Figure 1.5) which would provide NADPH for the reduction of fructose to produce mannitol. The polyol mannitol which has been shown to accumulate in *A. bisporus* under salt stress by Kalberer (1990) and functions in lowering the water potential osmotically below that of the external medium and creating a water potential gradient which allows an influx of water into the cells to increase the hydrostatic pressure and enable the cells to grow. In concomitant with this the highest concentration of mannitol was detected in mycelia grown on non-ionically modified media at -2.48 MPa.

There was a significant ($P<0.001$) water potential effect on the mannitol concentration whereby the mycelial mannitol concentration increased with increasing water stress and the mycelia grown on the most stressed medium -2.48 MPa had the highest concentration of mannitol. There was a significant ($P<0.001$) water potential and solute effect on the mycelial glycerol concentration which was only detected in mycelia grown on glycerol modified media and on un-modified media and was not present in mycelia grown on ionically or matrically modified media. The very high concentration of glycerol found in mycelia grown only on non-ionically modified media is probably as a result of the absorption of the polyol from the media. Magan (1997) reported that glycerol is the main polyol which accumulates in the mycelium of xerophilic filamentous fungi. There were significant water potential and solute effects on the mycelial erythritol concentration which could only be detected on un-modified media and on ionically and matrically modified media. The concentration of erythritol increased with increasing water stress and was highest on mycelia grown on matrically modified media. The fact that no erythritol could be detected on non-ionically modified media could be due to the fact that it had already accumulated a high concentration of the polyol glycerol.

At 25°C there was a significant ($P<0.001$) water potential effect on the glucose concentration which increased with increasing water stress and the highest concentration was detected on non-ionically modified media at -2.48 MPa. The levels of trehalose followed no particular trend and the water potential and solute had no effect on the mycelial trehalose concentration, the lowest trehalose concentration was detected on glycerol modified media at -0.98 MPa and the highest was found on the same solute adjusted media but at a water stress of -2.48 MPa. There was a significant water potential and solute effect on the mannitol concentration which increased with increasing water stress, and the same result effect was found at 18°C, and the lowest concentration of mannitol could be detected on un-modified media (-0.48 MPa) and the highest

concentration was found on non-ionically modified mycelia grown on the most stressed media (-2.48 MPa). Glycerol could only be detected on un-modified and non-ionically modified media and the highest concentration was found on non-ionically modified media at -2.48 MPa as was found in mycelia grown at 18°C. There were significant water potential and solute effects on the erythritol concentration which was only detected in un-modified media and ionically and matrically modified media and increased with increasing water stress.

There was only a significant temperature effect on the trehalose concentration which was lower for all solute treatments at 25°C than at 18°C except on non-ionically modified media at -2.48 MPa.

Table 3.3: Mean sugar/polyol concentrations (mM) in mycelia of *Agaricus bisporus* grown on malt extract agar/malt extract broth media at different water potentials at 18°C.

Sugars/ Polyols mM	-0.48 MPa	Solutes added to MEA/MEB lower the water potential -0.98 MPa			-2.48 MPa
	Un-modified media	KCl	Glycerol	PEG 8000	Glycerol
Glucose	51.85	40.63	52.21	56.39	191.38
Trehalose	74.66	77.26	118.80	36.22	7.68
Mannitol	31.37	112.34	45.99	199.08	244.87
Glycerol	20.27	0	417.22	0	2876.69
Erythritol	4.42	39.81	0	121.26	0

Table 3.4: Mean sugar/polyol concentrations (mM) in mycelia of *Agaricus bisporus* grown on malt extract agar/malt extract broth media at different water potentials at 25°C.

Sugar/ Polyols mM	-0.48 MPa	Solutes added to MEA/MEB to lower the water potential -0.98 MPa			-2.48 MPa
	Un-modified media	KCl	Glycerol	PEG 8000	Glycerol
Glucose	35.07	67.48	52.05	41.75	149.09
Trehalose	9.80	31.94	2.18	20.70	48.78
Mannitol	19.27	93.23	42.51	113.58	277.13
Glycerol	105.71	0	336.85	0	4518.97
Erythritol	12.28	35	0	140.74	0

3.3.3.2 Sugars and Polyols in CE/CYM

At 18 and 25°C the mean (of five replicates) concentration of the accumulated mycelial sugars and polyols grown on CE/CYM media are shown in Table 3.5 and 3.6 respectively. At 18°C there is no sugar or polyol analysis for mycelia grown on matrically modified media at –2.48 MPa because there was no mycelial growth at this lowest water stress treatment. No erythritol or arabitol could be detected in the mycelia grown on un-modified CE/CYM media (-0.28 MPa) or solute adjusted CE/CYM media at -0.48, -0.98 and -2.48 MP water stress treatments.

At 18°C there was a significant water potential effect on the glucose concentration which decreased with increasing water stress on ionically and non-ionically modified media except at -2.48 MPa where it increased compared to the glucose concentration at -0.98 MPa. The highest concentration of glucose was detected on non-ionically modified media at the lowest water stress treatment. The mycelial trehalose concentration showed no consistent trend of increasing or decreasing concentration for any of the solute water stress treatments. There was a significant water potential and solute effect on the mycelial mannitol concentration which increased with increasing water stress. Mycelia grown on matrically modified media had the lowest mannitol concentration compared with mycelia grown on ionically and non-ionically stressed media at all water potentials and also had the lowest osmotic and water potential. The mycelia was most sensitive to changes in matric potential as opposed to osmotic potential and this is reflected in the mycelia having the lowest growth rate on matrically modified media. However because the mycelia was most sensitive to changes in matric potential and at the lowest water stress treatment (-2.48 MPa) there was no mycelial growth, it would have been expected to accumulate the highest concentration of mannitol (as was found on malt extract media) to enable it to lower the water potential below that of the external medium and create a water potential gradient and allow an influx of water to enable cell

growth. There was a significant water potential and solute effect on the mycelial glycerol concentration which increased with increasing water stress. The levels of glycerol increased with increasing water stress on ionically and non-ionically modified media but were up to 80 times higher in mycelia grown on non-ionically modified media but this is probably because the mycelia is absorbing glycerol from the media which would result in the massive difference between the glycerol levels in mycelia grown on ionically or matrically modified media compared to non-ionically modified media.

At 25°C the levels of mycelial glucose increased and decreased irregularly between different water stresses regardless of the solute treatment. The highest glucose concentration was detected on matrically modified media at the highest water stress (-2.48 MPa). There was a significant water potential and solute effect on the mycelial trehalose concentration which increased on ionic and non-ionic water stress treatments between -0.48 and -0.98 MPa and decreased for the same solute media treatments between -0.98 and -2.48 MPa. No trehalose could be detected in mycelia grown on un-modified media and matrically modified media at -0.48 and -2.48 MPa. There was significant water potential and solute effect on the mannitol concentration which increased with increasing water potential stress. The lowest concentration of mannitol was detected in mycelia grown on matrically modified media as was found at 18°C. There was a significant ($P<0.001$) water potential and solute effect on the mycelial glycerol concentration which could not be detected in mycelia grown on matrically modified media and was significantly higher in mycelia grown on non-ionically modified media compared with ionically modified media and this is probably as a result of the absorption of glycerol from the media.

Table 3.5: Mean sugar/polyol concentrations (mM) in mycelia of *Agaricus bisporus* grown on compost extract/complete yeast extract media at different water potentials at 18°C.

	CE/CYM	Solutes added to CE/CYM to lower the water potential to:							
Sugars/Polyols	-0.28	-0.48 MPa			-0.98 MPa			– 2.48 MPa	
mM	MPa	KCl	Glycerol	PEG 8000	KCl	Glycerol	PEG 8000	KCl	Glycerol
Glucose	133.07	113.81	69.4	65.37	54.83	36.86	73.62	69.8	178.48
Trehalose	1.83	3	48.75	3.83	6.19	29.66	4.98	4.43	5.99
Mannitol	62.5	164.84	161.53	27.99	199.67	72.01	49.68	366.84	303.31
Glycerol	8.72	6.55	527.67	86.58	19.73	958.47	3.15	90.84	3217.16

Table 3.6: Mean sugar/polyol concentrations (mM) in mycelia of *Agaricus bisporus* grown on compost extract/complete yeast extract media at different water potentials at 25°C.

	CE/CYM	Solutes added to CE/CYM to lower the water potential to:								
Sugars/Polyols	-0.28	-0.48 MPa			-0.98 MPa			–2.48 MPa		
mM	MPa	KCl	Glycerol	PEG 8000	KCl	Glycerol	PEG 8000	KCl	Glycerol	PEG 8000
Glucose	54.14	23.77	17.89	33.54	20.38	8.31	21.78	57.47	83.8	227.9
Trehalose	0	0.64	130.62	0	2.57	118.51	4.79	8.02	5.83	0
Mannitol	121.21	148.07	66.75	8.77	196.59	91.74	46.19	423.54	377.1	226.43
Glycerol	80.92	14.92	1104.8	0	5.51	1487.29	0	54.06	4152.53	0

3.4 DISCUSSION

There was a significant temperature effect on the mycelial growth rates which were higher at 25°C compared to 18°C on both osmotically, with ionic (KCl) and non-ionic (glycerol) solutes, and matrically (PEG 8000) stressed media in the range -0.48 to –2.48 MPa and this is in agreement with results that Beecher (1999) found with *A. bisporus* mycelia when grown under the same solute induced water stresses on MEA/MEB media. Magan *et al*. (1995) found the optimum growth rate for *A. bisporus* (wild and hybrid strains) mycelium grown on agar medium was at a temperature of 20°C but in commercial practises the temperature is maintained at 25°C when the mycelium is colonising the compost. The type of media that the mycelia were grown on had an effect on the growth rate which was significantly higher on CE/CYM than on malt extract media and the mycelia were able to withstand lower water potentials when grown on CE/CYM than on MEA media. Compost extract (CE) was prepared from phase II compost and supplemented with complete yeast extract (CYM). The phase II compost is prepared from a mixture of cereal straw, animal manure and water to which gypsum is added and undergoes a two phase composting procedure which is detailed in Section 1.4.1. During the composting procedure a number of thermophilic fungi and actinomycetes proliferate and degrade the cellular structure of the cereal straw and after this composting procedure most of the carbon and nitrogen are in the form of polymers embedded in a nitrogen rich lignin humus complex (Gerrits *et al*., 1967; Atkey & Wood, 1983). This compost provides a selective and readily available nutrient source for the *A. bisporus* mycelium to colonise and grow. It may be likely that there are chemical and physical properties of the compost as yet undefined that encourage rapid mycelial growth and enable the mycelium to withstand water related stresses.

The mycelial growth rate decreased with increasing water stress regardless of the solute or the media. Although all the solutes were used to lower the water potential to

the same extent the type of solute used had an effect on the *A. bisporus* mycelial growth rate whereby it was significantly higher on ionically than on matrically modified media. Beecher (1999) also found that the *A. bisporus* mycelia grown on MEA/MEB media was most sensitive to changes in matric potential and had faster growth rate on non-ionically modified media compared to ionically or matrically modified media. This is in agreement with results obtained for mycelial growth rates of *A. bisporus* wild hybrid and commercial strains (Magan *et al*., 1995) and for *A. bisporus* (A12) sporophores and mycelium (Noble *et al*., 1999). Also *Pleurotus* mushrooms were found to be more sensitive to media modified matrically (PEG 8000) than osmotically (KCl) (Lee *et al*., 2000). The mycelial growth rates of 12 different species of temperate wood rotting basidiomycetes (Boddy, 1983) and tropical wood rotting basidiomycetes (Mswaka & Magan, 1999) were more sensitive to matrically than osmotically reduced water potential.

Overall the mycelial growth rates were higher on glycerol modified media than on KCl or matrically modified media and were more tolerant of osmotic potential stresses induced non-ionically than ionically. However at water potentials of -0.48 -0.98 and -1.48 MPa on CE/CYM media and -0.98 and -1.48 MPa on MEA at both 18 and 25°C the mycelial growth rates were marginally higher on KCl than glycerol modified media but with increasing water potential stresses the mycelia grew better on glycerol modified media and at the lowest water stress (-2.48 MPa) there was no mycelial growth at 18°C on ionically modified MEA media. This could be due to the polyol glycerol being very effective in preserving enzyme function at low levels of water availability and is a major polyol of the xerotolerant and xerophilic fungi (Boddy, 1983; Magan, 1997). At lower water potential stresses (low concentration of KCl in the media) the potassium ions are easily accumulated by fungal cells and can act as osmoregulators and can serve as compatible solutes (Mswaka & Magan, 1999). However, at high concentrations of KCl (increase in water potential stress) the potassium ions are probably toxic to the cells (Luard, 1983; Mswaka & Magan, 1999) and this is a possible reason why at the lowest water potential,

-2.48 MPa, on ionically modified media, the mycelial growth either ceased on MEA or was greatly reduced on CE/CYM medium. Mswaka & Magan (1999) found that tropical wood decaying basidiomycetes fungi ceased growing between -4 and -5 MPa on ionically modified malt agar media with KCl as the solute but could continue to grow up to a water potential of -7 MPa with glycerol as the solute. Magan *et al.* (1995) found that *A. bisporus* hybrid, wild and commercial strains ceased growing between -3 and -4 MPa and Lee *et al*. (2000) found that *Pleurotus* species also ceased growing between -3 and -4 MPa when they were grown on malt agar medium using KCl as the solute. However, none of the basidiomycete species were grown on compost derived substrates.

On both media the mycelial growth was more sensitive to matric than osmotic potentials, i.e. had lower growth rates on matrically modified media, but mycelial growth was less sensitive to changes in matric potential on CE/CYM than on malt extract media and the difference between the growth rate on these two media was less on CE/CYM than on MEA. The mycelia were able to withstand matrically reduced water potential on CE/CYM up to -2.48 MPa but on MEA media mycelial growth had ceased at this water potential. Magan *et al*. (1995) found that *A. bisporus* wild and hybrid strains could not grow below -3 MPa and growth was significantly reduced below -2 MPa on MEA modified matrically with PEG 6000. Nobel *et al*. (1999) found that sporophore production and mycelial growth rate is more closely related to the matric potential of the casing soil and un-related to the water holding capacity and the osmotic/chemical potential in the range -0.93 and -1.54 MPa. It is generally believed that water held by matric forces is due to the interaction of water with the surface of the solid phase such as cell walls and soil particles and is more tightly held than osmotic interactions. In compost and casing soil the water is likely to be held by matric interactions than osmotic therefore the mycelium and sporophore would more likely be sensitive to changes in matric than osmotic potential. Noble *et al*. (1999) found that the sporophores could withstand decreases in the casing soil matric potential to below -0.1 MPa without any adverse effect on the development of

the mushrooms. This would be a possible reason why the mycelium could withstand matrically reduced water potential on compost media below -1.48 MPa but not on malt extract agar. However, how the mycelium and sporophore are able to withstand changes in matric potential of the compost and casing layer and how these are linked to the physical and chemical properties of the compost are unknown (Noble *et al*., 1999). We can examine the effects of the reduced matric potential on the mycelium when grown on media derived from compost compared with laboratory based malt extract media by measuring the mycelial water, osmotic and turgor potential and their endogenous sugars and polyols under osmotic and matric stresses.

On MEA media all mycelia had lower water potential than the media they were grown on indicating that the mycelium generated a water potential gradient in response to the induced water stress treatment. To lower the water potential below the surrounding environment fungi accumulate osmolytes (osmotically active solutes) to create an osmotic potential that drives an influx of water into the cells which is necessary for hyphal growth (Davis *et al*., 2000). Under increasing water stress the mycelia need to constantly maintain lower water potential than the surrounding environment to maintain water influx and growth and this is termed osmotic adjustment (Davis *et al*., 2000). In *A. bisporus* sporophores the polyol mannitol has been shown to accumulate under increasing osmotic stress (NaCl) to maintain a lower water potential than the casing layer (Kalberer, 1990). At both temperatures the mycelia on matrically modified malt extract media at -0.98 MPa had the slowest growth rate compared to osmotically modified media indicating that the mycelia was under greater stress on matrically than osmotically modified media and in turn generated the lowest water and osmotic potential and the highest concentration of the osmolyte mannitol 113.58 mM compared to 93.23 and 42.51 mM on KCl and glycerol modified media respectively at 25°C.

On CE/CYM media at 18 and 25°C the mycelia grown on matrically modified media had slower growth rate than on osmotically modified media. On matrically induced

water potential stresses of -0.48 and -0.98 MPa the mycelial internal water potential was lower than the external media and had accumulated the lowest endogenous mannitol concentration at 8.77 mM compared to 66.75 and 148.07 mM on glycerol and KCl modified media respectively at -0.48 MPa/25°C. Noble *et al*. (1999) did report that the sporophore and mycelium could withstand decreases in the compost and casing soil matric potential below -0.1 MPa without any adverse effect on the mycelial growth rate. It is possible that the mycelium has adapted to decreases in the compost and casing soil matric potential and can withstand changes in matric potential to a certain lowered water potential value without having to create a high osmotic potential. Noble *et al.* (1999) found the lowest matric potential of the casing soil recorded was -1.06 MPa during sporophore production. At water stresses of -1.48 and -2.48 MPa the water potential was lower than the external media on matrically modified media but were still higher than those found on osmotically modified media. The mycelial tolerance to changes in matric potential are likely to be linked to the physical and chemical components of the compost and casing soil as suggested by Noble *et al*. (1999) because the mycelium grown on malt extract media showed no such tolerance to matric potential stresses and accumulated the highest concentration of mannitol at the matrically induced water stress of -0.98 MPa compared to mycelia grown on osmotically modified media.

Therefore it can be concluded that *A. bisporus* mycelium has adjusted to decreases in the matric potential of the compost and casing soil without it having to create an osmotic potential gradient, as has been shown to occur under induced osmotic stresses.

CHAPTER 4

SUGAR AND POLYOL CONTENT OF SPOROPHORE TISSUE OF *A. BISPORUS*

4.1 INTRODUCTION

Mono- and disaccharides are usually present in low concentrations in fungi with the exception of storage compounds (Wannet *et al.*, 2000). Trehalose and mannitol are the principal carbohydrates produced by species within the Kingdom Fungi (Jennings, 1984; Pfyffer *et al.*, 1990; Griffin, 1994; Hounsa *et al.*, 1998) but can vary between 1-10 % and 30-50% dry weight respectively during stages 2-7 of *A. bisporus* sporophore development (Hammond & Nichols, 1976). The polyol mannitol is thought to act as an osmoticum during fruit body development and functions in creating an osmotic potential between the compost, casing soil and the sporophore thus allowing for an influx of water into the cells and an increase in turgor pressure which drives water and solute through the mycelium and into the sporophore (Kalberer, 1987). Mannitol is synthesised by the reduction of fructose by the enzyme MtDH as indicated in Figure 1.5 and the activity of this enzyme was shown to increase in salt stressed sporophores (Stoop & Mooibroek, 1998). The other major carbohydrate is the disaccharide trehalose which is thought to be the major carbohydrate translocated from the mycelium to the sporophore (Hammond & Nichols, 1976; Wells *et al*., 1987; Wannet *et al*., 1999) and the enzyme trehalose phosphorylase which catalyses both the synthesis and degradation of trehalose was purified by Wannet *et al*. (1998).

As indicated in the carbohydrate pathway in *A. bisporus* (Figure 1.5) there are other carbohydrates involved in fungal metabolism namely glucose and fructose. Glucose was detected at low levels <1% dry weight by Hammond & Nichols (1976) and only low levels of fructose were detected by Wannet *et al*. (1999). There are possibly other polyols produced by *A. bisporus* sporophores besides mannitol, i.e. arabitol, erythritol and glycerol, which were detected in *A. bisporus* mycelium grown under osmotic and matric water stresses as shown in Chapter 3.

Although levels of soluble carbohydrates have been measured in the fruit body during developmental stages 1-7 in the cap, stipe and gills by Hammond & Nichols (1976) and in whole fruit bodies by Wannet *et al*. (2000) no distinction between the upper and lower stipe was made, where greater expansion of the upper stipe takes place during sporophore development compared to the lower stipe (Craig *et al.*, 1981) thus suggesting that there could be an increase in the amount of carbohydrates especially mannitol in the upper stipe region. So far only selected carbohydrates have been measured i.e. mannitol, glucose, trehalose and fructose. To get a greater picture of the changing carbohydrates in sporophore tissues during growth the following carbohydrates: glucose, trehalose, mannitol, arabitol, erythritol and glycerol were quantified in *A. bisporus* (A15) stages1-6 of sporophore development in the following tissues: upper and lower stipe (except at stage 1 where no distinction is possible), cap tissue (*pileus trama*), gills and cap skin (*pilei pellis*) (Figure 1.3) using high performance liquid chromatography (HPLC).

4.2 MATERIALS AND METHODS

Fruit bodies were harvested from stage 1 through to stage 6 (Hammond & Nichols, 1976) from a second flush crop of *A. bisporus* (A15) mushrooms grown at HRI mushroom unit. For each stage of development four mushrooms were taken and the following tissue: cap, gill, cap skin, upper and lower stipe was removed using a scalpel blade and placed in a 2 ml Eppendorf (Eppendorf AG), weighed and then frozen in liquid N_2 and stored at –70°C. For stage 1 of sporophore development there was no distinction between the upper and lower stipe tissue so simply the stipe tissue was removed. For all other stages of sporophore development the upper stipe tissue is that above where the cap was attached to the stipe by the velum and the stipe tissue below this is referred to as the lower stipe as indicated in Figure 1.3. The following solutes: trehalose, glucose, glycerol, erythritol,

arabitol and mannitol were quantified in all of the tissues using HPLC as described in Section 2.6.

4.3 RESULTS

The analysis of the data for the sugar and polyol concentration in the following tissues: cap (pileus), gills, skin and the upper stipe and lower stipe of all the stages of development was carried out using following statistical package, R Foundation for Statistical Computing Version 1.8.1. The following sugars glucose, trehalose and polyols mannitol, arabitol, erythritol and glycerol were not all detected in every tissue and this led to there being some zero values. The data was transformed for each sugar and polyol concentration using the following log transformation equation:

$$\log_{10}(y+\alpha)$$

y is sugar or polyol concentrations in mM

α is a value based on the log transformation of the sugar/polyols

α=1 for erythritol, trehalose, glycerol and arabitol

α=70 mannitol

α=1 glucose

A value of alpha (α) was chosen added to avoid problems with zero entries for each of the sugars and polyols based on the log transformation model which shows the best value for α for transformation of the data (Venables & Ripley, 2002) and these plots are shown in Appendix 3. However for the data of erythritol, arabitol and trehalose because of the number of zero values included in their respective data sets an optimal value of α was not found and in these cases a value of α=1 was used. The value of α for each data set was then fitted into the log transformation equation shown above. ANOVA test was performed on the log transformed data and the F test showed the most significant differences at $P<0.001$, significant differences $P<0.01$, least significant differences $P<0.05$ between

stages of sporophore development and for the tissues within each stage of sporophore development for each of the sugars and polyols. The residual plots for the log transformed data for each of the sugars and polyols are shown in Appendix 3.

The log transformed data for arabitol in the cap, gills, skin and upper and lower stipe tissue in stages 1-6 of sporophore development is shown in Figure 4.1. Arabitol could be detected in some of the tissue in the six stages of sporophore development and was not detected in the cap and stipe tissue at stage 1 nor could any be detected in the cap tissue in stage 2 and 4. The highest level of arabitol was found in stages 5 and 6.

The log transformed data for erythritol in the cap, gills, skin and upper and lower stipe tissue in stages 1-6 of sporophore development is shown in Figure 4.2. Erythritol could not be detected in all tissue in all of the stages. At stage 3 no erythritol could be detected in the cap, lower stipe and upper stipe tissue and in stage 4 none could be detected in the cap and lower stipe tissue. There was no erythritol in any of the tissue in stage 5. There were significant differences within stages ($P<0.01$) and these were found within stage 4 where there were differences between skin and gill tissue and the skin and upper stipe tissue with higher erythritol in the skin tissue. There were also differences within stage 6 between skin and cap, upper stipe and lower stipe tissue with higher levels of erythritol in the skin tissue.

The log transformed data for glucose in the cap, gills, skin and upper and lower stipe tissue in stages 1-6 of sporophore development is shown in Figure 4.3. Glucose was detected in all tissue in all of the stages and there were significant differences between stages ($P<0.001$). There were differences between stages 1 and 5 and 1 and 6 in all tissues with the lowest glucose in stage 1 tissue. There were also differences in glucose within stages of sporophore development ($P<0.001$). There were differences between cap and skin tissue within stage 3 with higher glucose in the skin tissue and between cap and gill tissue with higher glucose in the gill tissue. Within stage 5 there were differences between cap and skin and the cap and gill tissue with higher levels of

glucose in the cap tissue. In stage 6 there were also significant differences between cap and gill tissue and the cap and upper stipe tissue with higher levels of glucose in the cap tissue.

The log transformed data for glycerol in the cap, gills, skin and upper and lower stipe tissue in stages 1-6 of sporophore development is shown in Figure 4.4. Glycerol was detected in all stages of sporophore development and in all of the tissues. There were significant differences ($P<0.05$) between mushrooms but there were no significant differences within stages of sporophore development. Between stages 1 and 6 and stages 1 and 5 there were differences in the cap tissue with consistently lower levels of glycerol in stage 1 cap tissue.

The log transformed data for mannitol in the cap, gills, skin and upper and lower stipe tissue in stages 1-6 of sporophore development is shown in Figure 4.5. Mannitol was detected in all tissues in all of the stages. There were significant differences ($P<0.001$) within stages of sporophore development however there were no significant differences between stages of sporophore development. There were significant differences within all stages whereby the gill tissue had lower mannitol levels compared to all the other tissues.

The log transformed data for trehalose in the cap, gills, skin and upper and lower stipe tissue in stages 1-6 of sporophore development is shown in Figure 4.6. Trehalose was not detected in all tissues in all stages of sporophore development and could not be detected in the skin tissue in stages1, 4 and 5 of sporophore development. There was also no trehalose detected in cap and gill tissue in stage 5 and in the cap, lower stipe and skin tissue in stage 6. There were significant differences ($P<0.01$) within stages of sporophore development. In stage 1 there were differences between the gill and stipe tissue with higher trehalose in the gill tissue and in stage 4 there were differences between the lower stipe and cap tissue with higher trehalose in the lower stipe.

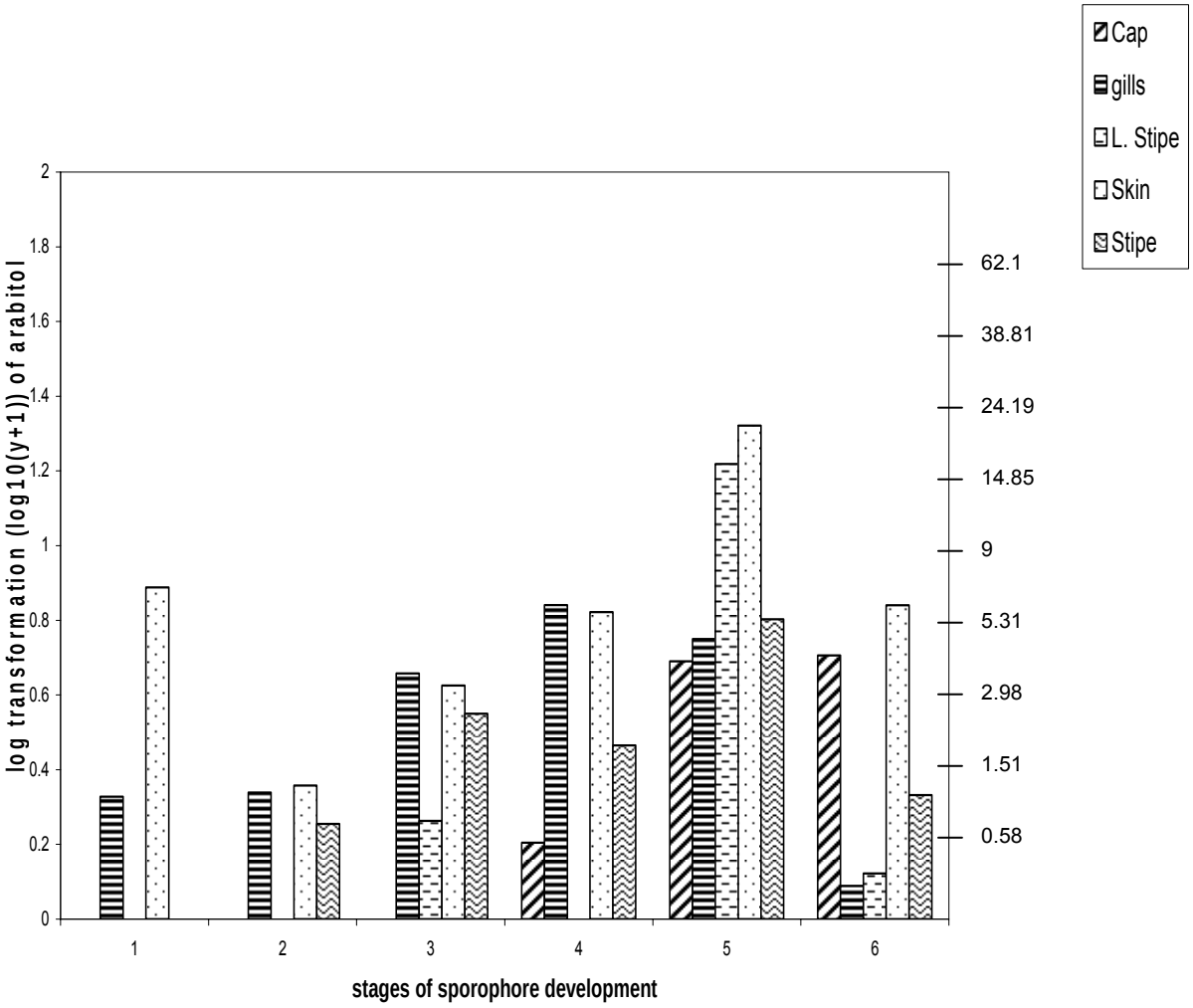

Figure 4.1: Log transformation of arabitol (mM) in the cap, gills, skin, upper and lower stipe tissue of stages 1-6 of sporophore development. In stage 1 sporophore there is no distinction between the upper and lower stipe and is labelled as the stipe in the legend but this refers to the upper stipe tissue for stages 2-6 where there is a distinction between the upper and lower stipe. The corresponding arabitol concentration (mM) for the log transformed values is shown on the right axis. There was no arabitol detected in the cap tissue of stage, 1, 2 and 3 sporophores, in the stipe tissue of stage 1 sporophores and in the lower stipe tissue of stage 2 and 4 sporophores.

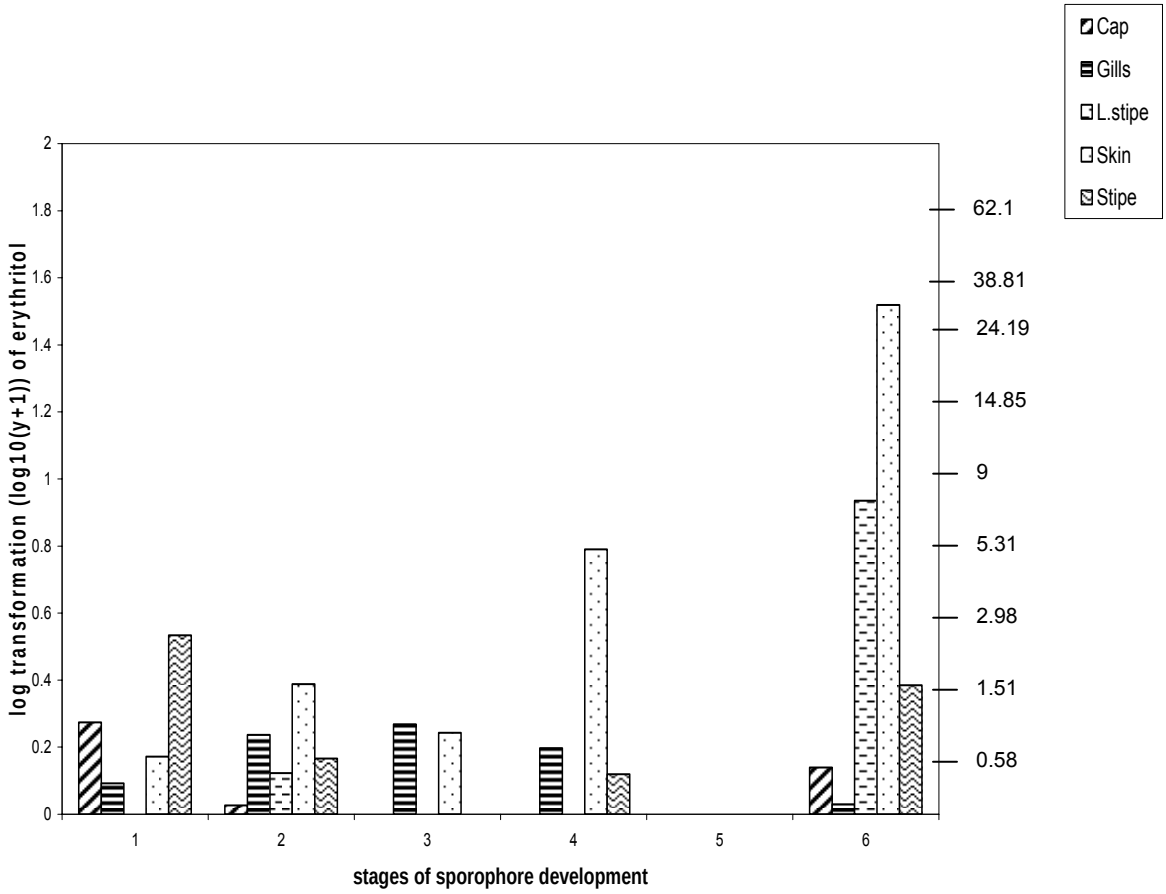

Figure 4.2: Log transformed data showing the amounts of erythritol in the cap, gills, skin, upper and lower stipe tissue of stages 1-6 of sporophore development. In stage 1 sporophore there is no distinction between the upper and lower stipe and is labelled as the stipe in the legend but this refers to the upper stipe tissue for stages 2-6 where there is a distinction between the upper and lower stipe. The corresponding erythritol concentration (mM) for the log transformed values is shown on the right axis. There was no erythritol detected in the cap and lower stipe tissue of stage 3, 4 and 5 sporophores, in the gill, skin and upper stipe tissue of stage 5 sporophores or in the upper stipe tissue of stage 3 sporophores.

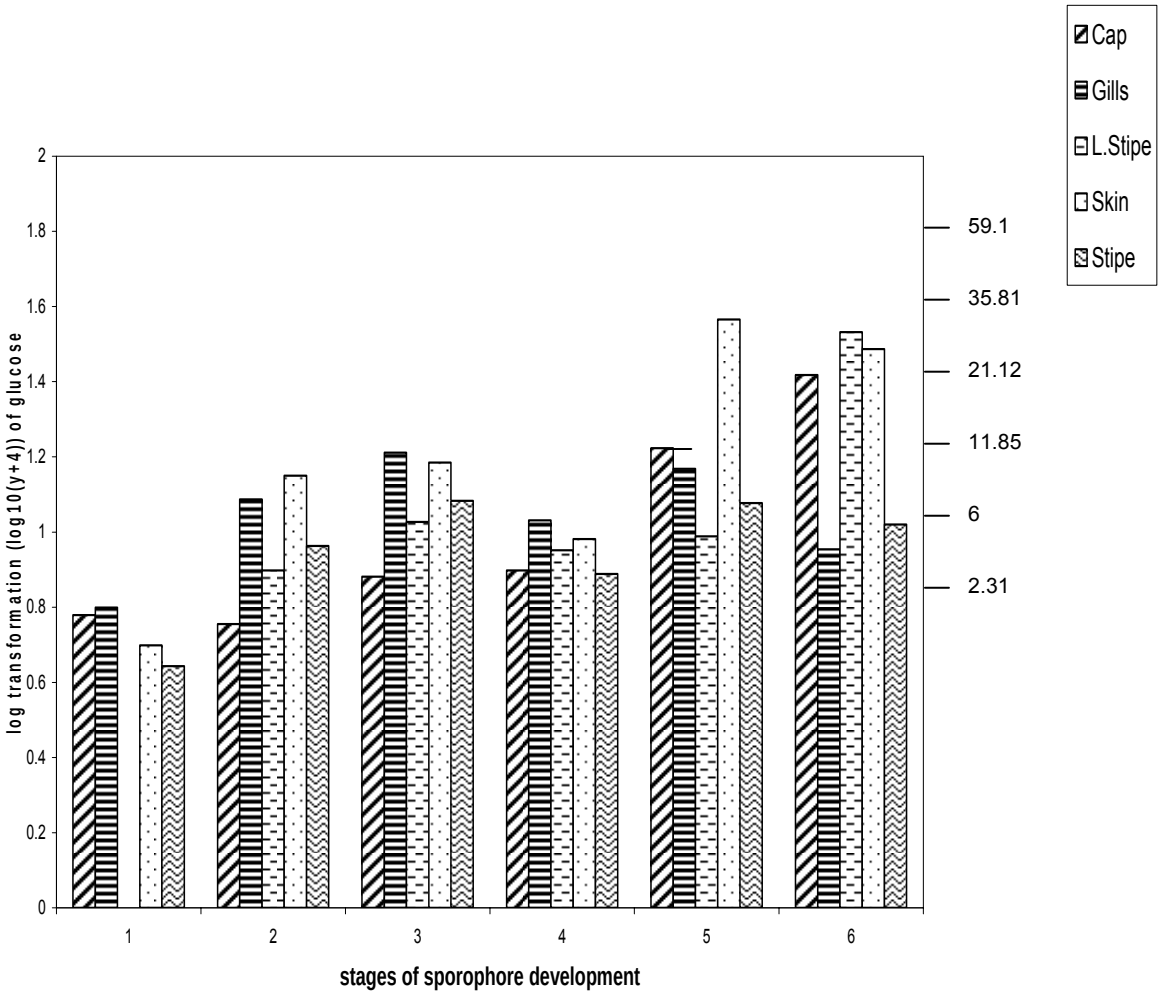

Figure 4.3: Log transformed data showing the amounts of glucose in the cap, gills, skin, upper and lower stipe tissue of stages 1-6 of sporophore development. In stage 1 sporophore there is no distinction between the upper and lower stipe and is labelled as the stipe in the legend but this refers to the upper stipe tissue for stages 2-6 where there is a distinction between the upper and lower stipe. The corresponding glucose concentration (mM) for the log transformed values is shown on the right axis. Glucose was detected in all the tissues during stages 1-6 of sporophore development.

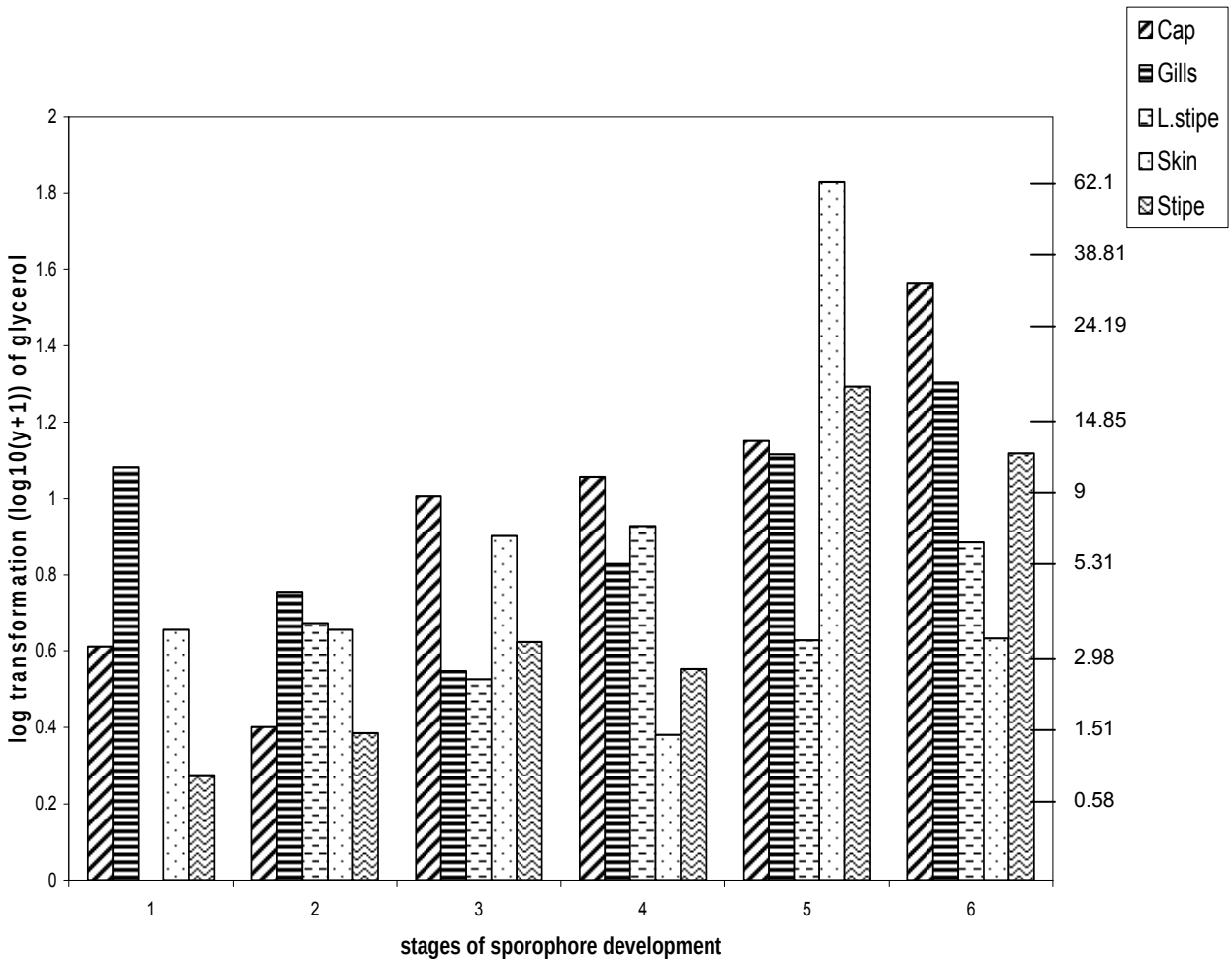

Figure 4.4: Log transformed data showing the amounts of glycerol in the cap, gills, skin, upper and lower stipe tissue of stages 1-6 of sporophore development. In stage 1 sporophore there is no distinction between the upper and lower stipe and is labelled as the stipe in the legend but this refers to the upper stipe tissue for stages 2-6 where there is a distinction between the upper and lower stipe. The corresponding glycerol concentration (mM) for the log transformed values is shown on the right axis. Glycerol was detected in all of the tissues during stages 1-6 of sporophore development.

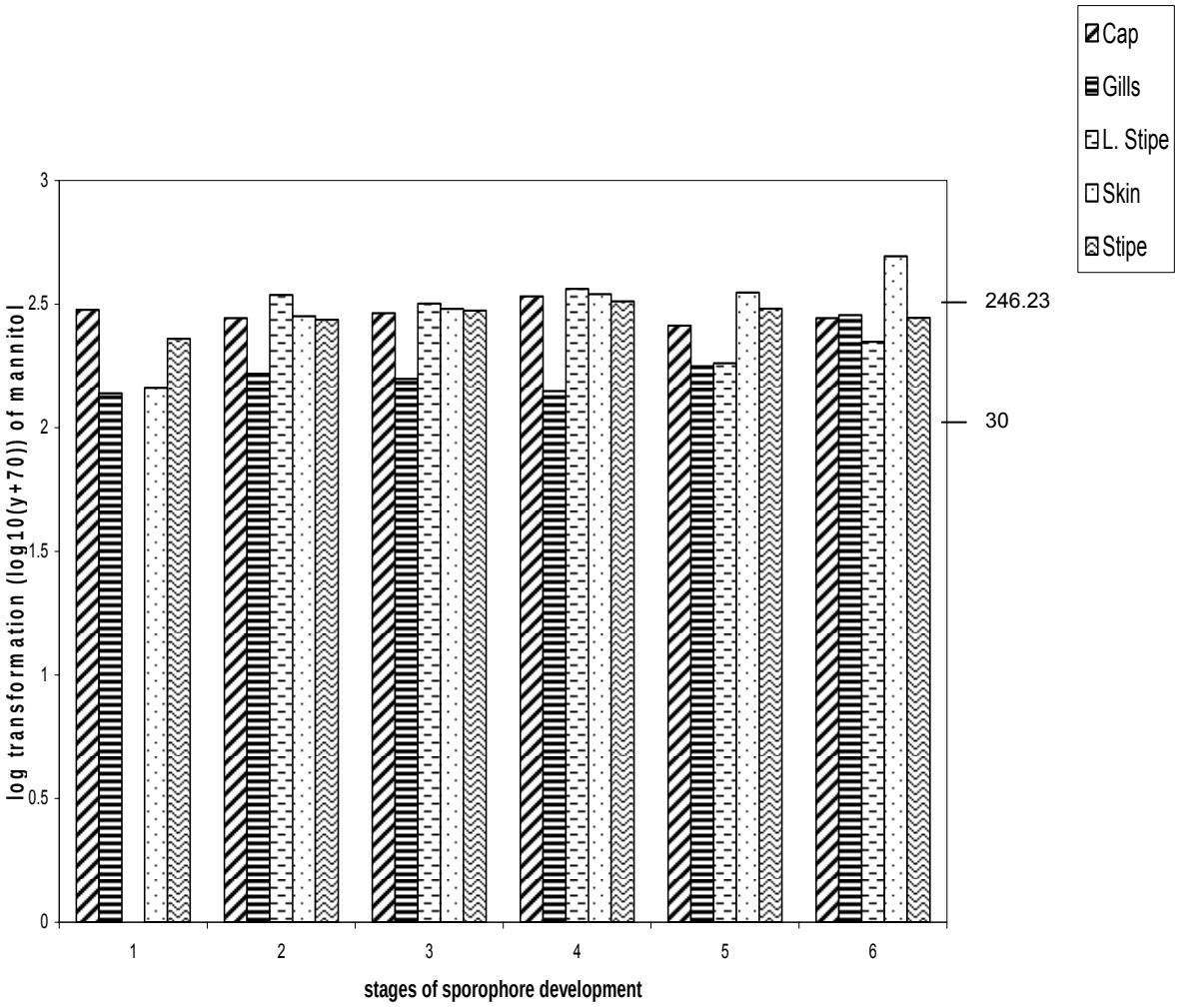

Figure 4.5: Log transformed data showing the amounts of mannitol in the cap, gills, skin, upper and lower stipe tissue of stages 1-6 of sporophore development. In stage 1 sporophore there is no distinction between the upper and lower stipe and is labelled as the stipe in the legend but this refers to the upper stipe tissue for stages 2-6 where there is a distinction between the upper and lower stipe. The corresponding mannitol concentration (mM) for the log transformed values is shown on the right axis. Mannitol was detected in all the tissues during stages 1-6 of sporophore development.

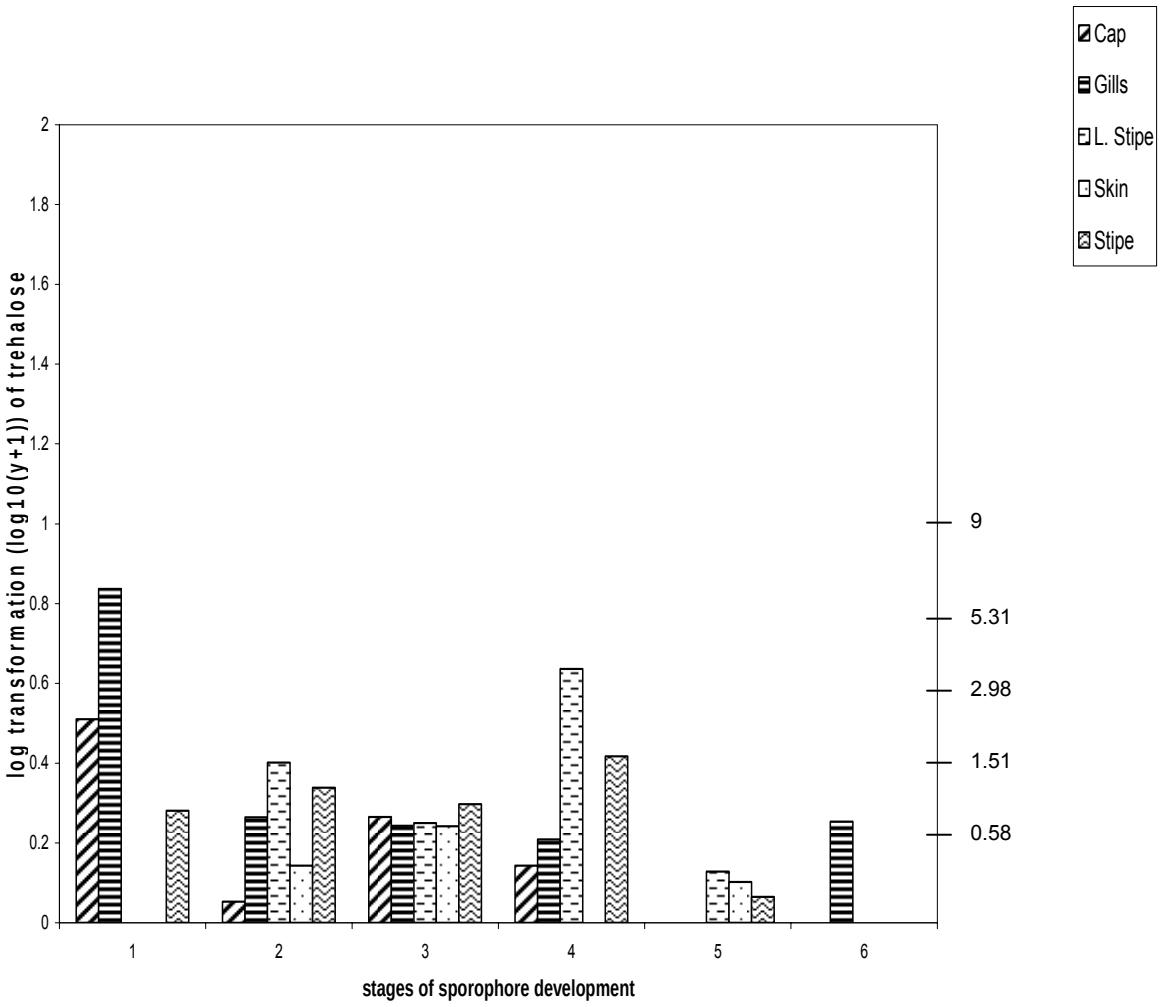

Figure 4.6: Log transformed data showing the amounts of trehalose in the cap, gills, skin, upper and lower stipe tissue of stages 1-6 of sporophore development. In stage 1 sporophore there is no distinction between the upper and lower stipe and is labelled as the stipe in the legend but this refers to the upper stipe tissue for stages 2-6 where there is a distinction between the upper and lower stipe. The corresponding trehalose concentration (mM) for the log transformed values is shown on the right axis. Trehalose was not detected in all tissues during all stages and no trehalose was detected in the skin tissue in stage 4 sporophore, in the cap and gill tissue in stage 5 sporophores or in the cap, upper and lower stipe and gill tissue of stage 6 sporophore.

4.4 DISCUSSION

The polyol erythritol was present in very small amounts in the sporophore tissue and were not detected in all stages of sporophore development. Arabitol was present in higher quantity than erythritol but was not detected in all tissues. Glucose was found in all tissues in all stages of sporophore development but at very low levels and this is in agreement with the findings of Hammond & Nichols (1976). Trehalose was not found in all of the stages of sporophore development while Wannet *et al.* (2000) did find low levels of trehalose in all stages of sporophore development. Mannitol and glycerol were found in all of the tissues in all stages of sporophore development but mannitol was found at much higher levels in all stages of sporophore development. The polyol glycerol is the major polyol in xerophilic and xerotolerant fungi and in yeasts (Magan, 1997). The other polyols erythritol and arabitol detected in very small amounts had not been previously identified but are probably only minor polyols.

The low levels of trehalose produced indicate a fast degradation of this disaccharide which has been suggested as the major carbohydrate translocated from the mycelium to the sporophore. Wannet *et al*. (1999) found the an increase in the degradative activity of trehalose phosphorylase in axenic fruit bodies of *A. bisporus* releasing glucose and glucose-1-phosphate and indicating a fast turnover of trehalose which is a possible reason why there were low levels of trehalose detected. The levels of glucose increased significantly and the activity of this enzyme increased during fruiting between stages 1 and 5 and 1 and 6 of sporophore development. Glucose could be used as a carbon and energy source where it could enter into the carbohydrate routing pathway as indicated in Figure 1.5. There was no trehalose detected at stages 5 and 6 of sporophore development and this could be because of a rapid increase in the TP degradative activity which would be in agreement with the increase in glucose at these stages.

Hammond & Nichols (1976) found that the mannitol levels increased during sporophore development from approximately 30-50% dry weight in the pileus and stipe but the data on the mannitol concentration measured during developmental stages 1-6 (Figure 4.6) did not show significant increases and remained steady during sporophore growth. The polyol mannitol has been postulated to act as an osmoticum in *A. bisporus* and create an osmotic gradient and lower the water potential below that of the compost and casing soil thus allowing for an influx of water into the cells which would increase the turgor pressure thus driving water and solutes into the hyphae and this pressure driven mass flow has been suggested as the mechanism which might explain water and solute transport in *A. bisporus* sporophores (Kalberer, 1987). As shown in Figure 3.6 the mycelium, when grown on compost extract media under increasing matric stresses, did not accumulate large amounts of mannitol but was able to withstand matric stresses without creating an osmotic potential gradient. Noble *et al*. (1999) showed that the mycelium could withstand decreases in compost and casing soil matric potential without having an adverse effect on mycelial growth and the production of sporophores is more closely related to the casing soil matric potential than osmotic potential. The water is held by the physical and chemical structure of the compost and casing soil causing a reduction in the water potential by matric effects. Therefore it is possible that the sporophores are able to extract water from the compost and casing soil without needing to constantly lower the water potential osmotically by increasing the polyol mannitol which would explain the constant levels of mannitol found during sporophore development. The differences between the mannitol concentration found during sporophore developmental stages in this chapter and the increasing levels observed by Hammond & Nichols (1976) could be due to the different cultivation techniques which are now in use which have changed greatly since the 1970's whereby the casing has sugar beet lime rather than mineral aggregate lime and the content of the casing layer is much wetter now (personal communication with Dr. K. Burton).

CHAPTER 5

MOLECULAR CHARACTERISATION OF THE SUGAR TRANSPORTER GENE *SUT1*

5.1 INTRODUCTION

In Chapter 3 the mechanism by which the mycelium extracts water from the compost and casing layer has been examined whereby the osmotically active substance mannitol accumulates to create an osmotic gradient under increasing water stress and is required for an influx of water into the mycelium to increase turgor pressure required for hyphal growth. In *A. bisporus* the polyol mannitol has been shown to accumulate in the mycelium (Chapter 3) and in the sporophores during various stages of development (Hammond & Nichols, 1976; Wannet *et al*., 2000) to create an osmotic gradient. In *A. bisporus* the process of translocation of water and solutes has been suggested by Kalberer (1987) to occur by osmotically derived pressure driven mass flow where mono- and disaccharides are actively transported to the sporophore and metabolised via the carbohydrate pathway (Figure 1.5) synthesising the osmoticum mannitol by the reduction of fructose by MtDH using NADPH produced through the PPP (Edmundowiz & Wriston, 1963). Mannitol accumulates creating an osmotic gradient thus allowing a flux of water into the hyphae which increases the turgor pressure and drives water and solutes into the hyphae.

The metabolism of carbohydrates involved in the metabolic pathway has been studied by Wannet (1999) and was shown to involve mono- and disaccharides. However the mechanism i.e. active primary transport using ATP or secondary transport using a proton or sodium motive force, by which sugars are transported from the compost into the mycelium and then through the sporophore and the particular sugars transported has not been extensively studied. To date the only known sugar transporter in *A. bisporus* was identified by Morales & Thurston (2003), from a partial cDNA sequence in *A. bisporus,* which showed homology to a hexose transporter (HXTA) from *Aspergillus parasiticus*. To understand the physiological process by which *A. bisporus* sporophores can grow and expand so rapidly, where it can develop from stage 1 to 7 within a week, it is necessary to identify the sugars that are actively transported into the sporophore and the type of

transporters involved and this would provide an insight into the mechanism of translocation. The sugar transporters could then be tested to try and manipulate the sporophore growth process using gene silencing methodologies such as RNAi which is currently being assessed to manipulate a number of important genes involved in postharvest quality at HRI (Eastwood *et al*., 2004). This process of gene silencing in *A. bisporus* could possibly slow the rapid growth process because mushrooms are required commercially at the closed button stage.

To identify genes involved in the rapid growth and expansion of the sporophore Dr. Sreenivasaprasad constructed a stage 4 cDNA library using the λ-ZAP Express vector (Stratagene) and from this library 3500 pBK-CMV phagemids were excised and ordered into 96 well microtitre plates and differentially screened using stage 2 and 4 cDNA probes (Sreenivasaprasad *et al.*, 2000b). This resulted in the identification of 31 upregulated cDNAs contained in the pBK-CMV phagemid vector which were sequenced using the T3 and T7 primers of the vector (Sreenivasaprasad *et al.*, 2000b). After this initial sequencing of the upregulated cDNAs and analysis of the sequences they were classified into five gene families and out of the 31 cDNAs 20 were found to be identical and identified as being a putative sugar transporter gene (Sreenivasaprasad *et al.*, 2000b). The cDNA sequence of one of the 20 sugar transporter cDNAs, STID4, was carried out by Krishnamurthy (1999) who identified it as being a sugar transporter, *SUT1*, gene belonging to the sugar transporter family of the MFS, although STID4 cDNA sequence did not have a complete open reading frame and included a number of stop codons. A 1.1 kb fragment of STID4 was then used to screen a cosmid genomic library constructed from a carboxin resistant mutant of *A. bisporus* C54-*carb*.8 (Challen *et al.*, 1996) and three cosmids 20A11, 25E6 and 32B10 were positively identified (Krishnamurthy, 1999).

This chapter details the sequencing of the *SUT1* cDNA and genomic sequence and determining the *SUT1* transcript level during sporophore developmental stages 1-7

as well as in stage 4 fruit body tissue. The full *SUT1* cDNA sequence was completed using STIIE2, which was one of the 20 cDNAs classified as being a sugar transporter gene and the cDNA sequence of STID4 and STIIE2 were compared. The three *SUT1* cosmids 20A11, 25E6 and 32B10 previously identified by Krishnamurthy (1999) were partially sequenced and cosmid 20A11 was used to complete the full genomic sequence of *SUT1*.

5.2 MATERIALS AND METHODS

5.2.1 Sequencing of *SUT1* cDNA STIIE2 and cosmids 20A11, 25E6 and 32B10

STIIE2 phagemid DNA was extracted as described in Section 2.4.1.1 and quantified as described in Section 2.4.3 on a 1% (w/v) agarose gel. The cDNA was sequenced by primer walking using the primers detailed in Appendix 2 and the sequencing reactions were performed as detailed in Section.2.4.5.

Cosmid DNA was extracted from *SUT1* cosmids 20A11, 25E6 and 32B10 as described in Section 2.4.1.2 and quantified as described in Section 2.4.3 on a 1% (w/v) agarose gel. The three cosmids were all sequenced using the primers STGF and STGR detailed in Appendix 2. The cosmid 20A11 was used to complete the full genomic sequence of *SUT1* mainly by primer walking using the primers detailed in Appendix 2.

To complete a small gap (200-300 bases) in the *SUT1* genomic sequence, an internal region of the *SUT1* cosmid 20A11 DNA was PCR amplified using *Pwo* DNA polymerase (Roche) a high fidelity enzyme. The 100 μl amplification reaction contained 0.5 μl *Pwo* DNA polymerase (5 $U\mu l^{-1}$), 50 μM each of 20A11 INT F and R primers, 10 μl of 10X PCR buffer with Mg^{2+} (Roche), 200 μM dNTP (Invitrogen), 2 μl of 20A11 cosmid DNA (25 $ng\mu l^{-1}$) and the volume was adjusted to 100 μl using PCR grade water. The PCR conditions were as described in Table 2.4 except that the annealing temperature used was 60°C and carried out in Hybaid MBS thermocycler. The PCR amplified DNA was then

loaded onto a 2% (w/v) agarose gel and separated by agarose gel electrophoresis as described in Section 2.4.2 along with Low DNA mass ladder (Invitrogen). The DNA was then extracted from the agarose gel as described in Section 2.4.7 using QIAquick gel extraction kit (Qiagen) and eluted from the column using 35 μl of buffer EB. The eluted DNA was then quantified as described in Section 2.4.3 on a 1% (w/v) agarose gel. The PCR fragment was sequenced (as detailed in Section 2.4.5) using the primers 20A11INT F and R.

5.2.2 Digestion of *SUT1* cosmid DNA and hybridisation

3 μg of DNA from *SUT1* cosmids 20A11, 25E6 and 32B10 was digested with *Hin*dIII restriction endonuclease in a total volume of 30 μl as described in Section 2.4.6. The digested DNA was then separated on a 1% (w/v) agarose gel along with DNA marker II (Boehringer Mannheim) as described in Section 2.4.2 and transferred onto Hybond-N nylon membrane (Southern blotting) as described in Section 2.4.9. Single stranded DNA of the 55 bp putative intron (highlighted in Figure 5.1), was synthesised using STID4 primers ID4PINTF and R as detailed in Appendix 2. Equal quantities (25 ng each) of the complimentary strands were mixed and radioactively labelled using ^{32}P as described in Section 2.4.9, except that the DNA was not denatured as it was already single-stranded and were used to probe the digested cosmid DNA. The hybridisation conditions were also as described in Section 2.4.9.

5.2.3 Digestion of STID4 and PCR amplification of STIIE2

STID4 phagemid DNA was extracted as described in Section 2.4.1.1 and diluted 1:10 with ultrapure water and quantified on a 1% (w/v) agarose gel as described in Section 2.4.3 and 4 μg STID4 cDNA was digested using *Eco*RI and *Xho*I restriction enzymes (Roche) in a total volume of 40 μl as described in Section 2.4.6. The digested DNA yielded a 0.7 kb *Eco*RI fragment and a 1.1 kb *Eco*RI/*Xho*I fragment following analysis on a 1% (w/v)

agarose gel as described in Section 2.4.2 along with DNA marker II and VI (Boehringer Mannheim) to determine the correct size of the fragments. The two fragments were then extracted from the agarose gel as described in Section 2.4.7 using QIAquick gel extraction kit (Qiagen). The 1.1 kb and 0.7 kb DNA fragments were then quantified as described in Section 2.4.3 on a 1% (w/v) agarose gel.

Two separate regions (ST1P1 and ST1P2) of *SUT1* cDNA STIIE2 were amplified using two pairs of primers. ST1P1 primer pair was designed to amplify a 0.11 kb region of STIIE2 that codes for a region from TMD IV to V (highlighted in Figure 5.5) and this region is highly conserved amongst the SP family of the MFS and therefore is non-specific to SUT1. ST1P2 primer pair was designed to amplify a 0.2 kb region which is highly specific to *SUT1* and codes for the sugar transporter protein hydrophilic carboxyl-terminus (SUT1-C) after TMD XII (highlighted in Figure 5.5). The conditions used for the PCR amplification reactions were as described in Section 2.4.4 using a Hybaid MBS thermocycler, except that the annealing temperature used for the amplification reactions using the primer pairs for ST1P1 and ST1P2 regions were 64 and 62°C respectively. The 100 μl PCR reactions contained STIIE2 cDNA 4 μl (12 ngμl^{-1}), 1 μM each of forward and reverse primers, 50 μl Red *Taq* Readymix with $MgCl_2$ (Sigma-Aldrich) and the volume was adjusted to 100 μl with PCR grade water. Both ST1P1 and ST1P2 PCR products were examined by gel electrophoresis on a 1% (w/v) agarose gel as described in Section 2.4.2 along with DNA marker VI (Boehringer Mannheim) to verify the size of each fragment. Each PCR product was extracted from the agarose gel using QIAquick gel extraction kit (Qiagen) as described in Section 2.4.7. ST1P1 and ST1P2 PCR products were quantified as described in Section 2.4.3 on a 1% (w/v) agarose gel.

5.2.4 Determination of the transcript level of *SUT1* in compost, vegetative mycelium and during sporophore development

A. bisporus vegetative mycelium (VM) and fruit bodies from stage 1-7 of strains A15 and U3 and tissue (upper and lower stipe, cap, gills and skin) of stage 4 sporophore (A15) and compost taken from a second flush crop of mushrooms (A15) supporting stage 2 and 4 sporophores were removed from the -70°C freezer. Total RNA was extracted from compost, VM, whole sporophores (stage 1-7 of sporophore development) and stage 4 sporophore tissue (upper and lower stipe, cap, gills and skin of cap) as described in Section 2.4.10 and the amount of ground tissue used is detailed in Table 5.1 and 5.2 and the same amount of compost was used as for VM. However compost and VM was extracted twice using phenol:chloroform. RNA was quantified and separated by agarose electrophoresis as described in Section 2.4.11 and 2.4.12 respectively and equal amounts of RNA (approx. 11 μg) were loaded for each sample. RNA was transferred onto Hybond-N nylon membrane (Northern blotting) as described in Section 2.4.13. Hybridisation of the blots was carried out as described in Section 2.4.13. Northern blots were probed using a 28s rRNA fragment (to determine that equal quantities of RNA were loaded onto the gel). Northern blots were probed with the following SUT1 probes: ST1P1 and STIP2 (described in Section 5.2.3), a 55 bp putative intron (highlighted in Figure 5.1) of cDNA STID4 using oligonucleotides ID4PINTF and R, a 700 bp and a 1.1 kb fragment from STID4 as described in Section 5.2.3. All probes were radioactively labelled using ^{32}P as described in Section 2.4.9.

Table 5.1: Amount of tissue used for RNA extractions from vegetative mycelium and sporophore developmental stages 1-7 of *Agaricus bisporus* strains A15 and U3.

A. bisporus Strain	Stages of mushroom development							
	VM	1	2	3	4	5	6	7
A15	44 g	5.5 g	5.5 g	5.5 g	5.5 g	11 g	11 g	11 g
U3	40 g	5.5 g	5.5 g	5.5 g	5.5 g	11 g	11 g	11 g

VM-vegetative mycelium

Table 5.2: Amount of tissue used for RNA extraction from sporophore developmental stage 4 of *Agaricus bisporus* strain A15

A. bisporus (A15) tissue	Sporophore tissue from *A. bisporus* stage 4 of mushroom development				
	Upper stipe	Lower stipe	Cap	Gills	Skin
Tissue	11 g	11 g	11 g	11 g	11 g

5.2.5 Restriction digestion of PC1 genomic DNA to analyse copy number of *SUT1* gene

A. bisporus homokaryotic strain PC1 (*A. bisporus* homokaryotic uracil auxotroph =C28u) (Moore *et al.*, 2001) was cultured by placing agar plugs, stored under sterile conditions in water at 4°C, in the centre of 9 cm Petri dishes containing CE/CYM agar and the plates were incubated at 25°C. After approximately four weeks the plates were removed from the incubator and 7 mm agar plugs were removed using a no. 4 cork borer from the growing margin of the mycelial culture. Into each 9 cm Petri dish three agar plugs were placed and squashed using a sterile spatula and then flooded with MYPG broth (approx. 20 ml was added per Petri dish). The plates were then incubated at 25°C for 4 weeks until a mycelial mat had formed on the surface. The mycelium was removed from the surface of each Petri dish and dried by placing it between six sheets of student grade Whatman paper and excess broth was removed by rolling it with a glass rod. The dried mycelium from each Petri dish was placed in 1.5 ml microcentrifuge tube (Treff Lab) and frozen in liquid N_2. The Eppendorfs containing the mycelial samples were then opened and freeze-dried overnight using a refrigerated freeze drier (Edwards Modulyo) and either used immediately or stored at –20°C.

PC1 genomic DNA was isolated from the freeze-dried mycelium and quantified as described in Sections 2.4.1.3 and 2.4.3 respectively. PC1 genomic DNA (4 μg) was digested using the following restriction enzymes *Bam*HI, *Hin*dIII, *Pst*I, *Kpn*I and *Xho*I as described in Section 2.4.6 in a total volume of 30 μl and spermidine was added to each digestion reaction from a 10 mM stock to give a final concentration of 1 mM. *Kpn*I restriction digest required a final concentration of 100 $\mu g ml^{-1}$ of bovine serum albumin (10 $mg ml^{-1}$, Promega). Three 30 μl digestion reactions were set up for each restriction digest.

The digested PC1 DNA was loaded onto a 0.8% (w/v) agarose gel along with DNA markers II and VI (Boehringer Mannheim) and electrophoresis was carried out in

0.5X TBE at 45V overnight. The agarose gel was split in three so that there were three identical gels and these were individually transferred onto Hybond-N^+ membrane (Amersham Pharmacia) as described in Section 2.4.9. Hybridisation of one membrane was carried out as described in Section 2.4.9 and ST1P2 (as described in Section 5.2.3) was radioactively labelled using ^{32}P and used to probe the membrane. Two ST1P1 (as described in Section 5.2.3) probes were radioactively labelled using ^{32}P and used to probe the two other membranes as described in Section 2.4.9 except that the hybridisation temperature and post-hybridisation washes varied and are described below. One membrane was hybridised at 55°C and washed with solutions PHW I for 15 minutes and with PHW II for 30 minutes. The second membrane was hybridised at the less stringent temperature of 50°C and washed 2 X 15 minutes with PHW I and 1 X 15 minutes with PHW II.

5.3 RESULTS

5.3.1 Analysis of *SUT1* cDNAs STID4 and STIIE2

The nucleotide sequence of STID4, including the putative 55 bp intron is detailed in Figure 5.1. The presence of the putative intron leads to errors in the predicted peptide sequence with a number of stop codons starting from within the intron region despite being read in the correct frame (based on homology to other sugar transporters). To positively identify this 55 bp region as an intron it was used to probe a Southern blot, containing the three SUT1 cosmids: 20A11, 25E6 and 32B10 (which were digested with *Hin*dIII restriction enzyme) and a Northern blot of stages 3, 4 and 5 of *A. bisporus* (A15) sporophore development. The 55 bp intron probe was found to hybridise to the Southern blot containing SUT1 cosmid DNA (Figure 5.2) but did not hybridise to the RNA samples in the Northern blot of stages 3, 4 and 5 of *A. bisporus* (A15) sporophore development (result not shown). However a 700 bp *Eco*RI fragment of cDNA STID4 (which included the

55 bp intron) positively hybridised to a Northern blot of stages 3, 4 and 5 of sporophore development (Figure 5.3), thus indicating that the 55 bp nucleotide sequence was an intron. The 5' sequence of STID4 up to the 55 bp intron (Figure 5.1) is identical to STIIE2 (Figure 5.4) but the inclusion of the intron in STID4 causes a frame shift and therefore the peptide sequence reads differently from that of STIIE2. After this 55 bp intron sequence was removed from STID4 its nucleotide sequence was aligned with that of STIIE2 (Figure 5.4) and they matched base for base except that STIIE2 had a full ORF encoding a predicted peptide of 551 amino acids with a calculated molecular mass of 61.2 kDa and an isoelectric point of 9.23.

Probes generated from the two cDNAs, STIIE2 and STID4 were used to determine the transcript level of the *SUT1* gene in mycelium colonised compost, vegetative mycelium, in stage 4 tissue and during stages of sporophore development.

Figure 5.1: The nucleotide and predicted peptide sequence of SUT1 encoded by cDNA STID4. The nucleotide sequence is shown in lower case with the predicted peptide sequence in upper case underneath the coding region in one letter code and the poly A tail is highlighted in blue. The 55 bp intron is highlighted in red and its inclusion in STID4 sequence causes a frame shift which leads to a number of stop codons which begin within this intron region and are found throughout the sequence denoted by the '-' underneath the nucleotide sequence.

```
                acgcctatattttgacggcatcggcttgcttgggaagtgtgttt
                 Y  A  Y  I  L  T  A  S  A  C  L  G  S  V  F
cacgggtgggatgtgcaagtatccttttgacgctggctcatcccttgtttgctgatggtt
 H  G  W  D  V  Q  V  S  F  -  R  W  L  I  P  C  L  L  M  V
aatttgtagaggccttataggaggcatactctcgctacggtcatttcaagaatatctcgg
 N  L  -  R  P  Y  R  R  H  T  L  A  T  V  I  S  R  I  S  R
gatcaatacaaaaaatgccgtcaagaaagcgattctagacggaaacatcatctctgtgct
 D  Q  Y  K  K  C  R  Q  E  S  D  S  R  R  K  H  H  L  C  A
ccaagccggatgttttttggcgcgcttggaaccggatatctctctagtcgattcggccg
 P  S  R  M  F  F  W  R  A  W  N  R  I  S  L  -  S  I  R  P
aagaccctgtcttattgcatctggtattgtgtatataactggcggtttgctgcaatgcac
 K  T  L  S  Y  C  I  W  Y  C  V  Y  N  W  R  F  A  A  M  H
tgtcggtttgggaccctcgcaagctgctgctctacacgtgttctatattggcaggttcat
 C  R  F  G  T  L  A  S  C  C  S  T  R  V  L  Y  W  Q  V  H
ttctggtatcggtgttgggatggtgtccactctcgtgcctttgtatatttcggagtgtgt
 F  W  Y  R  C  W  D  G  V  H  S  R  A  F  V  Y  F  G  V  C
ccctaggactatacgcgggcgctgtactggaacactccaatttgcgactaacagtggtct
 P  -  D  Y  T  R  A  L  Y  W  N  T  P  I  C  D  -  Q  W  S
gatgctgggcttctgggtcaactacagcgtgtcgaaaaacgtgccctttggtgaaatgca
 D  A  G  L  L  G  Q  L  Q  R  V  E  K  R  A  L  W  -  N  A
atggcgaattccgttaattatccaaatgattccgagcctcttgttcatcatagccatgtt
 M  A  N  S  V  N  Y  P  N  D  S  E  P  L  V  H  H  S  H  V
tttccaaccagaatcgccgagatggcttgttgaacacgggaaacacaaggaagctgcgac
 F  P  T  R  I  A  E  M  A  C  -  T  R  E  T  Q  G  S  C  D
ggtactggcgcgtactggcggcaaggatgttgatcatcctagtgttgtacagacactgga
 G  T  G  A  Y  W  R  Q  G  C  -  S  S  -  C  C  T  D  T  G
ggagatcaagcaagaatttgtggcgagtaaacaaccatcgtttttaaagcagattcgcct
 G  D  Q  A  R  I  C  G  E  -  T  T  I  V  F  K  A  D  S  P
ggtcggtgaatcgagggctgttgccctgaggtgctttataccaccgctagtgatgttctt
 G  R  -  I  E  G  C  C  P  E  V  L  Y  T  T  A  S  D  V  L
ccagcagtggacgggtacaaatgccatcaacctttatagtcccgaagtattccgtcatct
 P  A  V  D  G  Y  K  C  H  Q  P  L  -  S  R  S  I  P  S  S
tggaatccatggcaccagcggggctctcttcgctactggtgtttatggcgtggtgaaggt
 W  N  P  W  H  Q  R  G  S  L  R  Y  W  C  L  W  R  G  E  G
tgtttcagttgcacttgccctcacttttgctgtcgaacgctttggacgcaagagagggtt
 C  F  S  C  T  C  P  H  F  C  C  R  T  L  W  T  Q  E  R  V
gatttttggtggtatcggccaagcacttatgatgttttggttgggaggttatagtgccac
 D  F  W  W  Y  R  P  S  T  Y  D  V  L  V  G  R  L  -  C  H
ccaccaagacggtactgtcagtcctgcgagtcatgtttccattgttgcactctacttgta
 P  P  R  R  Y  C  Q  S  C  E  S  C  F  H  C  C  T  L  L  V
tggtgcattcttctccatgggatggggcccattaccatgggtcgtcgctggagaggttgc
 W  C  I  L  L  H  G  M  G  P  I  T  M  G  R  R  W  R  G  C
acctaaccatgtccgctccttcgccctctccatcgccgttggaactcattggctcttcgg
 T  -  P  C  P  L  L  R  P  L  H  R  R  W  N  S  L  A  L  R
gtttgtgatatcaaaagtgacgccaattatgttggaccgtatcaaatatggcacattcct
 V  C  D  I  K  S  D  A  N  Y  V  G  P  Y  Q  I  W  H  I  P
actcttcggattctgttgcatgatagtagcgacatgggcttatttctgtctacctgagac
 T  L  R  I  L  L  H  D  S  S  D  M  G  L  F  L  S  T  -  D
aagtgggttcgctctggaggacatcaaatatctgttcgagcgagacgtcatcattcgttc
 K  W  V  R  S  G  G  H  Q  I  S  V  R  A  R  R  H  H  S  F
attgcaggacgctcccggtggaaaaatattcttgggggggaggcgtgtggaatctgtagc
 I  A  G  R  S  R  W  K  N  I  L  G  G  E  A  C  G  I  C  S
ttcgttgaaagagaggcgcgttggagtcgctggtgagcagggtgagaagataactggtct
 F  V  E  R  E  A  R  W  S  R  W  -  A  G  -  E  D  N  W  S
aaattcggaattggaagatgtttcctcaaaaaaatcaacattgaaggaaacttcatccgt
 K  F  G  I  G  R  C  F  L  K  K  I  N  I  E  G  N  F  I  R
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 L  I  Y  S  L  Q  I  L  L  –  C  M  F  P  N  S
aaaaaa
```

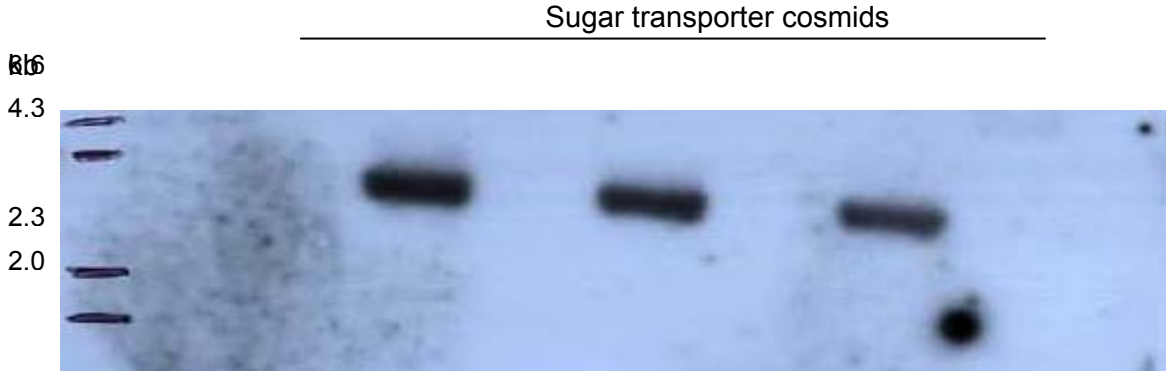

Figure 5.2: Southern blot showing positive hybridisation of DNA from *SUT1* cosmids: 20A11, 25E6 and 32B10 with the 55 bp putative intron (highlighted in Figure. 5.1) from cDNA STID4. The cosmid DNA (3 µg) were digested with *Hin*dIII restriction endonuclease and equal amounts of digested cosmid DNA were separated on a 0.8% agarose gel, transferred onto nylon membrane and hybridised with ^{32}P α-dCTP labelled 55 bp sequence. The 55 bp putative intron probe did not hybridise with a Northern blot containing equal amounts of total RNA from stages 3, 4 and 5 of *Agaricus bisporus* (A15) sporophore development (result not shown).

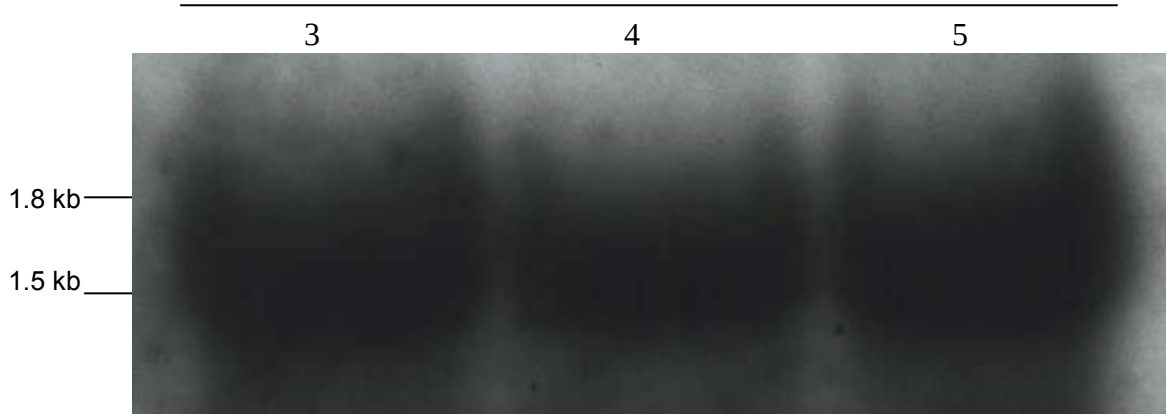

Figure 5.3: Northern blot showing positive hybridisation of *SUT1* during stages 3, 4 and 5 of sporophore development of *Agaricus bisporus* (A15). Equal amounts of total RNA were loaded for each of stages 3, 4 and 5 of sporophore development and the blot was probed with ^{32}P α-dCTP labelled 700 bp *Eco*RI fragment (including the 55 bp putative intron) of cDNA STID4. The *SUT1* transcript was detected equally in stages 3, 4 and 5 of sporophore development.

ggctgagctctattcatcatggcgtcggaacgacagattgaagaacttcccagttataag
M A S E R Q I E E L P S Y K

tacgcctatattttgacggcatcggcttgcttgggaagtgtgtttcacgggtgggatgta
tacgcctatattttgacggcatcggcttgcttgggaagtgtgtttcacgggtgggatgta
Y A Y I L T A S A C L G S V F H G W D V

ggccttataggaggcatactctcgctacggtcatttcaagaatatctcgggatcaataca
ggccttataggaggcatactctcgctacggtcatttcaagaatatctcgggatcaataca
G L I G G I L S L R S F Q E Y L G I N T

aaaaatgccgtcaagaaagcgattctagacggaaacatcatctctgtgctccaagccgga
aaaaatgccgtcaagaaagcgattctagacggaaacatcatctctgtgctccaagccgga
K N A V K K A I L D G N I I S V L Q A G

tgtttttttggcgcgcttggaaccggatatctctctagtcgattcggccgaagaccctgt
tgtttttttggcgcgcttggaaccggatatctctctagtcgattcggccgaagaccctgt
C F F G A L G T G Y L S S R F G R R P C

cttattgcatctggtattgtgtatataactggcggtttgctgcaatgcactgtcggtttg
cttattgcatctggtattgtgtatataactggcggtttgctgcaatgcactgtcggtttg
L I A S G I V Y I T G G L L Q C T V G L

ggaccctcgcaagctgctgctctacacgtgttctatattggcaggttcatttctggtatc
ggaccctcgcaagctgctgctctacacgtgttctatattggcaggttcatttctggtatc
G P S Q A A A L H V F Y I G R F I S G I

ggtgttgggatggtgtccactctcgtgcctttgtatatttcggagtgtgtccctaggact
ggtgttgggatggtgtccactctcgtgcctttgtatatttcggagtgtgtccctaggact
G V G M V S T L V P L Y I S E C V P R T

atacgcgggcgctgtactggaacactccaatttgcgactaacagtggtctgatgctgggc
atacgcgggcgctgtactggaacactccaatttgcgactaacagtggtctgatgctgggc
I R G R C T G T L Q F A T N S G L M L G

ttctgggtcaactacagcgtgtcgaaaaacgtgcccttggtgaaatgcaatggcgaatt
ttctgggtcaactacagcgtgtcgaaaaacgtgcccttggtgaaatgcaatggcgaatt
F W V N Y S V S K N V P F G E M Q W R I

ccgttaattatccaaatgattccgagcctcttgttcatcatagccatgtttttccaacca
ccgttaattatccaaatgattccgagcctcttgttcatcatagccatgtttttccaacca
P L I I Q M I P S L L F I I A M F F Q P

gaatcgccgagatggcttgttgaacacgggaaacacaaggaagctgcgacggtactggcg
gaatcgccgagatggcttgttgaacacgggaaacacaaggaagctgcgacggtactggcg
E S P R W L V E H G K H K E A A T V L A

cgtactggcggcaaggatgttgatcatcctagtgttgtacagacactggaggagatcaag
cgtactggcggcaaggatgttgatcatcctagtgttgtacagacactggaggagatcaag
R T G G K D V D H P S V V Q T L E E I K

caagaatttgtggcgagtaaacaaccatcgttttaaagcagattcgcctggtcggtgaa
caagaatttgtggcgagtaaacaaccatcgttttaaagcagattcgcctggtcggtgaa
Q E F V A S K Q P S F L K Q I R L V G E

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S R A V A L R C F I P P L V M F F Q Q W

acgggtacaaatgccatcaacctttatagtcccgaagtattccgtcatcttggaatccat
acgggtacaaatgccatcaacctttatagtcccgaagtattccgtcatcttggaatccat
T G T N A I N L Y S P E V F R H L G I H

```
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 G  T  S  G  A  L  F  A  T  G  V  Y  G  V  V  K  V  V  S  V

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 F  S  M  G  W  G  P  L  P  W  V  V  A  G  E  V  A  P  N  H

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 F  C  C  M  I  V  A  T  W  A  Y  F  C  L  P  E  T  S  G  F

gctctggaggacatcaaatatctgttcgagcgagacgtcatcattcgttcattgcaggac
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 A  L  E  D  I  K  Y  L  F  E  R  D  V  I  I  R  S  L  Q  D

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gctcccggtggaaaaatattcttgggggggaggcgtgtggaatctgtagcttcgttgaaa
 A  P  G  G  K  I  F  L  G  G  R  R  V  E  S  V  A  S  L  K

gagaggcgcgttggagtcgctggtgagcagggtgagaagataactggtctaaattcggaa
gagaggcgcgttggagtcgctggtgagcagggtgagaagataactggtctaaattcggaa
 E  R  R  V  G  V  A  G  E  Q  G  E  K  I  T  G  L  N  S  E

ttggaagatgtttcctcaaaaaaatcaacattgaaggaaacttcatccgtttgatatata
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 L  E  D  V  S  S  K  K  S  T  L  K  E  T  S  S  V  -

gtctccaaattctattgtaatgccattttcccaattcaaaaaaaaaaaaaaaaaaa
gtctccaaattctattgtaatgccattttcccaattcaaaaaaaaaaaaaaaaaaa
```

Figure 5.4: Nucleotide and predicted peptide sequence of *SUT1* cDNAs STIIE2 and STID4. The nucleotide sequence of STID4 (without the 55 bp intron) encoding a partial cDNA transcript and STIIE2 encoding a full open reading frame are highlighted in red and black respectively. The predicted peptide sequence is in upper case below the coding region in one letter code. The 12 putative transmembrane domains, underlined in black, were predicted according to the hidden Markov model (HMM) membrane topology prediction method (Tusnady & Simon, 1998). The '– ' denotes the stop codon.

5.3.2 Analysis of *SUT1* cDNA and genomic sequences

Topology prediction of the putative peptide sequence (551 amino acids) of SUT1, revealed it to contain 12 TMDs and was achieved using the hidden Markov model (HMM) topology prediction method (http://www.enzim.hu/hmmtop) (Tusnady & Simon, 1998) and a model of the secondary structure of SUT1 protein has been produced based on this topology prediction (Figure 5.5). The 12 TMDs of SUT1 were linked by hydrophilic loops of between 8 and 20 amino acids with the exception of a larger cytoplasmic loop of 65 amino acids between TMD VI and VII (Figure 5.5). The duplicated pentameric motif RXGRR (where R can be replaced by K and X is usually hydrophobic) was found between TMD II and III and VIII and IX as RFGRR and RFGRK respectively in the SUT1 protein (highlighted in Figure 5.5), and this motif is found duplicated in these positions in nearly all members of MFS (Maiden *et al.*, 1987; Baldwin & Henderson, 1989; Sato & Mueckler, 1999; Hvorup & Saier, 2002). Also conserved motifs PESPRXL and PETKGXXXE are found at the ends of the sixth and twelfth TMDs respectively in nearly all members of the sugar transporter family of the MFS (Griffith, 1992) and these were also found in these positions in the SUT1 peptide sequence as PESPRWL and PETSGFALE. These conserved sequences and 12 TMDs found in SUT1 are distinguishing features of members of the MFS.

All three cosmids: 20A11, 32B10 and 25E6 which positively hybridised to *SUT1* cDNA STID4 using a 1.1 kb fragment (Krishnamurthy, 1999) were partially sequenced (1 kb) and were found to be identical, therefore one cosmid 20A11 was used to generate full genomic sequence of *SUT1* and the ORF is 2055 bp long (from the predicted translation start ATG to the stop TGA). From comparison of the *SUT1* genomic and cDNA sequences, seven introns were identified in the genomic sequence of between 52 and 62 bp in length (Figure 5.6).

The copy number of the *SUT1* gene was determined using both ST1P1 (non-gene specific probe) and ST1P2 (gene-specific probe) which are PCR amplified regions of the *SUT1* gene (highlighted in Figure 5.5). The *SUT1* gene has been determined as a single copy gene when ST1P2 probe was used under standard high stringency hybridisation conditions (Figure 5.7 A). However when ST1P1 was used to probe two identical membranes under low stringency hybridisation conditions: one membrane was hybridised at 50°C and the other at 55°C (Section 5.2.5), more than one band was detected in at least three of the five lanes for both membranes (Figure 5.7 B), thus indicating the presence of one or more sugar transporter(s) with homology to the *SUT1* gene in *A. bisporus*. Only the result for hybridisation at 55°C with ST1P1 is shown as hybridisation at both temperatures gave the same result.

The predicted peptide sequence of *SUT1*, using the cDNA STIIE2 sequence (Figure 5.4) was used to search the Swiss-Prot /TrEMBL database (webserver, http://us.expasy.org/) for homology to other proteins and identify a possible function of this protein. The putative SUT1 protein showed homology (28-33% identity and 46-50% similarity) to a number of fungal proteins belonging to the sugar transporter family of the MFS (Table 5.3). For example, 28-30% identity and 46-48% similarity were observed with the monosaccharide transporters such as HXT2, MSTA and MST1 from *S. cerevisiae*, *Aspergillus niger* and *A. muscaria*. Similarly 32-33% identity and 48-50% similarity were observed with the quinate permeases from ascomycetes *N. crassa, Aspergillus nidulans* and *Podospora anserina*. However, both the quinate permeases and the monosaccharide transporters amongst themselves showed considerably higher levels of homology. SUT1 did not show high level of homology to a specific sugar transporter and therefore it was difficult to assign a specific sugar of transport to this protein based on sequence analysis. The sequence homology between SUT1 and quinate transporters and monosaccharide transporters is probably due to the fact that these are all distantly related fungal proteins

belonging to the sugar transporter family of the MFS showing some of the characteristic features such as conserved motifs and the 12 TMD secondary structure as described earlier. From a multiple alignment (Figure 5.8) of SUT1 predicted protein sequence with MST1 from *A. muscaria*, QA-Y from *N. crassa* and QUTD from *A. nidulans* it can be seen that these proteins share a number of conserved motifs found amongst the sugar transporter family of the MFS and are therefore likely to be distantly related.

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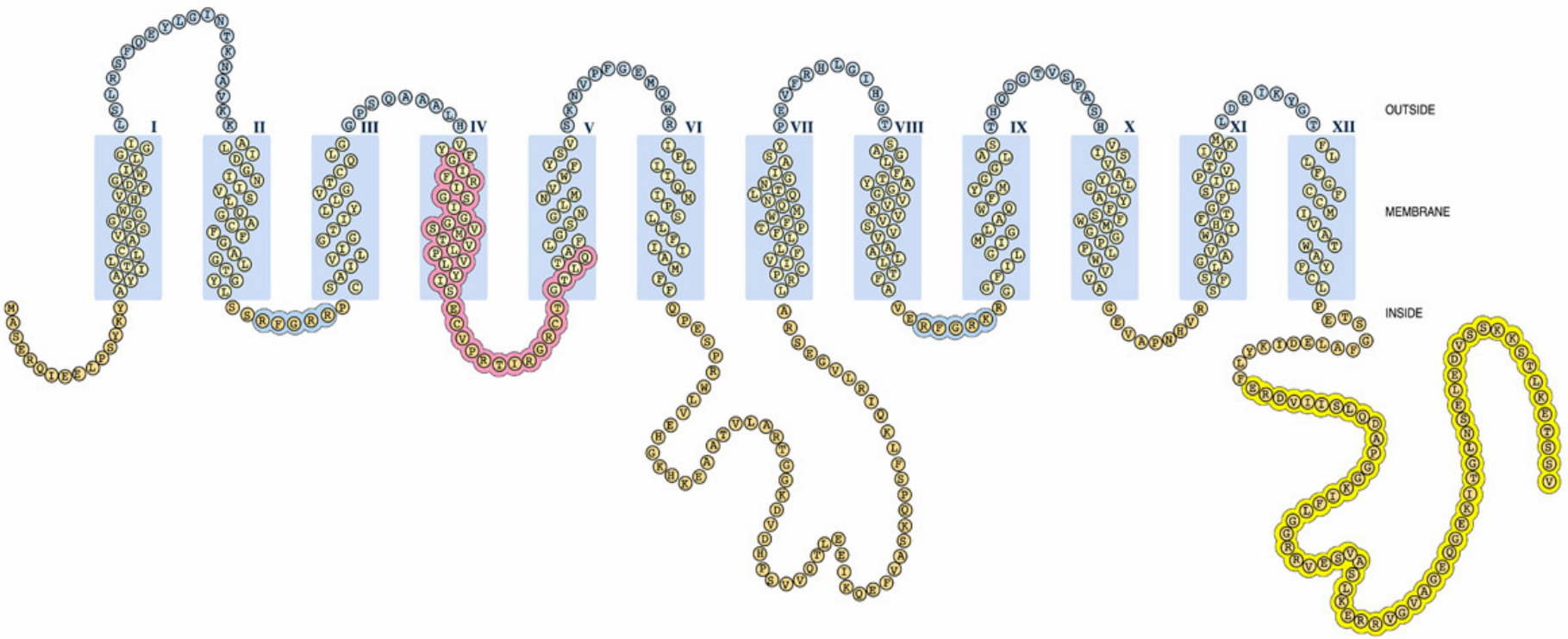

Figure 5.5: Topology prediction model of *Agaricus bisporus* sugar transporter protein (SUT1) using the predicted peptide sequence encoded by the cDNA STIIE2. The topology prediction was achieved using the hidden Markov model (HMM) (Tusnady & Simon, 1998), revealing 12 transmembrane domains (TMD) (I-XII), which are shown in shaded blue boxes. Two repetitive motifs RFGRR and RFGRK, which link TMD II and III and TMD VIII and IX respectively and are found amongst members of the major facilitator superfamily (MFS) of transporters, are highlighted in blue. ST1P1 (a conserved region amongst the sugar transporter family of the MFS) and ST1P2 (a region which is highly specific to SUT1) are highlighted in red and yellow, respectively.

Figure 5.6: Analysis of *SUT1* genomic sequence of *Agaricus bisporus* using cosmid 20A11 where coding regions are indicated by upper case letters and introns (in italics) and flanking regions are in lower case. The amino acid sequence is given underneath the coding regions in one letter code. The amino acid sequences of the 12 putative transmembrane domains (TMD) (deduced from the topology prediction model of the *SUT1* cDNA sequence) are boxed and duplicated motifs RFGRR and RFGRK found between TMD II and III and VIII and IX respectively are highlighted in red. Conserved motifs PESPRWL and PETSGFALE found immediately after the sixth and twelfth TMDs are highlighted in green. Seven introns italicised in lower case and numbered 1-7 were identified from the comparison of the cDNA (STIIE2) and genomic (20A11) sequences. Putative TAATA and CAAT sites are underlined in the promoter and terminator regions. ⏩indicates start of the cDNA transcript, ▸ indicates the poly A tail in the cDNA transcript, ◂ indicates the end of the cDNA transcript and – indicates the stop codon.

-318 ttaatactaattggaagtttcagttgttggatgagcaa -281

agttggtgcagatagaaactagaattcggattcccatatctgaggtacctttttccttccgctggcaatcc -211

tggccacttcgacgtggtgacgcagagggcgcgtgctattgttagcacatgccatatggatcgacgttgc -141

ctctcgtacttcgcgcctaggctcgctcatgcctcgatgcatctttcaattcgggcgttgcgtctcccag -71

gtgcctgttaaaagggcgaactttagtgtaattgtactaacacagtccctcgggctgagctctattcatc -1

ATGGCGTCGGAACGACAGATTGAAGAACTTCCCAGTTATAAGTACGCCTATATTTTGACGGCATCGGCTT 70
M A S E R Q I E E L P S Y K Y A Y I L T A S A

GCTTGGGAAGTGTGTTTCACGGGTGGGATGTgcaagtatccttttgacgctggctcatcccttgtttgct 140
C L G S V F H G W D V

gatggttaatttgtagAGGCCTTATAGGAGGCATACTCTCGCTACGGTCATTTCAAGAATATCTCGGGAT 210
G L I G G I L S L R S F Q E Y L G I

CAATACAAAAAATGCCGTCAAGAAAGCGATTCTAGACGGAAACATCATCTCTGTGCTCCAAGCCGGATGT 280
N T K N A V K K A I L D G N I I S V L Q A G C

TTgtaagtcgtagcactcgttcgaccagcttactttcttttactaacaacgtgttgacctagTTTTGGCG 350
F F G

CGCTTGGAACCGGATATCTCTCTAGTCGATTCGGCCGAAGACCCTGTCTTATTGCATCTGGTATTGTGTA 420
A L G T G Y L S S R F G R R P C L I A S G I V Y

TATAACTGGCGGTTTGCTGCAATGCACTGTCGGTTTGGGACCCTCGCAAGCTGCTGCTCTACACGTGTTC 490
I T G G L L Q C T V G L G P S Q A A A L H V F

TATATTGGCAGGTTCATTTCTGGTATCGGTGTTGGGATGGTGTCCACTCTCGTGCCTTTGTATATTTCGG 560
Y I G R F I S G I G V G M V S T L V P L Y I S

AGTGTGTCCCTAGGACTATACGCGGGCGCTGTACTGGAACACTCCAATTTGCGACTAACAGTGGTCTGAT 630
E C V P R T I R G R C T G T L Q F A T N S G L M

GCTGGGCTgtaagtgtacctttgttatgctccggggacgatactaaaagtagtctgtagTCTGGGTCAA 700
L G F W V N

CTACAGCGTGTCGAAAAACGTGCCCTTTGGTGAAATGCAATGGCGAATTCCGTTAATTATCCAgtacgtt 770
Y S V S K N V P F G E M Q W R I P L I I Q

attggatgcaaagtgaagcatatgctgaaattagacttgtgtcgtactttccagAATGATTCCGAGCCTC 840
M I P S L

TTGTTCATCATAGCCATGTTTTTCCAACCAGAATCGCCGAGATGGCTTGTTGAACACGGGAAACACAAGG 910
L F I I A M F F Q P E S P R W L V E H G K H K

AAGCTGCGACGGTACTGGCGCGTACTGGCGGCAAGGATGTTGATCATCCTAGTGTTGTACAGACACTGGA 980
E A A T V L A R T G G K D V D H P S V V Q T L E

GGAGATCAAGCAAGAATTTGTGGCGAGTAAACAACCATCGTTTTTAAAGCAGATTCGCCTGGTCGGTGAA 1050
E I K Q E F V A S K Q P S F L K Q I R L V G E

TCGAGGGCTGTTGCCCTGAGGTGCTTTATACCACCGCTAGTGATGTTCTTCCAGCAGgtattgaatctcc 1120
S R A V A L R C F I P P L V M F F Q Q

ttaatggaaagatgcttaccgtcgaactcactccatccgtccctttagTGGACGGGTACAAATGCCATC 1190
W T G T N A I

```
AACCTTTATAGTCCCGAAGTATTCCGTCATCTTGGAATCCATGGCACCAGCGGGGCTCTCTTCGCTACTG  1260
 N  L  Y  S  P  E  V  F  R  H  L  G  I  H  G  T  S  G  A  L  F  A  T

GTGTTTATGGCGTGGTGAAGGTatactcaatatgttcatcgctatgaagtctcctaatcgttattgatgt  1330
G  V  Y  G  V  V  K  V

tacaggtTGTTTCAGTTGCACTTGCCCTCACTTTTGCTGTCGAACGCTTTGGACGCAAGAGAGGGTTGAT  1400
          V  S  V  A  L  A  L  T  F  A  V  E  R  F  G  R  K  R  G  L  I

TTTTGGTGGTATCGGCCAAGCACTTATGATGTTTTGGTTGGGAGGTTATAGTGCCACCCACCAAGACGGT  1470
  F  G  G  I  G  Q  A  L  M  M  F  W  L  G  G  Y  S  A  T  H  Q  D  G

ACTGTCAGTCCTGCGAGTCATGTTTCCATTGTTGCACTCTACTTGTATGGTGCATTCTTCTCCATGGGAT  1540
 T  V  S  P  A  S  H  V  S  I  V  A  L  Y  L  Y  G  A  F  F  S  M  G

GGGGCCCATTACCgtaggtcgtcattcgctactattgagttggcgactgaagaatatttcttgttagATG  1610
W  G  P  L  P                                                       W

GGTCGTCGCTGGAGAGGTTGCACCTAACCATGTCCGCTCCTTCGCCCTCTCCATCGCCGTTGGAACTCAT  1680
  V  V  A  G  E  V  A  P  N  H  V  R  S  F  A  L  S  I  A  V  G  T  H

TGGCTCTTCGGGTTTGTGATATCAAAAGTGACGCCAATTATGTTGGACCGTATCAAATATGGCACATTCC  1750
 W  L  F  G  F  V  I  S  K  V  T  P  I  M  L  D  R  I  K  Y  G  T  F

TACTCTTCGGATTCTGTTGCATGATAGTAGCGACATGGGCTTATTTCTGTCTACCTGAGACAAGTGGGTT  1820
L  L  F  G  F  C  C  M  I  V  A  T  W  A  Y  F  C  L  P  E  T  S  G  F

CGCTCTGGAGGACATCAAATATCTGTTCGAGCGAGACGTCATCATTCGTTCATTGCAGGACGCTCCCGGT  1890
  A  L  E  D  I  K  Y  L  F  E  R  D  V  I  I  R  S  L  Q  D  A  P  G

GGAAAAATATTCTTGGGGGGGAGGCGTGTGGAATCTGTAGCTTCGTTGAAAGAGAGGCGCGTTGGAGTCG  1960
 G  K  I  F  L  G  G  R  R  V  E  S  V  A  S  L  K  E  R  R  V  G  V

CTGGTGAGCAGGGTGAGAAGATAACTGGTCTAAATTCGGAATTGGAAGATGTTTCCTCAAAAAAATCAAC  2030
A  G  E  Q  G  E  K  I  T  G  L  N  S  E  L  E  D  V  S  S  K  K  S  T

ATTGAAGGAAACTTCATCCGTTTGAtatatagtctccaaattctattgtaatgccattttcccaattcaa  2100
  L  K  E  T  S  S  V  -                                              ▸

aaggacccgctctcgaaccgggtcagatgcaattttggtcagcaatggtttatgttgtttccccgtaagt  2170
                ◂

atgcactagaagagaacaaaacgtcactatttgctcaatgcaggatgcacctggcgagataatattcttg  2240

cggtgaagtcgaacaacgtctgtagtcctgtaaaaatatacagtgagtagagggatgatgccgatgtgga  2310

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catgcgtcatgacgcaagctgtcaattttcattttccagttcggtcccattctcactctcgcccctccta  2730

atgtcttccagaaaa 2745
```

Figure 5.7: Southern blots showing the copy number of the *SUT1* gene. Genomic DNA was isolated from *Agaricus bisporus* PC1 homokaryotic strain and approximately 4 μg of PC1 DNA was digested with the following restriction enzymes: *Bam*HI (lane1), *Hin*dIII (lane 2), *Pst*I (lane 3), *Kpn*I (lane 4), *and XhoI* (lane 5). The DNA fragments were separated on a 0.8% agarose gel and transferred onto nylon membrane. Southern blot (A) was probed with ST1P2 (hybridisation temperature 65°C) which is a PCR amplified 0.2 kb region of *SUT1* cDNA STIIE2 which codes for the SUT1 hydrophilic carboxyl-terminus after transmembrane domain (TMD) XII and is highly specific to SUT1. Southern blot (B) was probed with ST1P1 (hybridisation temperature 55°C) which is a PCR amplified 0.11 kb region of STIIE2 that codes for a region from TMD IV to V and is conserved amongst the sugar transporter family of the MFS, therefore it is a non-specific region of SUT1. The probes were labelled with ^{32}P α-dCTP. A single band could be detected in each lane in blot A indicating the presence of a single copy of the *SUT1* gene but in blot B two bands could be detected in lanes 2, 4 and 5 indicating the presence of more than one sugar transporter gene in *A. bisporus*.

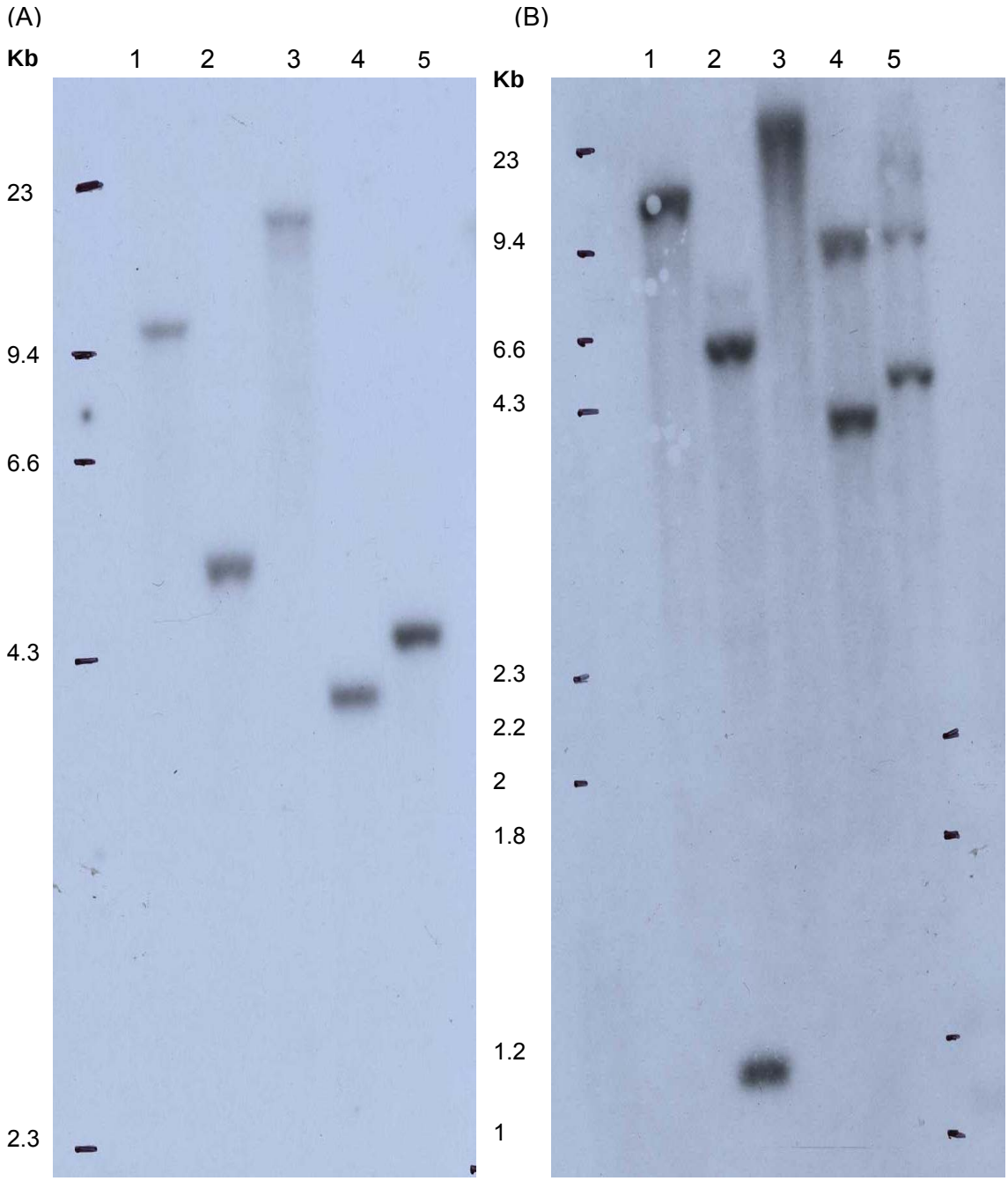
(A)
Kb
1
2
3
4
5
23
9.4
6.6
4.3
2.3
(B)
Kb
1
2
3
4
5
23
9.4
6.6
4.3
2.3
2.2
2
1.8
1.2
1

Table 5.3: Identity and similarity of the SUT1 protein, using the STIIE2 cDNA putative peptide sequence, to other proteins based on blastp search of the databases.

Protein	Organism	Identity (%)	Positives (%) (similarity)	Accession no
QA-Y Quinate permease	*Neurospora crassa*	33	48	P116306
QUTD Quinate permease	*Aspergillus nidulans*	32	50	P15325
Quinate permease	*Podospora anserina*	33	49	Q86ZM8
MSTA Monosaccharide transporter	*Aspergillus niger*	29	48	Q8JOV1
MST1 Monosaccharide transporter	*Amanita muscaria*	30	48	O13411
HXT2 Glucose transporter	*Saccharomyces cerevisiae*	28	46	P23585

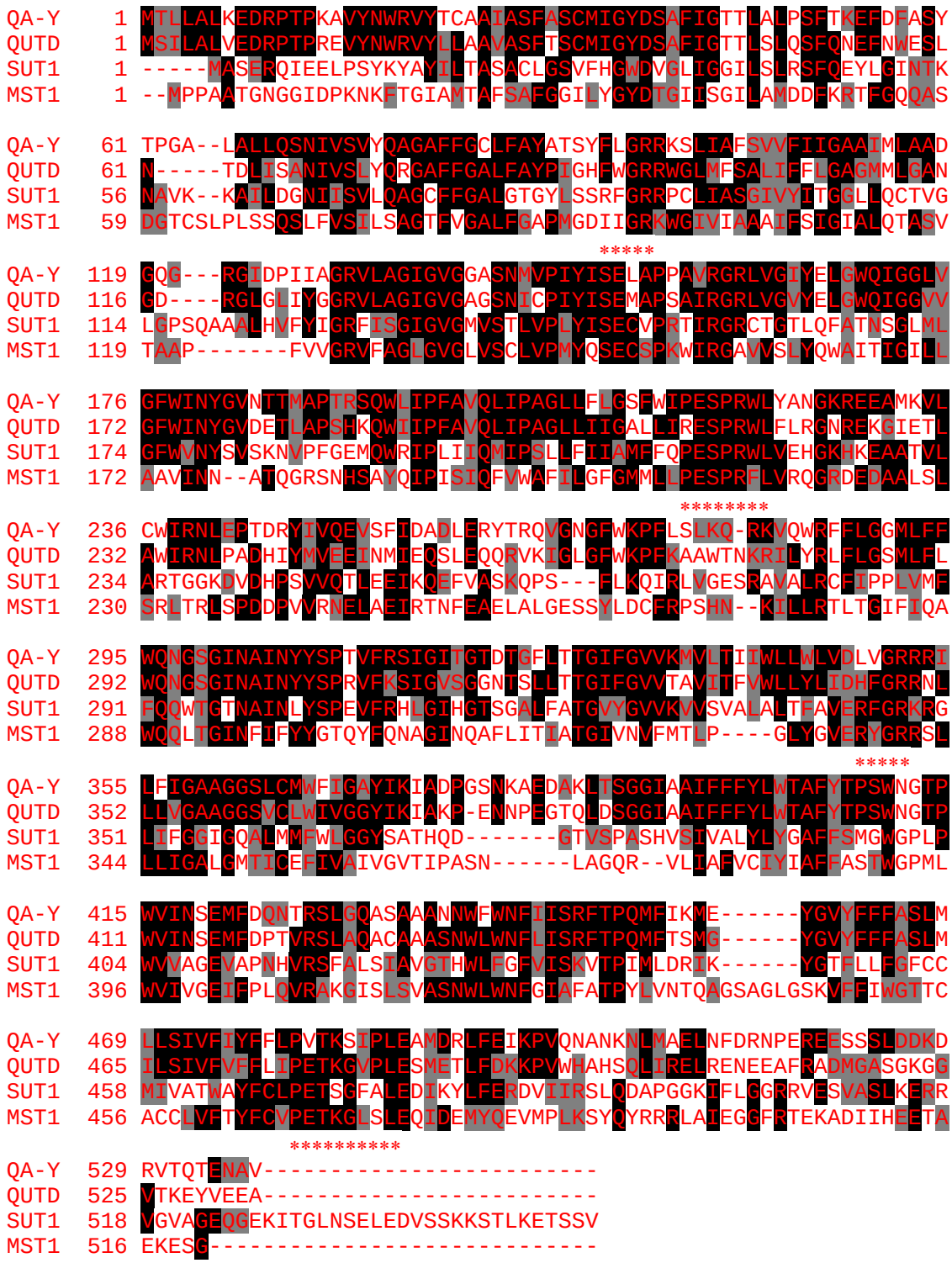

Figure 5.8: Multiple sequence alignment of SUT1 amino acid sequence from *Agaricus bisporus* with other members of the fungal sugar transporter family of the major facilitator superfamily (MFS). The multiple sequence alignment was generated using Clustal W and box shade and regions highlighted in black are identical and those shaded in grey are similar. Alignment was generated between the SUT1 amino acid sequence and quinate permeases QA-Y and QUTD from *Neurospora crassa* and *Aspergillus nidulans* respectively and the monosaccharide transporter MST1 from *Amanita muscaria*. Highly conserved and duplicated motifs found amongst members of the MFS are denoted by asterix ∗.

5.3.3 Analysis of *SUT1* gene expression during sporophore developmental stages, and in sporophore tissues

Northern blots were prepared containing equal amounts of total RNA from VM, compost colonised by mycelium taken from a second flush crop of mushrooms (strain A15) supporting stage 2 and 4 sporophores, stages 1-7 of sporophore development of both U3 and A15 strains of *A. bisporus* and stage 4 tissue (upper stipe, lower stipe, cap, gills and cap skin) from *A. bisporus* strain A15. All the blots were probed with a 28S rRNA control probe and with a 1.1 kb fragment of *SUT1.*

No *SUT1* transcript could be detected by Northern analysis in compost colonised by mycelium which was supporting stage 2 and 4 sporophores (result not shown) indicating the absence of this specific sugar transporter in the compost and suggesting that one or more other sugar transporters are involved in transporting sugars from the mycelium to the sporophore. In both *A. bisporus* strains A15 and U3 no *SUT1* transcript was detected in VM, with increasing levels during sporophore development (stages 1-7) as indicated in Figure 5.9 and 5.10 respectively. However there were differences in the level of transcript of *SUT1* detected between the two *A. bisporus* strains (U3 & A15) with U3 there was a large increase in transcript from stage 2-7 but with A15 no transcript could be detected at stage 2 and the increase from stage 3 onwards was not as great as with U3. For both A15 and U3 strains the *SUT1* transcript was barely detectable at stage 1. This difference in expression patterns between the two strains, A15 and U3, could be due to the growing conditions of the sporophores of the different strains and the fact that they were part of different crops. Although growing conditions are standardised at HRI mushroom unit some variation can be expected between strains even though the sporophores from A15 and U3 were taken from a second flush crop. However the overall pattern of expression of *SUT1* observed between the two strains is

similar where there is an increase in transcript after stage 3 through to the final stage 7 of sporophore development.

Because the initial transcript of the *SUT1* gene was determined using a 1.1 kb fragment of cDNA STID4 there was a possibility that the level of transcript detected could also include other possible sugar transporter genes with sequence similarity found within this 1.1 kb sequence. Therefore to ensure that the transcript levels detected were only of the *SUT1* gene, two probes, ST1P1 (non gene specific) and ST1P2 (gene specific), highlighted in Figure 5.5, were generated from *SUT1* and used to probe Northern blots of *A. bisporus* strain A15 containing VM and stages 1-7 of sporophore development. There was no difference in the level of gene transcript during sporophore development when ST1P1 and ST1P2 probes were used (Figure 5.11) and the expression was similar to that found with the 1.1 kb fragment of *SUT1* indicating that this 1.1 kb probe from *SUT1* was in fact only detecting expression of *SUT1*.

In Northern blots of *A. bisporus* (A15) stage 4 tissues: upper stipe, lower stipe, cap, gills and cap skin greatest levels of *SUT1* transcript were detected in the upper and lower stipe with lower transcript levels in the cap, gills and cap skin (Figure 5.12).

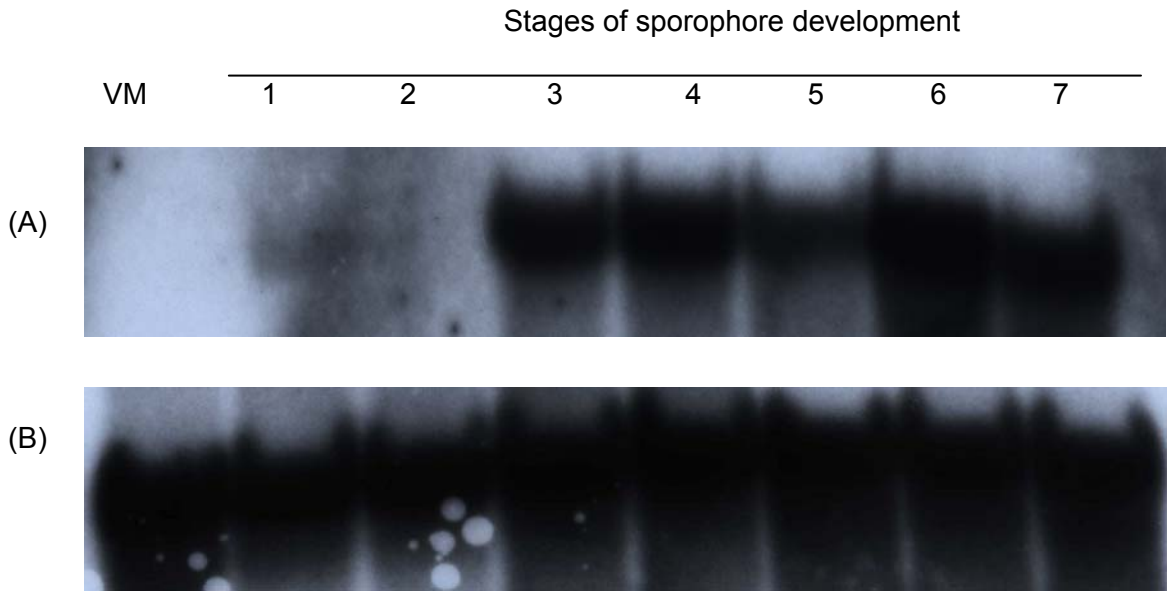

Figure 5.9: Northern hybridisation of RNA from *Agaricus bisporus* (strain A15) vegetative mycelium (VM) and stages 1-7 of sporophore development with *SUT1.* In each lane 11 μg of total RNA was loaded. Northern blots were probed with a *SUT1* gene fragment generated from 1.1 kb region of cDNA STID4 (A) and with 28S rRNA gene fragment as a loading control (B). The probes were labelled with ^{32}P α-dCTP. Northern blots indicate that *SUT1* transcripts could be detected from stages 3-7 of sporophore development with lower transcript at stage 1 and no transcript could be detected in VM or at stage 2 (A) and that equal amounts of RNA were loaded in each lane (B).

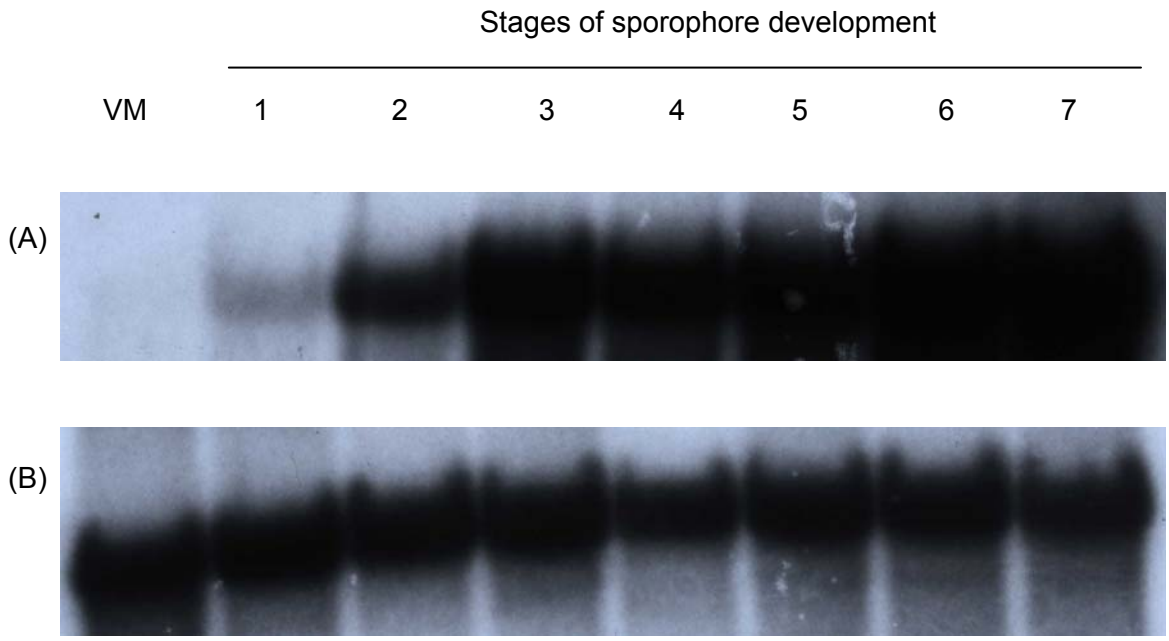

Figure 5.10: Northern hybridisation of RNA from *Agaricus bisporus* (strain U3) vegetative mycelium (VM) and stages 1-7 of sporophore development with *SUT1.* In each lane 11 μg of total RNA was loaded. Northern blots were probed with a *SUT1* gene fragment generated from 1.1 kb region of cDNA STID4 (A) and with 28S rRNA gene fragment as a loading control (B). The probes were labelled with ^{32}P α-dCTP. Northern blots indicate that increasing level of *SUT1* transcripts could be detected with sporophore development from stages 2-7 with very low levels of transcript at stage 1 and no transcript could be detected in VM (A) and that equal amounts of RNA were loaded in each lane (B).

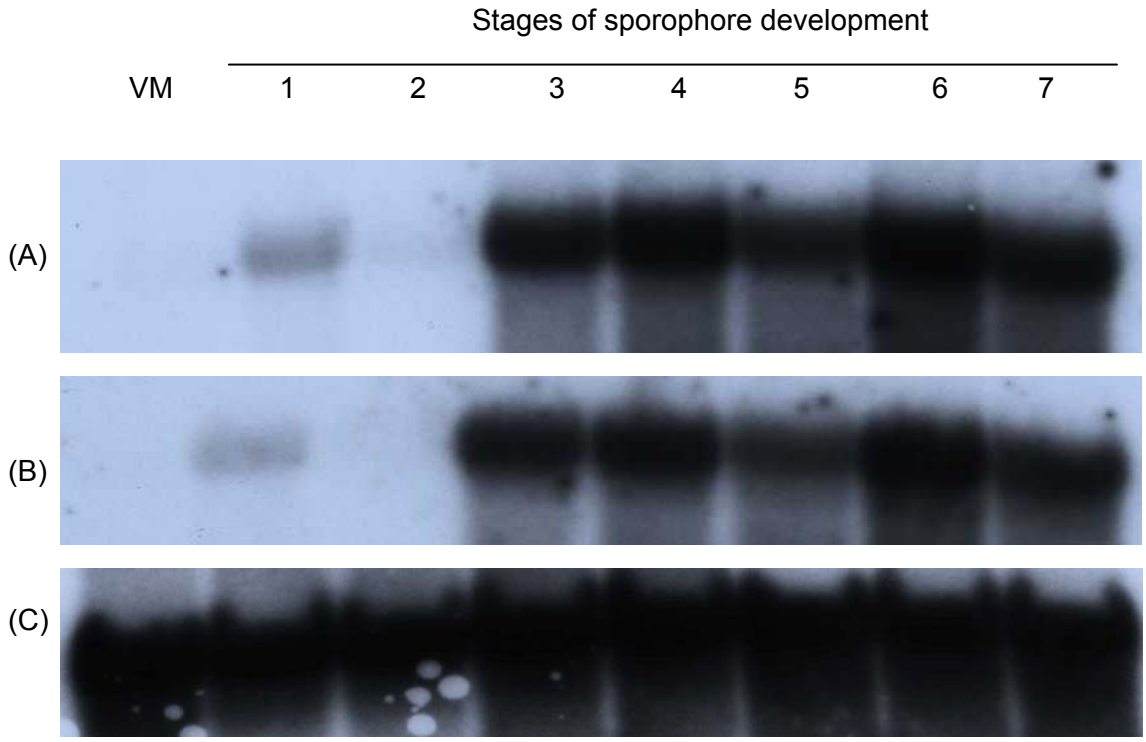

Figure 5.11: Northern hybridisation of RNA from *Agaricus bisporus* (strain A15) vegetative mycelium (VM) and stages 1-7 of sporophore development with *SUT1* gene specific and non-specific fragments. In each lane 11 μg of total RNA was loaded. Northern blots were probed with: ST1P1 a PCR amplified 0.11 kb region of *SUT1* which codes for a region from transmembrane domain (TMD) IV-V and is conserved amongst the sugar transporter family of the major facilitator superfamily, therefore is non-specific to *SUT1* (A), STIP2 a PCR amplified 0.2 kb fragment of *SUT1* and codes for the SUT1 hydrophilic carboxyl-terminus after TMD XII and is highly specific to *SUT1* (B) and 28S rRNA gene fragment as a loading control to ensure equal amounts of RNA were loaded for each sample (C). The probes were labelled with ^{32}P α-dCTP. There were no differences in level of transcript of *SUT1* using the gene specific and non-specific probe ST1P1 and ST1P2 respectively during VM and stages 1-7 of sporophore development with no *SUT1* transcript detected in VM or at stage 2 but could be detected between stages 3 and 7 with lower transcript detected at stage 1 (A) and (B). Equal amounts of RNA were loaded for each sample as indicated in blot C.

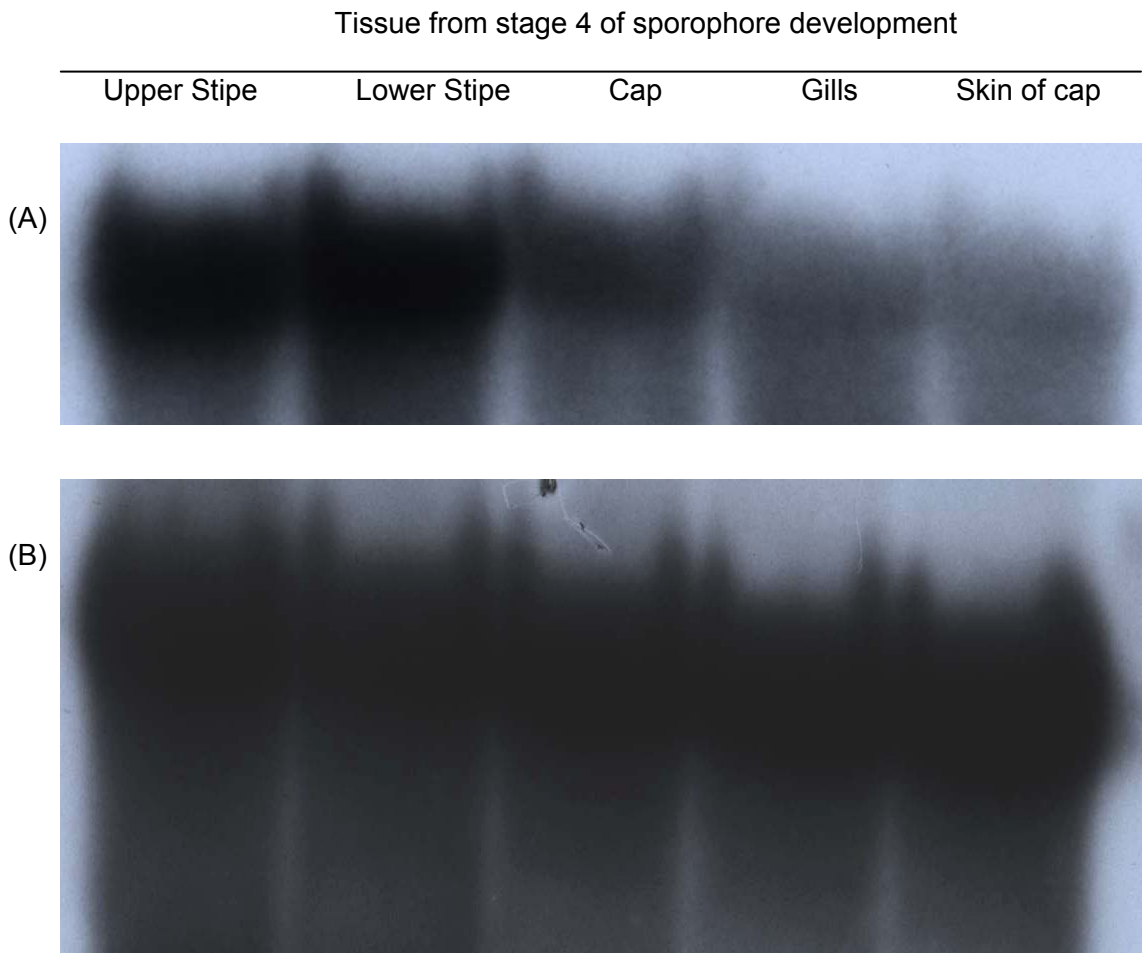

Figure 5.12: Northern hybridisation of RNA from *Agaricus bisporus* (strain A15) stage 4 tissue (upper stipe, lower stipe, cap, gills and skin of cap) with *SUT1*. In each lane 11 μg of total RNA was loaded. Northern blots were probed with a *SUT1* gene fragment generated from 1.1 kb region of cDNA STID4 (A) and 28S rRNA gene fragment as a loading control (B), to ensure each equal amounts of total RNA were loaded for all the sporophore tissues. The probes were labelled with ^{32}P α-dCTP. There were higher but equal levels of *SUT1* transcript in the upper and lower stipe with lower levels detected in the cap, gills and skin tissue. There were equal amounts of RNA loaded for each sample.

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5.4 DISCUSSION

The STIIE2 cDNA was fully sequenced and was found to have a complete ORF unlike STID4 although both were identified as belonging to the *SUT1* gene but STID4 had an incomplete sequence and based on predicted peptide sequence contained a number of stop codons (Figure 5.1). Upon comparing the two cDNA sequences STID4 and STIIE2, STID4 was found to contain an additional 55 bp sequence, which was identified as an intron. This 55 bp intron prepared from oligonucleotides synthesised to this sequence was found to positively hybridise to *SUT1* genomic sequences of cosmids 20A11, 25E6 and 32B10 (Figure 5.2) but did not hybridise to a Northern blot containing RNA from mushroom developmental stages 3, 4 and 5. However a 700 bp *Eco*RI fragment from cDNA STID4 containing the 55 bp sequence hybridised to the same Northern blot (Figure 5.3) indicating that it was an intron present due to improper processing of mRNA.

The nucleotide sequence of the two cDNAs STIIE2 and STID4 (without the 55 bp intron sequence) were aligned (Figure 5.4) and found to be identical except that STIIE2 had a full ORF which encoded a predicted peptide of 551 amino acids with a molecular weight of 61.2 kDa. The predicted peptide sequence of SUT1 has according to the HMM topology prediction method (Tusnady & Simon, 1998) 12 TMDs. There is a highly conserved pentameric motif RFGRR duplicated between TMD II and III and VIII and IX in SUT1 (Figure 5.5) which is found amongst nearly all members of the sugar transporter family of the MFS. The presence of this conserved motif RXGRR (where R can be replaced by K and X is usually a large hydrophobic residue) in equivalent positions in the two halves of the protein implies that they arose by an internal gene duplication event and are thought to form a β-turn (Maiden *et al.*, 1987; Baldwin & Henderson, 1989; Hvorup & Saier, 2002;). This conserved motif is found in nearly all members of the MFS and it is unlikely to play a role in the transport of specific substrates but has been hypothesised to function as a cytoplasmic anchor and its function was

determined using the human glucose transporter Glut1 (Sato & Mueckler, 1999). Using site-directed mutagenesis on the conserved motifs of Glut1 it was found that the positive charges within the motif were critical for membrane topology and their removal led to the shifting of the loop into the exoplasmic domain therefore suggesting the role of this duplicated motif as a cytoplasmic anchor and the charged residues required for maintaining the correct orientation of the loop (Sato & Mueckler, 1999). Also two conserved motifs PESPRXL and PETKGXXXE can be found at the ends of the sixth and twelfth transmembrane helices respectively in the sugar transporter family of the MFS (Griffith, 1992) and these motifs are also found in these positions in SUT1 as PESPRWL and PETSGFALE (Figure 5.8). Two of the most distinguishing features of members of the MFS are the 12 TMDs and the highly conserved pentameric motifs both of which are contained in the SUT1 protein and indicate that this sugar transporter belongs to the MFS.

The putative peptide sequence of SUT1 encoded by cDNA STIIE2 was used to search the EMBL database for sequence homology to other known transporters. The putative SUT1 protein showed homology (28-33% identity and 46-50% similarity) to a number of fungal proteins belonging to the sugar transporter family of the MFS (Table 5.3). For example, 28-30% identity and 46-48% similarity were observed with the monosaccharide transporters such as HXT2, MSTA and MST1 from *S. cerevisiae*, *A. niger* and *A. muscaria*. Similarly 32-33% identity and 48-50% similarity were observed with the quinate permeases from ascomycetes *N. crassa*, *A. nidulans* and *P. anserina*. However, both the quinate permeases and the monosaccharide transporters amongst themselves show considerably higher levels of homology. The quinate genes of *N .crassa* (Geever *et al*., 1989) and *A. nidulans* (Hawkins *et al*., 1988) are part of a cluster of quinic acid utilisation genes and show high percentage identity between each other where the quinate permease QUTD from *A. nidulans* has 61 and 60% identity to quinate permeases from *P*. *anserina* and *N. crassa* respectively, indicating that these are a group of highly

distinct proteins within the sugar transporter family and are only distantly related to the SUT1 protein and the level of homology does not infer similar substrate of transport. However the SUT1 predicted peptide sequence does show identity if slightly less (29-30%) to monosaccharide transporters MST1 (glucose/hexose transporter) of *A. muscaria* (also a member of the order Agaricales) and MSTA from *A. niger*. The level of homology between SUT1 and other sugar transporters is low but because they are all members of the SP family of the MFS and have a similar function, i.e. active secondary transport of sugars they share some homology between each other and contain conserved motifs as indicated in the multiple sequence alignment in Figure 5.8. The identity of the sugar transported by SUT1 is not apparent from homology to other sugar transporters and does not show any clear identity with a particular sugar transporter. However from the predicted secondary structure of SUT1 based on the topology prediction model it was shown to have 12 TMDs and highly conserved motifs which are characteristics of members of the MFS and SUT1 shows homology to sugar transporters belonging to the SP family of the MFS, therefore it can be inferred from this that SUT1 belongs to this family.

Recently, a second sugar transporter SUT2 was identified in *A. bisporus* and has 65% identity to hexose/glucose transporter MST1 from *A. muscaria* (over 220 amino acids) and 48% identity to HXT1 a hexose/glucose transporter of *U. fabae* (over 220 amino acids) (Fleming-Archibald *et al.*, 2003) both of which are well characterised basidiomycete fungal sugar transporters and have been shown using a *S. cerevisiae* mutant hexose strain to preferentially transport glucose. Therefore it is likely that the *SUT2* gene codes for a hexose transporter. The predicted peptide sequence of SUT1 and SUT2 were used to search the *Coprinus cinereus* genome sequence located at: http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/ and identity of 28% for SUT1 (over 305 amino acids) and 45% for SUT2 (over 163 amino acids) were found in the *C. cinereus* genome sequence. Within *C. cinereus* so far there are no reports of any

sugar transporters but bioinformatic analyses of the genome sequence indicates the presence of several sugar transporters belonging to the SP family of the MFS (pers. communication, Dr. Sreenivasaprasad).

The transcript of *SUT1* could not be detected in compost colonised by mycelium supporting *A. bisporus* stage 2 and 4 sporophores but was detected in the sporophore of these stages indicating that the *SUT1* gene is not active in transporting sugar from the mycelium to the sporophore during these stages of sporophore development but is active in sugar transport within the sporophore. It is possible that *SUT1* could be active in the mycelium during different stages of sporophore development or could be present at a very low level and not detected. The *SUT1* transcript could not be detected in vegetative mycelium but during *A. bisporus* stages of development the transcript of *SUT1* increased between stages 3 and 7 (strain A15) (Figure 5.9) with higher level of transcript detected in strain U3 during the same stages (Figure 5.10). This indicates that the transport of this sugar by SUT1 is developmentally regulated and linked to the rapidly expanding growth phase of the sporophores and the increase in the supply of the polyol mannitol (Hammond & Nichols, 1976; Wannet *et al*., 2000) and the sugar transported is likely to be involved in the metabolic pathway (Figure 1.5) which synthesises the major osmoticum mannitol. Transcription of *SUT1* was detected in all sporophore tissues: upper and lower stipe, cap, gills and skin (Figure 5.12) with lower levels detected in the cap, gills and skin compared to the stipe tissue. During sporophore growth greater expansion occurs in the upper stipe and cap tissue (Craig *et al.*, 1977; Craig *et al*., 1981) but because there were near equal levels of the *SUT1* transcript in the upper and lower stipe this indicates that the sugar transporter is not involved in transporting sugars to expanding tissue but it is more likely that it is involved in long distance translocation from the stipe to the cap.

Because SUT1 shows no more than 33% identity to any sugar transporter in the database the sugar of transport of SUT1 cannot be determined from its sequence.

However by examining the carbohydrate metabolism pathway and the sugars involved in this pathway in *A. bisporus* in conjunction with the characteristics of the SUT1 transporter it is possible to predict a possible sugar of transport by SUT1. The two major sugars which are involved in the carbohydrate metabolism in *A. bisporus* are glucose and trehalose. Glucose is metabolised by the PPP pathway and supplies NADPH for the reduction of fructose to mannitol however SUT1 shows limited identity (28-30%) to hexose transporters while SUT2 has 65% identity to the basidiomycete hexose/glucose transporter of *A. muscaria* indicating that SUT2 is likely to be a hexose/glucose transporter and SUT1 is unlikely to be a hexose transporter. The other major sugar involved in carbon metabolism is the disaccharide trehalose which has been suggested as the major sugar translocated from the mycelium to the fruit body in *A. bisporus* (Hammond & Nichols, 1976; Hammond & Nichols, 1979; Wells *et al.*, 1987; Wannet *et al.*, 1999). The enzyme trehalose phosphorylase (TP) purified by Wannet *et al.* (1998) catalyses both the synthesis and degradation of trehalose and was measured in *A. bisporus* fruit body initials grown under axenic conditions and in the mycelium between fruiting by Wannet *et al.* (1999) who found that the degradative activity of TP was 2-3 times higher in the fruit body initials than in the surrounding mycelium and the synthetic activity of the enzyme increased in the mycelium between fruiting. This disaccharide could be a possible candidate for the SUT1 transporter as *SUT1* transcript increased during sporophore development in agreement with the increase in the translocation of trehalose and the increase in the degradative activity of TP but there was no SUT1 transcript detected in the mycelium supporting stage 2 and 4 sporophores although trehalose has been suggested as being translocated from the mycelium to the sporophore. However Wannet *et al*. (1999) only measured TP activity in the mycelium during axenic fruiting of *A. bisporus* and not in compost colonised by mycelium supporting sporophore development stages or in the interflush period and trehalose has only been measured during sporophore development stages (1-7) and not in the mycelium.

Therefore to understand the pattern of trehalose movement and synthesis and degradation in *A. bisporus* it is necessary to measure both the TP degradative and synthetic activity and the concentration of trehalose in the mycelium and during sporophore development. Although no *SUT1* transcript was detected in the mycelium supporting stage 2 and 4 sporophores it is possible that SUT1 is more active at different stages in the mycelium and that there is a large increase in sugar transport from the mycelium at other stages of development and during stage 2 and 4 it could be present at an undetectable level. It is also possible that there is a different sugar transporter involved in transporting trehalose from the mycelium to the sporophore and that SUT1 is only involved in long distance transport from the stipe to the cap within the sporophore where the highest SUT1 transcript was found in the stipe tissue (Figure 5.12).

CHAPTER 6

PRODUCTION AND PURIFICATION OF SUT1-C RECOMBINANT FUSION PROTEIN FOR POLYCLONAL ANTIBODY PRODUCTION

6.1 INTRODUCTION

The characterisation of the sugar transporter SUT1 from *A. bisporus* has been detailed in Chapter 5. The SUT1 has an open reading frame (ORF) of 551 amino acids which encodes a protein with a calculated molecular mass of 61.2 kDa and topology prediction of the SUT1 putative peptide sequence revealed it to contain 12 transmembrane domains. The predicted peptide sequence of SUT1 only showed 33% identity to any other sugar transporter in the database and did not give a clear indication of the sugar of transport. Therefore it is not easy to identify the sugar of transport of SUT1 by functional complementation using mutant yeast strains such as *S. cerevisiae* and *S. pombe* which have been engineered with deficiencies in specific transport pathways and allow for the functional characterisation of transporters. In other basidiomycete fungi such as the rust fungus, *Uromyces fabae,* a sugar transporter was identified specifically expressed in the haustoria (the structure where nutrient exchange between the plant and fungus might occur) which showed high homology to a number of other glucose transporters (Voegele *et al.*, 2001). Therefore by functional complementation using a mutant hexose strain of *S. cerevisiae* a substrate preference for glucose was assigned to the transporter (Voegele *et al.*, 2001). Because membrane proteins are notoriously difficult to study due to their highly hydrophobic nature and difficulties with their expression, purification and crystallisation (Wilson, 2001; Werten *et al.*, 2002) the functional analysis of membrane proteins is often more achievable. While 30% of all sequenced genes code for membrane proteins only 30 unique structures of membrane proteins have been resolved to atomic resolution and this is due to the fact that they are usually only expressed at low levels and constitute less than 0.1% of total cell protein (Ward *et al.*, 1999).

The *SUT1* transcript was not detected in the mycelium supporting fruit body development but was detected in sporophores at increasingly higher levels after stage 2 of sporophore development (Figure 5.10). However the determination of the *SUT1* gene

transcript levels does not necessarily reflect the corresponding SUT1 protein abundance. Sometimes mRNA abundance is a poor indicator of the levels of the corresponding protein (Pradet-Balade *et al.*, 2001). Analyses to examine a possible correlation of mRNA and protein expression levels have been carried out in yeast and have shown that the abundance of the corresponding protein might vary by up to 30-fold and concludes that the deduction of protein abundance from mRNA levels alone appears unreliable (Pradet-Balade *et al.*, 2001). To examine the SUT1 protein during sporophore developmental stages and in sporophore tissue it is necessary to produce a SUT1 antibody which could be used to detect the SUT1 protein using Western analysis. To produce the SUT1 protein a number of heterologous expression systems were examined. The use of heterologous expression systems has allowed for the expression and purification of proteins from their corresponding genes and several heterologous expression systems can be used and these include, *E. coli*, yeast, mammalian cell lines and insect cells. Heterologous expression vectors have been developed that convert gene sequences to purified proteins and perform this by the genetic attachment of purification tags to recombinant proteins which allow for the purification of tagged proteins. The most common problems associated with membrane protein expression are the failure of the expressed protein to fold correctly and many membrane proteins seem to be toxic when expressed in *E. coli* (Dreyer *et al*., 1999)

The *E. coli* heterologous expression system was used to purify the SUT1 protein using the pET vector system (Novagen). However because of the difficulties in expressing and purifying a protein containing 12 TMD only the hydrophilic C-terminal region of the SUT1 protein (highlighted in Figure 5.5) was used and produced as a fusion protein. The method of expressing only the C-terminal region of the 12 TMD transporter was also performed on the hexose transporter HXT1 protein of the rust fungus *U. fabae* which was expressed as part of a fusion protein (Voegele *et al.*, 2001).

A 0.2 kb fragment of the *SUT1* cDNA STIIE2 coding for the C-terminus hydrophilic loop (highlighted in Figure 5.5) was cloned into the multiple cloning site of the pET-43.1c(+) expression vector (Novagen). This SUT1 C-terminus region showed very little identity (Figure 5.8) to the following proteins in a multiple sequence alignment: quinate permeases QA-Y and QUTD from *N. crassa* and *A. nidulans* respectively and monosaccharide transporter, MST1, from *A. muscaria* (all belonging to the SP family of the MFS). The C-terminus had a high hydrophilicity value when analysed using the hydrophilicity scale devised by Hopps & Wood (1981) located at http://us.expasy.org/tools/protscale.html and they reported that stretches of amino acids with high hydrophilicity are located in or immediately adjacent to antigenic determinants. Therefore because the C-terminal region is highly specific to SUT1 and also has a high probability of being antigenic it was used as an antigen to produce a SUT1 antibody. The pET-43.1c(+) vector which belongs to the pET-43.1a-c(+) vector series was chosen to express the SUT1-C protein because it would maintain the correct reading frame of the *SUT1*-C sequence. The pET-43.1c(+) vector contains an N-terminal Nus/His/S●Tag fusion sequence followed by a multiple cloning site with a C-terminal HSV●Tag (Figure 6.1). The Nus●Tag sequence encodes a 54.8 kDa NusA protein which has been shown to have a high probability (>90%) of being soluble when expressed in *E. coli* and has successfully been shown to increase the solubility of target proteins when fused with them (Novy & Drott, 2002). The His●Tag enables the purification of the expressed protein using immobilised metal affinity chromatography (IMAC). The expression of the fusion protein encoded by the pET-43.1c(+) vector is regulated by the T7 promoter and the binding of T7 RNA polymerase which causes transcription of the fusion protein. The T7 RNA polymerase is produced in the expression *E. coli* strain BL21(DE3) and is regulated by the L8-UV5 *lac* promoter and the addition of IPTG induces transcription of T7 RNA polymerase by binding to the *lac* repressor which prevents it binding downstream of the promoter and reducing transcription (Novy & Morris, 2001). The efficient transcription of

T7 RNA polymerase also requires the presence of cyclic AMP and the cyclic AMP receptor protein (CRP) which binds upstream of the promoter and stimulates transcription. The presence of glucose reduces cAMP levels and so transcription is reduced and this is called catabolite repression.

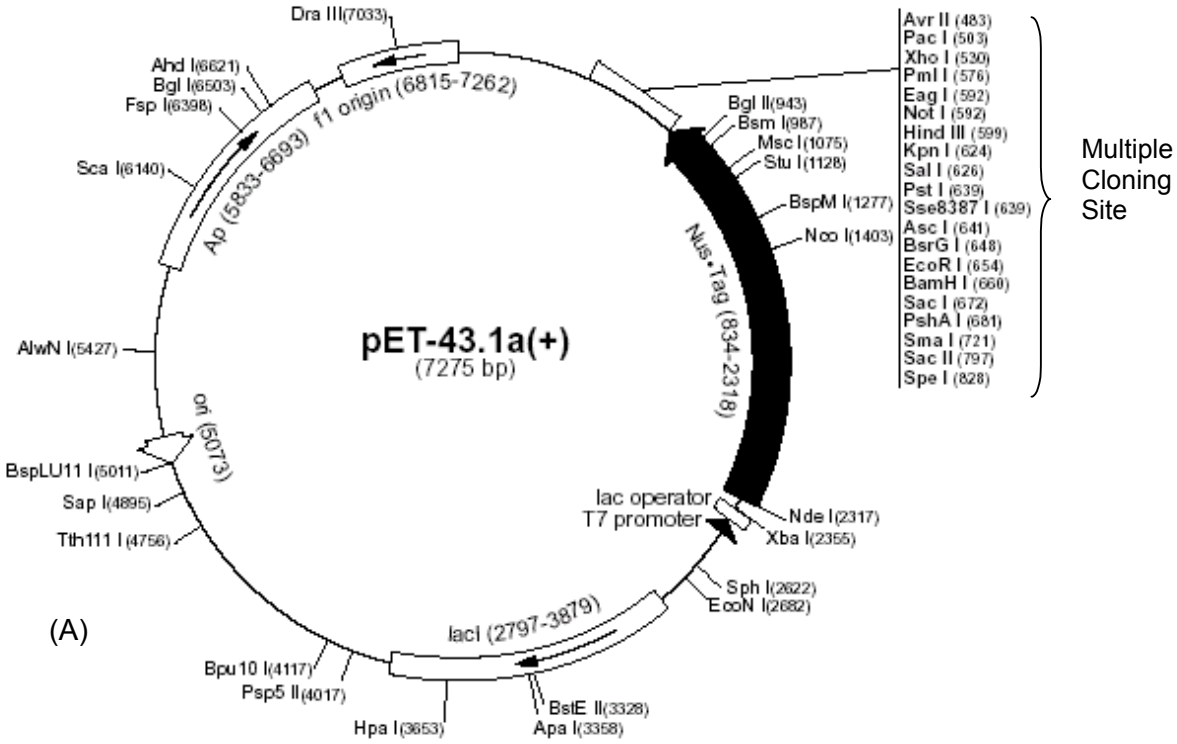
This chapter details the expression and purification of the SUT1-C protein as a fusion protein encoded by the pET-43.1c(+)::SUT1-C recombinant vector and its use as an immunogen to produce a polyclonal antibody. In addition a number of techniques were be attempted to try and extract the SUT1 membrane protein in a soluble state from sporophore tissue and to detect it in Westerns using the SUT1-C antibody and to identify changes in the SUT1 protein during sporophore development.

6.2 MATERIALS AND METHODS

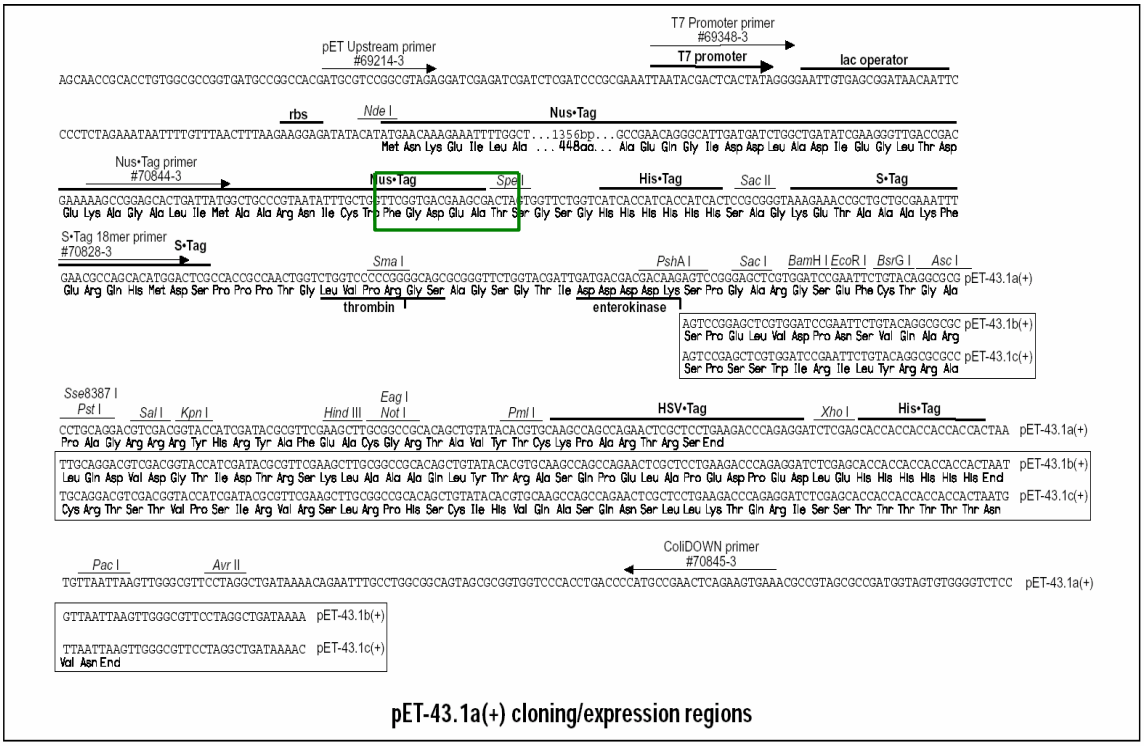
6.2.1 PCR amplification and gel extraction of *SUT1*-C terminus fragment

Primers SUT1-CtermF and SUT1-Cterm R (Appendix 2) incorporating a *Bam*HI and *Xho*I site respectively were designed to amplify the *SUT1*-C terminus (Figure. 5.5). PCR amplification of the *SUT1*-C fragment was carried out in a Hybaid multi block system (MBS) thermocycler under the cycling conditions outlined in Table 2.4 except that the annealing temperature used was 55°C. The PCR reaction was carried out using *Pwo* DNA polymerase (Roche) a high fidelity DNA polymerase and the components of the PCR reaction are detailed in Table 6.1. The PCR amplified *SUT1*-C DNA was loaded onto a 1% (w/v) agarose gel along with DNA marker VI (Boehringer Mannheim) to verify the product size and separated in 0.5X TBE at 50V as described in Section 2.4.2 The DNA was then extracted from the agarose gel as described in Section 2.4.7 using QIAquick gel extraction kit (Qiagen), and eluted from the QIAquick column using Buffer EB (provided with the kit). Restriction enzymes *Bam*HI and *Xho*I were used to digest and clone the *SUT1*-C PCR amplified fragment into the pET-43.1c(+) (Novagen) multiple cloning site (Figure.6.1).

Figure 6.1: The diagrams of the circular map of the pET-43.1a(+) vector (A) and the cloning/expression sequence of the pET-43.1 a-c(+) series of vectors (B) were taken from the Novagen website. The pET-43.1(+) vector has three versions denoted by the addition of a letter suffix a, b or c following the pET vector name. These letters denote the reading frame relative to the *Bam*HI cloning site and the vector with suffix 'a' expresses from the GGA triplet, the 'b' expresses from the GAT triplet and for the suffix 'c' from the ATC triplet therefore these vectors will encode different peptide sequence and they have different C-Terminal sequences. The multiple cloning site (MCS) and C-Terminal sequence of the pET-43.1 b & c vectors are boxed in black (B). All the pET-43.1(+) vector series 'a-c' contain an N-terminal Nus/His/S•Tag fusion sequence followed by a thrombin protease cleavage site and a MCS. The pET-43.1c(+) vector has an additional His•Tag in its C-Terminal sequence for purification. The *SUT1*-C gene was cloned into the *Bam*HI/*Xho*I site of the 'c' version of the pET-43.1 (+) vector because it would maintain the correct reading frame of the *SUT1*-C sequence. The pET-43.1c forward primer sequence is shown boxed in green (B).



(A)



(B)

pET-43.1a(+) cloning/expression regions

Table 6.1: Components of the PCR amplification reaction of *SUT1*-C fragment incorporating *Bam*HI and *Xho*I restriction sites

Reagents	Volume per 100 μl reaction	Final concentration
Pwo DNA polymerase 5 $U\mu l^{-1}$ (Roche)	0.5 μl	2.5 U
PCR grade water	79.7 μl	
PCR buffer (10X $+Mg^{2+}$) (Roche)	10 μl	1X
DNA 10 $ng\mu l^{-1}$	4 μl	40 ng
dNTP 25 mM (Stratagene)	0.8 μl	200 μM
F primer 20 μM	2.5 μl	2 μMl
R primer 20 μM	2.5 μl	2 μM
Total volume	100 μl	

6.2.2 Cloning of the *SUT1*-C fragment into the pET-43.1c(+) vector

*Bam*HI and *Xho*I restriction enzymes (Boehringer Mannheim) were used to digest both the *SUT1*-C PCR amplified DNA (1.2 μg) and the pET-43.1c(+) vector (1 μg) (Novagen). The digestion reactions were performed as detailed in Section 2.4.6.

The digested pET-43.1c(+) vector was loaded onto a 1% (w/v) agarose gel and electrophoresis of the gel was carried at 65V as described in Section 2.4.2. The digested vector was excised from the agarose gel using QIAquick gel extraction kit (Qiagen) as described in Section 2.4.7 and eluted from the QIAquick column using 35 μl Buffer EB (provided with the kit). The *SUT1*-C digested DNA was analysed by agarose gel electrophoresis as described above and purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer instructions and eluted from the spin column using 35 μl of Buffer EB (supplied with the kit). Both the digested: pET-43.1c(+) vector and *SUT1*-C DNA were quantified on a 1% (w/v) agarose gel as described in Section

2.4.2 along with DNA marker II (Boehringer Mannheim) and Low DNA mass ladder (Invitrogen).

The digested pET-43.1c(+) vector and *SUT1*-C DNA were ligated using T4 DNA ligase (Roche). The 20 μl ligation reaction contained 6.5 μl (20 ngμl^{-1}) digested and gel purified pET-43.1c(+) vector, 4.5 μl (8 ngμl^{-1}) digested and purified *SUT1*-C DNA, 1U T4 DNA ligase (Roche), 1X T4 DNA ligase buffer (Roche) and 4 μl distilled water. The ligation reaction was carried out overnight at 16°C.

6.2.3 Transformation of pET-43.1c(+)::*SUT1*-C into *E. coli* strain DH5α

The plasmid construct pET-43.1c(+)::*SUT1*-C was transformed into the non-expression *E. coli* strain DH5α (Invitrogen) as described in Section 2.4.8 and aliquots of 100, 250 and 550 μl of the transformed cells were spread plated onto Petri dishes containing LB agar supplemented with 50 μgml^{-1} ampicillin. The Petri dishes were allowed to dry before being inverted and incubated overnight at 37°C.

6.2.4 Screening colonies to identify pET-43.1c(+)::*SUT1*-C recombinants

Twenty-four putative recombinant colonies were randomly selected from the three Petri dishes, containing the transformants and were streaked onto two Petri dishes, each were divided into 12 sections, containing LB agar supplemented with 50 μgml^{-1} ampicillin and the putative recombinants were numbered 1-24. The Petri dishes were incubated overnight at 37°C.

From the Petri dishes containing the putative recombinants, eight colonies were selected at random from the sections of the Petri dishes and each colony was transferred into 5 ml LB broth supplemented with 50 μgml^{-1} ampicillin. The cultures were incubated overnight at 37°C in an orbital shaker at 225 rpm. Plasmid DNA was extracted from 3 ml of these overnight cultures using Qiaprep 8 plasmid extraction kit (Qiagen) according to

the protocol and the DNA was eluted using 80 μl of buffer EB. The plasmid DNA was then analysed and quantified by electrophoresis on a 0.8% (w/v) agarose gel as described in Section 2.4.3.

The primers SUT1-CtermF and SUT1-CtermR used to amplify *SUT1*-C region of the *SUT1* gene as described in Section 6.2.1 were used to identify recombinants. Plasmid DNA isolated from the eight putative recombinants were PCR amplified as using as described in Section 2.4.4 except that the annealing temperature used was 55°C. As a negative control the pET-43.1c(+) vector was also PCR amplified under the same conditions. A PCR control was also carried out in which DNA was substituted with water. The composition of the PCR reaction was as follows: 12.5 μl Red *Taq* Readymix with $MgCl_2$ (Sigma-Aldrich), 20 μM of each forward and reverse primers, 2 μl plasmid DNA of putative recombinant clones (20 $ng\mu l^{-1}$), and the volume was adjusted to 25 μl with PCR grade water. The PCR amplified DNA was then separated on a 1% (w/v) agarose gel as described in Section 2.4.2 along with Low DNA mass ladder (Invitrogen).

From the PCR reaction all eight putative recombinants were positively identified as containing the *SUT1*-C DNA and to ensure that they were recombinants, two were selected and sequenced using the pET-43.1c forward primer (designed 182 bp upstream from the *Bam*HI cloning site of the pET-43.1c(+) vector and highlighted in Figure 6.1(B). Sequencing reactions were performed as described in Section 2.4.5. After sequencing both plasmids were found to be recombinants and contained the *SUT1*-C DNA in the correct frame. One of the two recombinants was then selected and transformed into the *E. coli* expression strain BL21(DE3) (Novagen).

6.2.5 Transformation of pET-43.1c(+)::*SUT1*-C recombinant and pET-43.1c(+) vector into the *E. coli* strain BL21(DE3)

The pET-43.1c(+)::*SUT1*-C recombinant and the pET-43.1c(+) vector were transformed into the expression *E. coli* strain BL21(DE3) (Novagen) as described in Section 2.4.8. Aliquots of 50, 100, 200, and 500 μl of the transformed cells were spread plated onto Petri dishes containing LB agar supplemented with 50 μgml^{-1} ampicillin. The Petri dishes were allowed to dry before being inverted and incubated overnight at 37°C.

6.3. EXPRESSION AND PURIFICATION OF THE HIS-TAGGED SUT1-C FUSION PROTEIN

All protein purification was carried out at 4°C to prevent protein degradation by proteases. The SUT1-C fusion protein encoded by the pET-43.1c(+)::*SUT1*-C construct and the His-Tagged fusion protein encoded by pET-43.1c(+) vector that were transformed into *E. coli* strain BL21(DE3) will be described simply as the His-Tagged SUT1-C and the His-Tagged protein respectively.

6.3.1 Induction and repression of the His-Tagged SUT1-C protein and His-tagged protein

The *E. coli* strain BL21(DE3) containing the plasmid construct pET-43.1c(+)::*SUT1*-C and pET-43.1c(+) vector, were streaked onto Petri dishes containing LB agar supplemented with 50 μgml^{-1} ampicillin and incubated overnight at 37°C. A loopful of BL21(DE3) cells were spread plated onto Petri dishes containing LB agar and incubated overnight at 37°C. Each of 3 X 50 ml of LB broth cultures, supplemented with 100 μgml^{-1} ampicillin in 250 ml conical flasks, were individually inoculated with a single colony from the Petri dish containing the pET-43.1c(+)::*SUT1*-C colonies. A single colony from the Petri dish

containing only the pET-43.1c(+) vector was inoculated into each of 3 X 50 ml of LB broth cultures supplemented with 100 μgml^{-1} ampicillin in 250 ml conical flasks. A single colony was removed from the Petri dish containing BL21(DE3) expression strain and inoculated into 50 ml of LB broth supplemented with 100 μgml^{-1} ampicillin in 250 ml conical flasks. The broth cultures were incubated at 37°C in an orbital shaker at 225 rpm until the cultures reached an A_{600} of 0.6 and then to the broth cultures containing the pET-43.1c(+)::*SUT1*-C and pET-43.1c(+) vector, isopropyl-β-D-thiogalactopyranoside (IPTG, Melford Labs) was added from a 100 mM stock to give a final concentration of 1 mM to induce the His-tagged fusion protein. To another set of the same broth cultures, glucose (Fischer scientific) was added to give a final concentration of 1% (v/v), which causes decreased levels of transcription of the T7 RNA polymerase thus reducing expression of the fusion protein which is under the control of the T7 promoter. To the third set of the same broth cultures and the broth culture containing only BL21(DE3) expression strain nothing was added. All the broth cultures were incubated for a further 3 hours at 37°C in an orbital shaker at 225 rpm.

The cells were harvested by placing the samples on ice for 5 minutes followed by centrifugation at 5,000 *g* for 5 minutes at 4°C. The supernatant was removed and placed in pre-chilled 50 ml round bottomed centrifuge tubes (Nalgene) and stored at -70°C. The pellet was resuspended in 0.25 culture volume of cold 20 mM Tris-HCl pH 8.0 was added and centrifuged at 5,000 *g* for 5 minutes at 4°C. The supernatant was removed (soluble fraction) and the pellet (insoluble fraction) was frozen at -70°C until the protein fractions were ready to be solubilised as described in Section 6.3.4. Both the soluble and insoluble protein fractions were analysed by SDS-PAGE as described in Section 2.5.1.

6.3.2 Time course of induction of the His-Tagged SUT1-C protein and His-Tagged protein and analysis of the soluble and insoluble protein fractions

The His-Tagged SUT1-C and His-Tagged proteins were expressed as described in Section 6.3.1 except that after induction of the proteins with 1 mM of IPTG 5 ml samples were removed after 3 and 6 hours and placed into sterile 14 ml graduated polypropylene tubes (Greiner). The cells were harvested as described in Section 6.3.1. The cells were then disrupted by sonication as described in Section 6.3.4 and the soluble and insoluble protein fractions were analysed by SDS-PAGE as described in Section 2.5.1.

6.3.3 Time course of induction of His-Tagged SUT1-C protein

To determine the time at which the highest concentration of His-Tagged SUT1-C protein would be expressed a time course of the expression of the protein was carried out every hour over a 6 hour period. The His-Tagged SUT1-C protein was expressed as described in Section 6.3.1. After induction of the His-Tagged SUT1-C protein, 5 ml samples were removed every hour for a total of six hours and placed in sterile 14 ml graduated polypropylene tubes (Greiner). The cells were harvested as described in Section 6.3.1 and solubilised as described in Section 6.3.4 and the soluble fractions were analysed by SDS-PAGE as described in Section 2.5.1. The concentration of the soluble protein fractions harvested at hourly intervals was determined using the Bradford assay based on the dye-binding method.

6.3.4 Solubilisation of the protein fractions

The frozen insoluble pellets of the His-Tagged SUT1-C protein and His-Tagged protein cells were removed from the -70°C freezer and suspended in one tenth of the original culture volume of cold 20 mM Tris-HCl pH 8.0. The cells were then sonicated using a Soniprep 150 (MSE) sonicator set at 15 μm amplitude for approximately 30 seconds and then frozen in liquid N_2 and thawed at 37°C. This sonicating freeze thawing cycle was

repeated three times for each sample, after which the suspension was centrifuged at 10,000 *g* for 10 minutes at 4°C. The supernatant was removed and labelled the soluble fraction and the pellet of insoluble debris was suspended in 0.10 of the culture volume of cold 20 mM Tris-HCl pH 8 and labelled the insoluble fraction. Both fractions were kept on ice and analysed by SDS-PAGE as described in Section 2.5.1 using 12%Tris–HCl polyacrylamide gels (separating gel of 12% acrylamide and a 4% stacking gel).

6.3.5 Purification of the His-Tagged SUT1-C fusion protein using immobilised metal affinity chromatography (IMAC)

All buffers used in protein purification were ice-cold and prepared as 8X concentration except for the column elution and stripping buffer, which was prepared as 4X concentration and the buffers were diluted to 1X using cold sterile water and their composition are detailed in Appendix 1.The protein purification procedure was followed as outlined in the His•Bind Kit protocol (Novagen).

The expression of the His-Tagged SUT1-C protein was as described in Section 6.3.1 except a single colony was removed from the Petri dishes containing pET-43.1c(+)::SUT1-C recombinant transformed into BL21(DE3) cells and used to inoculate 8 X 50 ml of LB broth supplemented with 100 $\mu g ml^{-1}$ ampicillin in 250 ml conical flasks. Each 50 ml of LB broth containing the expressed His-Tagged SUT1-C protein was transferred into pre-chilled 50 ml round bottomed centrifuge tubes (Nalgene) centrifuged at 10,000 *g* for 10 minutes at 4°C after 3 hrs post-induction of the protein with IPTG and the supernatant was removed and the pellet was stored at -70°C until required.

The frozen pellets of the cells (from 50 ml of LB broth) were removed from the -70°C freezer and suspended in 4 ml of ice-cold 1X binding buffer (5 mM Imidazole). The cells were sonicated twice using a Soniprep 150 (MSE) sonicator set at 15 μm amplitude for approximately 30 seconds each time and placed on ice between sonications and after

the second sonication the cells were frozen in liquid N_2 and then thawed at 37°C. This duplicate sonicating and freeze thawing of the cells was repeated three times for each sample, after which they were centrifuged at 14,000 *g* for 20 minutes at 4°C. The supernatant was removed and filtered through a 0.22 μm filter (diam. 25mm) (Millipore Corp.) and kept on ice.

Protein purification using immobilised metal affinity chromatography (IMAC) was carried out at 4°C. Onto a 20 ml chromatography column (Pharmacia Biotech) 10 ml slurry of the iminodiacetic acid (IDA) affinity chromatography based His•Bind resin (Novagen) was added and left overnight to settle. This resulted in a settle bed volume of 5 ml which can theoretically purify up to 40 mg of protein (Novagen). To the column bed 30 ml of sterile distilled water was added and 25 ml of 1X charge buffer (50 mM $NiSO_4$) (Fischer Scientific) was added to charge the column i.e. the Ni^{2+} ions bind to the IDA matrix chromatography. Excess nickel was removed by adding 25 ml of 1X binding buffer (5 mM imidazole) to the charged column. The flow rate of the column was adjusted to approximately $1mlmin^{-1}$. The column was then loaded with 25 ml of the induced His-Tagged SUT1-C soluble protein extract (suspended in 1X binding buffer) and the eluate was collected in glass test tubes. For all subsequent column washes the eluate was collected in 30 ml glass test tubes. The column was then washed with 50 ml of 1X binding buffer (5 mM imidazole) to remove any unbound protein. The bound protein was eluted with a series of wash buffers containing a linear stepped (increments of 10 mM) imidazole gradient (10 mM-60 mM). To the column 30 ml of 1X wash buffer (10 mM imidazole) was added followed with 30 ml of a series of 1X wash buffers containing 20, 30, 40, 50 and 60 mM of imidazole. Then 30 ml of 1X elute buffer (1 M imidazole) was added. The column was stripped of Ni^{2+} ions and protein by adding 40 ml of 1X stripping buffer (100 mM EDTA).

The eluted protein fractions and the original protein extract (before it was added to the His-Bind resin column) were analysed by SDS-PAGE as described in Section 2.5.1 to determine the fraction that contained the purified recombinant protein.

6.3.6 Dialysis of the purified protein

The protein fractions containing the purified His-Tagged SUT1-C protein were dialysed against 1X phosphate buffered saline (PBS) over 24 hours at 4°C. The protein was poured into 25.4 mm dialysis tubing (Medicell International) which was pre-treated (as described in Appendix 1) and sealed at both ends using medicell clips (Medicell International). The dialysis tubing was placed in a 2 litre glass beaker which was filled with 1X PBS and stirred over 24 hours with 2 changes of 1X PBS at 4°C.

The concentration of the dialysed His-Tagged SUT1-C purified protein was determined using the Bradford assay based on a dye-binding Bradford (1976). The assay was carried out according to the manufacturer instructions (Sigma-Aldrich), the 96 well plate assay protocol was followed. The concentration of the His-Tagged SUT1-C purified protein was 0.26 $mgml^{-1}$.

6.3.7 Protein concentration by ultrafiltration and quantification of protein

The dialysed His-Tagged SUT1-C purified protein was concentrated using an Amicon stirred cell fitted with an Amicon PM 10 ultrafiltration membrane disc (diam. 25 mm) with a molecular weight limit of 10 kDa (supplied by Millipore Corp.). Before use the ultrafiltration membrane was placed in a beaker of distilled water for 1 hour with three changes of water to remove the pre-treated coating of glycerin. The membrane was then placed in the stirred cell device to which the protein was added and concentrated under an operating pressure of 55 psi (following the manufacturer instructions).

The concentration of the dialysed His-Tagged SUT1-C purified protein, after it was concentrated, was determined using the Bradford assay based on a dye-binding

Bradford (1976). The assay was carried out according to the manufacturer instructions (Sigma-Aldrich), the 96 well plate assay protocol was followed. The protein had a concentration of 1.75 $mgml^{-1}$.

The purified His-Tagged SUT1-C purified protein was used as an antigen for polyclonal antibody production (carried out at Harlan Sera-Lab, Leicester, UK). A New Zealand white rabbit was given six intraperitoneal injections each of 0.525 mg of His-Tagged SUT1-C purified protein mixed with an equal volume of Freund's adjuvant every two weeks over a 12 weeks period. Pre-immune serum was taken from the rabbit before the first injection and serum was taken 7 days after injections 3, 4 and 5. A final bleed was taken 7 days after injection 6 and was used as the serum used for immunological experiments and was termed the polyclonal anti-rabbit SUT1-C antibody.

6.4 METHODS FOR THE EXTRACTION AND DETECTION OF TRANSMEMBRANE SUGAR TRANSPORTER, SUT1, PROTEIN IN *A. BISPORUS* SPOROPHORES

A. bisporus (strain A15) sporophores were harvested from stage 1 through to stage 7 (Hammond & Nichols, 1976) from a second flush crop of mushrooms grown at the mushroom unit, HRI as described in Section 2.1.2 and stored at -70°C.

6.4.1 Extraction of membrane protein using detergents CHAPS and N-Octlyglucoside

The detergents CHAPS (3[(3-Cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate, Calbiochem) and N-Octlyglucoside (N-Octyl-β-D-glucopyranoside, Calbiochem) were used as recommended by Hjelmeland & Chrambach (1984) in an attempt to solubilise the SUT1 membrane protein. Experiments for membrane protein solubilisation were carried out using modifications of the methods of Hjelmeland & Chrambach (1984).

A stage 4 sporophore was removed from the freezer and allowed to defrost at room temperature and weighed. The extraction buffer contained 50 mM Tris/HCl pH 8 and 0.15 M KCl and was pre-chilled at 4°C prior to adding to the sporophore tissue and the following protease inhibitors were added to the indicated final concentration: of 0.075 $mgml^{-1}$ of phenylmethylsulfonyl fluoride (PMSF), 0.02 $mgml^{-1}$ of Pepstatin A (Sigma) and 0.1 mM EDTA. The sporophore was placed in pre-chilled centrifuge tubes to which the extraction buffer at a 2:1 ratio (v:w) was added. The sporophore was homogenised using a Polytron homogeniser at half the maximal speed and the homogenate was divided equally between six 50 ml Nalgene round bottomed centrifuge tubes and centrifuged at 18,000 rpm for 20 minutes at 4°C. The supernatant was removed and pellets were suspended in half the buffer volume and the individual aliquots were placed into glass beakers containing stir bars. The two detergents CHAPS and N-Octylglucoside were prepared as 10% (w/v) solutions in extraction buffer and were added to the suspended pellets to give the following detergent concentration: 0.1%, 1%, and 3% and to test the effect on solubilising the membrane protein in the insoluble pellet phase. The individual aliquots were stirred gently with a magnetic stirrer for 1 hr at 4°C, after which they were centrifuged at 105,000 *g* at 4°C for 1 hr. The resultant supernatant was removed from each sample and the pellet re-dissolved in the same volume of extraction buffer containing the protease inhibitors. Both the supernatant and re-suspended pellet for each detergent concentration were analysed by SDS-PAGE as described in Section 2.5.1 along with the SDS VII-B (Sigma) and the SDS-PAGE standard pre-stained low range marker (Bio-Rad). The SDS-PAGE gels were transferred by Western blotting to nitrocellulose membrane as described in Section 2.5.2. The membranes were probed with the polyclonal anti-rabbit SUT1-C antibody and detected with goat anti-rabbit labelled horseradish peroxidase (HRP) using the substrate chloronapthol (CN) as described in Section 2.5.3.

6.4.2 Extraction of membrane protein using chloroform/methanol

Extraction of hydrophobic proteins using different ratios of chloroform/methanol (C:M) were attempted using a method based on that described by Seigneurin-Berny *et al.* (1999) and Ferro *et al.* (2000) who used C:M for the extraction of hydrophobic chloroplast membrane proteins. Initially only three different ratios of C:M were used and these were 2:7, 4:5, and 8:1 to try and extract membrane proteins from *A. bisporus* sporophore (stage 4). These ratios were chosen based on results from previous work carried out by Seigneurin-Berny *et al.* (1999) where a 12 transmembrane protein was extracted from spinach chloroplast envelope membranes mainly in the above mentioned C:M ratios. A stage 4 mushroom was removed from the -70°C freezer and allowed to thaw at room temperature. The following series of C:M ratios were prepared: 2:7, 4:5, and 8:1 (v/v) in glass pyrex tubes and placed on ice. The initial extraction procedure was as described in Section 6.4.1 whereby the sporophore was homogenised using a Polytron homogeniser at half the maximal speed and the homogenate was divided between three 50 ml Nalgene centrifuge tubes and centrifuged at 18,000 rpm for 20 minutes at 4°C. The supernatant was removed and pellets were dissolved in half the buffer volume and homogenised using a Silverson polytron at half maximal speed and the above prepared cold C:M ratios were added to the homogenised pellet, 1:10 (aqueous:solvent), and placed on ice for 15 minutes and shaken twice during the incubation time after which the mixtures were centrifuged at 12,000 *g* for 20 minutes at 4°C.

Proteins insoluble in the organic phase were recovered as a white pellet at the bottom of the tube (for C:M solutions 2:7 and 4:5) or at the interface of the organic and aqueous phase (for C:M solutions 8:1) and were placed in glass pyrex tubes. The organic phase which contains proteins soluble in C:M solutions were removed and placed in glass pyrex tubes and both the soluble and the insoluble fractions were placed under a stream of N_2 to remove any solvent. To re-suspend the proteins 3 ml of re-suspension buffer was

added which contained: 50 mM of Tris/HCl pH 8, 0.1% SDS, 0.02 mg/ml of Pepstatin A and 0.1 mM EDTA. The soluble and insoluble samples from each C:M ratio and the His-Tagged SUT1-C fusion protein (used as an antigen) were analysed by SDS-PAGE along with pre-stained low range marker (Bio-Rad) as described in Section 2.5.1 and two replicate SDS-PAGE gels containing the same samples were prepared and transferred by Western blotting to nitrocellulose membrane as described in Section 2.5.2. One of the membranes was cross-linked using glutaraldehyde after protein transfer to nitrocellulose membrane to prevent the protein eluting from the membrane during various stages of Western staining. The other membrane was not treated with glutaraldehyde. To cross-link the proteins, the membrane was immersed in 0.25% (v/v) glutaraldehyde (EM grade, Agar Scientific) in TBS for 45 minutes and then rinsed six times in TBS. Both membranes were probed with the polyclonal anti-rabbit SUT1-C antibody and detected with goat anti-rabbit labelled horseradish peroxidase (HRP) using the substrate chloronapthol as described in Section 2.5.3.

6.5 RESULTS

6.5.1 Analyses of expression of the His-Tagged SUT1-C protein

The His-Tagged SUT1-C fusion protein expressed by the pET-43.1c(+)::*SUT1*-C construct transformed into BL21(DE3) cells has a predicted MW of 69.8 kDa and the His-Tagged fusion protein encoded by the pET-43.1c(+) vector has a predicted MW of 67.2 kDa.

In Figure 6.2 the expression profile of the His-Tagged and His-Tagged SUT1-C proteins under different conditions are shown. The results show that the His-Tagged and the His-Tagged SUT1-C fusion proteins were expressed in the presence of the inducer IPTG and the molecular weight of the proteins are close to their predicted MW. There was expression of the fusion proteins without the addition of IPTG indicating that the T7 RNA polymerase is being transcribed from the *lac* promoter even when the *lac* repressor binds upstream of the promoter indicating that the *lac* promoter is not tightly regulated by the *lac* repressor. However the level of expression of the fusion proteins was slightly reduced when grown in cultures to which no IPTG was added compared to those which were grown in the presence of the inducer IPTG. The addition of glucose caused complete repression of the His-Tagged and His-Tagged SUT1-C proteins suggesting that the *lac* promoter is influenced by catabolite repression whereby in the presence of glucose cAMP levels are low and the CAP/cAMP complex can't bind upstream of the lac promoter and stimulate transcription. To determine which protein fraction contained the expressed protein both the soluble and insoluble fractions were analysed and there was very low level of expression of both the His-Tagged and His-Tagged SUT1-C proteins in the insoluble fraction (Figure 6.2 (B)).

The expression profile of the His-Tagged SUT1-C and His-Tagged protein were determined 3 and 6 hours after induction with IPTG and are shown in Figure 6.3. The proteins were expressed at two different time points 3 and 6 hours after induction to

determine if the level of the expressed protein increased with time. Both the soluble and insoluble proteins fractions were analysed and expression of the His-Tagged SUT1-C protein could only be detected in the soluble protein. The His-Tagged protein could be detected in both the soluble and insoluble fractions with reduced level of expression in the insoluble protein. There appeared to be no major increase in the level of expression of the His-Tagged SUT1-C protein at 6 hours compared to 3 hours post-induction with IPTG.

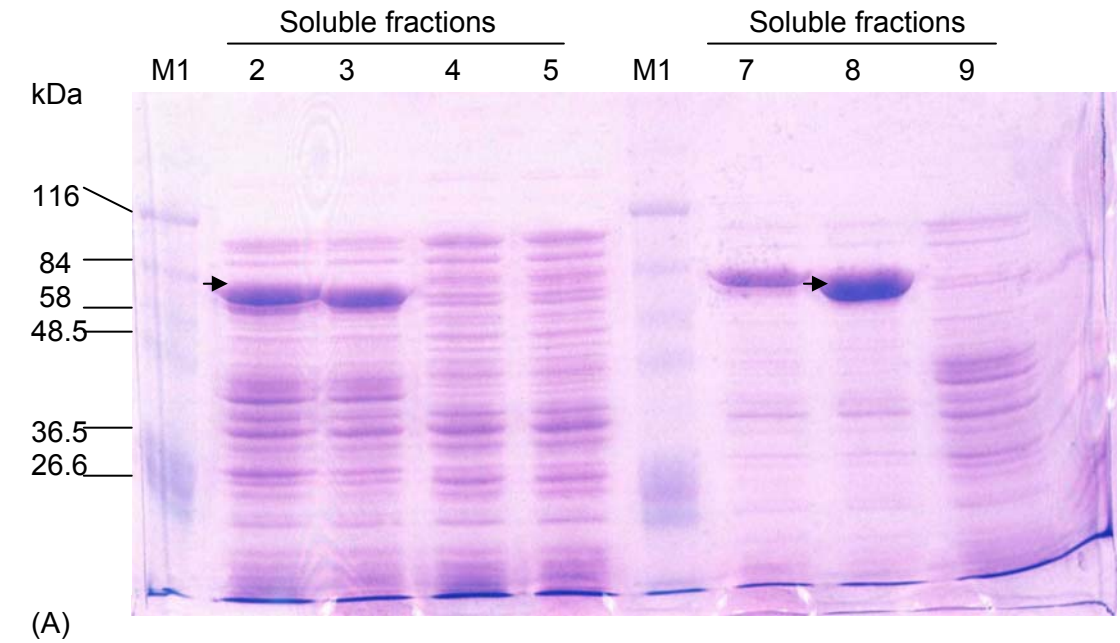
To determine the optimal time point at which there would be the greatest yield of the His-Tagged SUT1-C protein a time course of expression of the protein was carried out every hour for 6 hours and the expression profile of the protein at hourly intervals in the soluble fraction is shown in Figure 6.4. The protein concentration was measured using the Bradford assay and the highest protein concentration was found 3 hours post-induction with IPTG.

To purify the His-Tagged SUT1-C protein expressed by the pET-43.1c(+)::*SUT1*-C construct, transformed into *E. coli* BL21(DE3) cells, the cells containing the transformed construct were harvested 3 hours after induction with 1 mM of IPTG. The soluble fraction of the induced protein were suspended in 1X binding buffer (5 mM imidazole) and added to a chromatography column containing Ni^{2+} charged His•Bind resin (Novagen). The protein fractions were eluted from the column with a series of wash buffers containing a linear stepped (increments of 10 mM) imidazole gradient (10 mM-60 mM). To determine which eluted fraction contained the purified His-Tagged SUT1-C protein all the six fractions were analysed along with the original soluble protein from the induced pET-43.1c(+)::*SUT1*-C construct and are shown in Figure 6.5. The majority of the His-Tagged SUT1-C protein was eluted using 40 and 50 mM of imidazole.

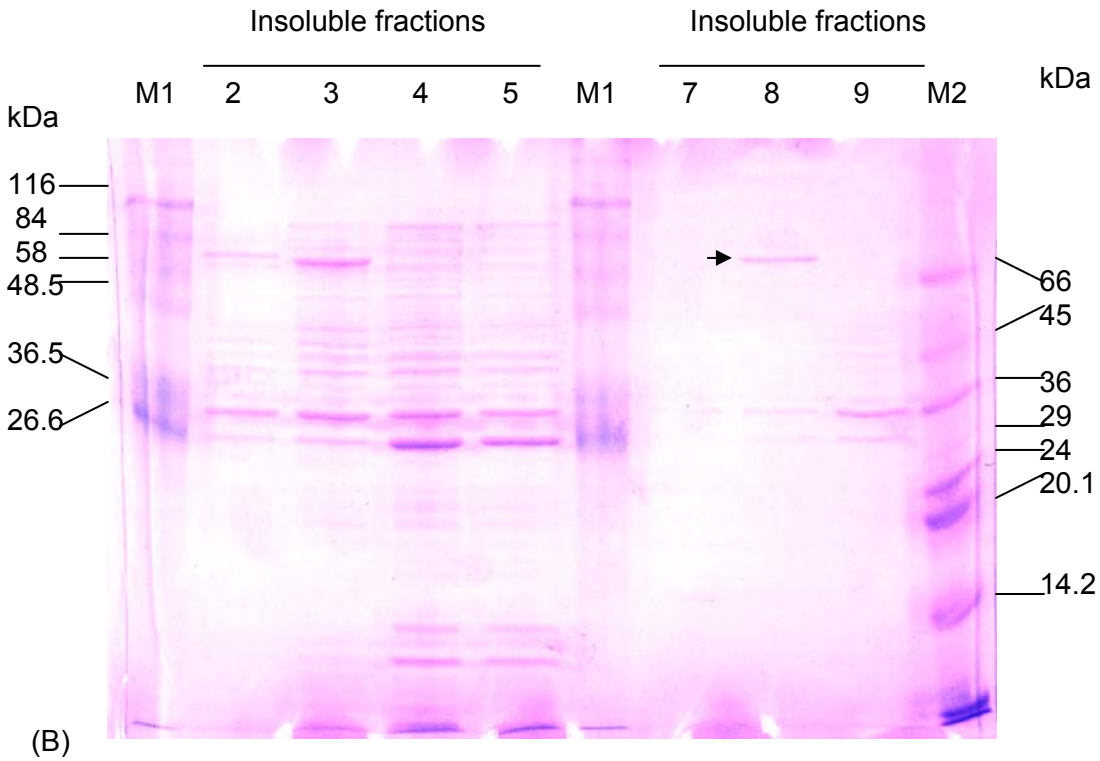
The protein fractions eluted with 40 and 50 mM of imidazole containing the His-Tagged SUT1-C protein were pooled together and the concentration of the protein was 0.26 $mgml^{-1}$ and was determined using the Bradford assay. The His-Tagged SUT1-C

protein was concentrated using the Amicon stirred cell (as described in Section 6.3.7) after which the protein concentration was 1.75 $mgml^{-1}$ and was determined using the Bradford assay.

Figure 6.2: Coomassie blue stained SDS-PAGE of the protein fractions encoded by the pET-43.1c(+) vector and the pET-43.1c(+)::SUT1-C construct containing His-Tagged and His-Tagged SUT1-C fusion proteins respectively after the induction and repression of the fusion proteins indicating the (A) soluble and (B) insoluble protein fractions. Marker 1 (M1) is SDS-7B (Sigma) and marker 2 (M2) is SDS-7L (Sigma). In lanes 2 and 3 the non-induced His-Tagged SUT1-C and the His-Tagged protein respectively are indicated by an arrow. In lanes 4 and 5 the His-Tagged SUT1-C and the His-Tagged protein respectively are shown (indicated an arrow) after being supplemented with 1% glucose. In lanes 7 and 8 the His-Tagged SUT1-C and His-Tagged protein respectively are shown (indicated by arrow) after the addition of 1mM IPTG. Lane 9 contains the protein profile of the expression strain BL21(DE3). The expression of His-Tagged SUT1-C and His-Tagged protein was detected in both the soluble (indicated by arrow) and insoluble fraction (indicated by arrow) with higher expression in the soluble fraction. The protein fractions to which glucose was added had much lower expression level of His-Tagged and His-Tagged SUT1-C protein and this was due to the addition of glucose to the cultures which causes catabolite repression and decreases the rate of transcription of T7 RNA polymerase which is required for transcription of the His-Tagged fusion protein by the pET-43.1c(+) vector. The expression of the His-Tagged proteins were detected when grown in cultures to which no IPTG was added indicating the transcription of T7 RNA polymerase by the *lac* promoter even in the presence of the *lac* repressor gene. The level of expression of the His-Tagged protein was lower when grown in culture to which no IPTG was added but there was very little difference in the expression level of the His-Tagged SUT1-C protein in cultures to which no IPTG was added compared to those to which the inducer IPTG was added.

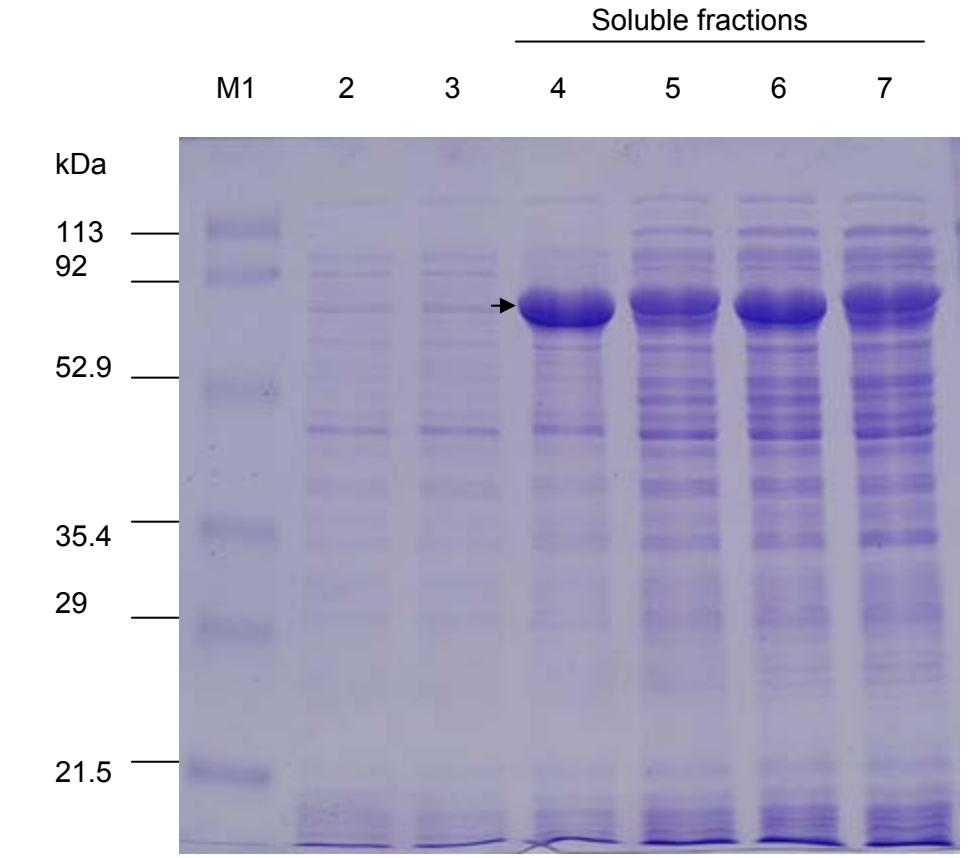

Soluble fractions
Soluble fractions
M1
2
3
4
5
M1
7
8
9
kDa
116
84
58
48.5
36.5
26.6

(A)

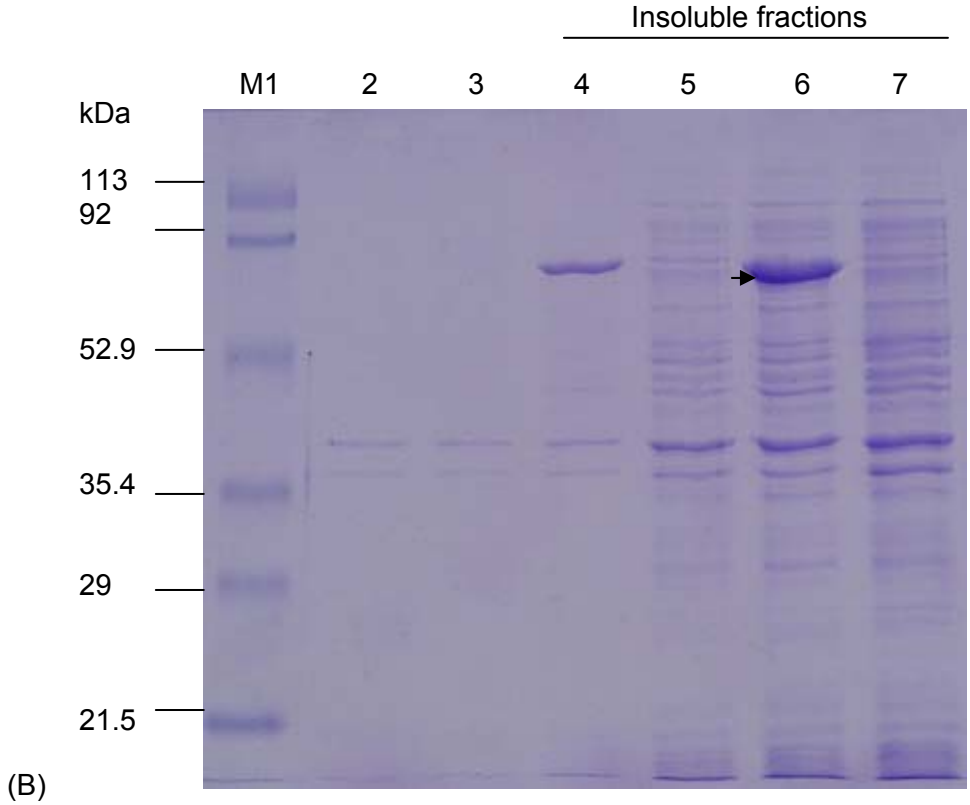

Insoluble fractions
Insoluble fractions
M1
2
3
4
5
M1
7
8
9
M2
kDa
kDa
116
84
58
48.5
36.5
26.6
66
45
36
29
24
20.1
14.2

(B)

Figure 6.3: Coomassie blue stained SDS-PAGE of the protein fractions encoded by the pET-43.1c(+) vector and the pET-43.1c(+)::SUT1-C construct containing the His-Tagged and His-Tagged SUT1-C fusion proteins respectively after the induction and repression of the fusion proteins indicating the (A) soluble and (B) insoluble protein fractions. Marker 1 (M1) is low range pre-stained standard (Bio-Rad). In lanes 2 and 3 the His-Tagged and His-Tagged SUT1-C fusion protein respectively are shown before the addition of the inducer IPTG (t=O). In lanes 4 and 5 the His-Tagged and His-Tagged SUT1-C protein are indicated by an arrow 3 hrs after the addition of IPTG and lanes 5 and 6 are 6 hrs after the addition of IPTG respectively. No expression of the His-Tagged SUT1-C protein could be detected in the insoluble fraction but the His-Tagged protein could be detected in both the soluble and insoluble fractions but at lower levels in the insoluble fraction (indicated by arrow)


Soluble fractions
M1
2
3
4
5
6
7
kDa
113
92
52.9
35.4
29
21.5

(A)


Insoluble fractions
M1
2
3
4
5
6
7
kDa
113
92
52.9
35.4
29
21.5

(B)

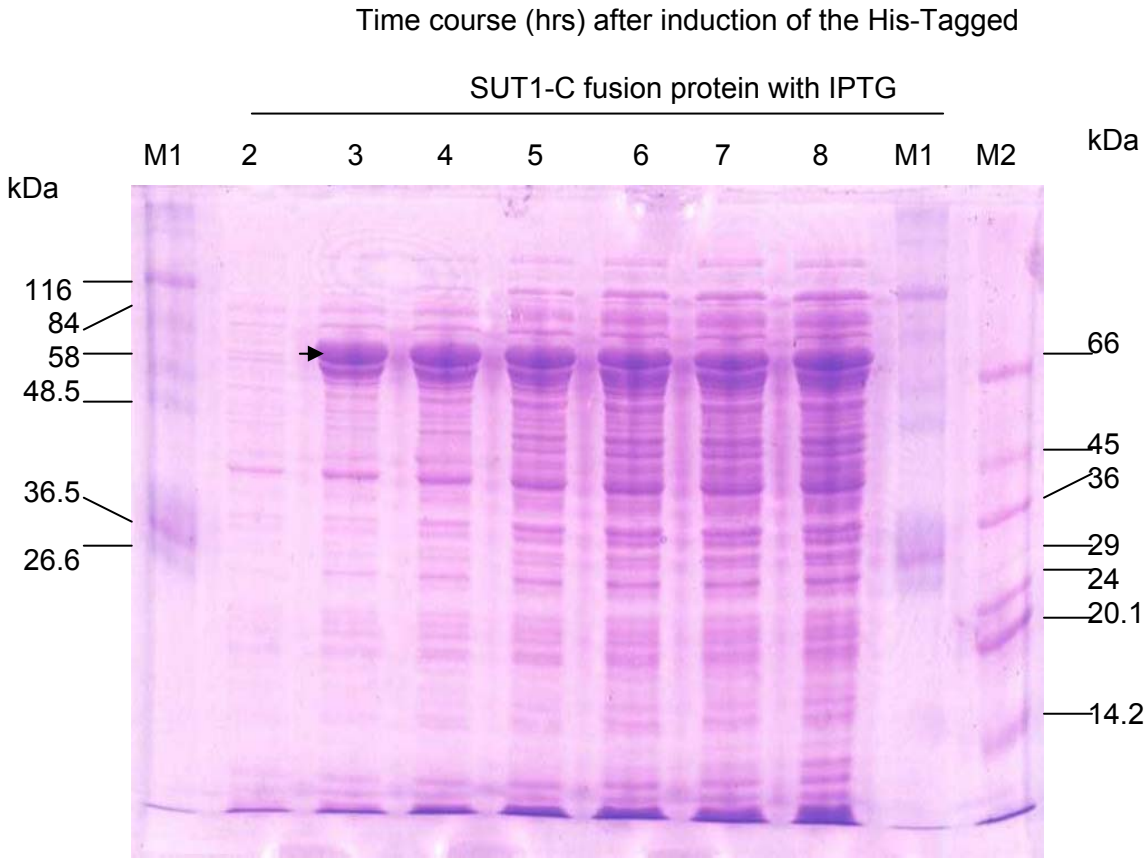


Figure 6.4: Coomassie blue stained SDS-PAGE of a time course of 1-6 hrs after the induction with IPTG of the His-Tagged SUT1-C fusion protein encoded by the pET-43.1c(+)::SUT1-C construct that was transformed into the *E. coli* BL21(DE3) cells. The induction of the His-Tagged SUT1-C protein (indicated by arrow) was achieved with the addition of IPTG to the culture and samples were removed every hour for 6 hours post-induction and these protein samples are shown in lanes 3-8. Lane 2 represents the protein encoded by pET-43.1c(+)::SUT1-C construct before the addition of the inducer IPTG. Marker 1 (M1) is SDS-7B and marker 2 (M2) is SDS-7L (Sigma).

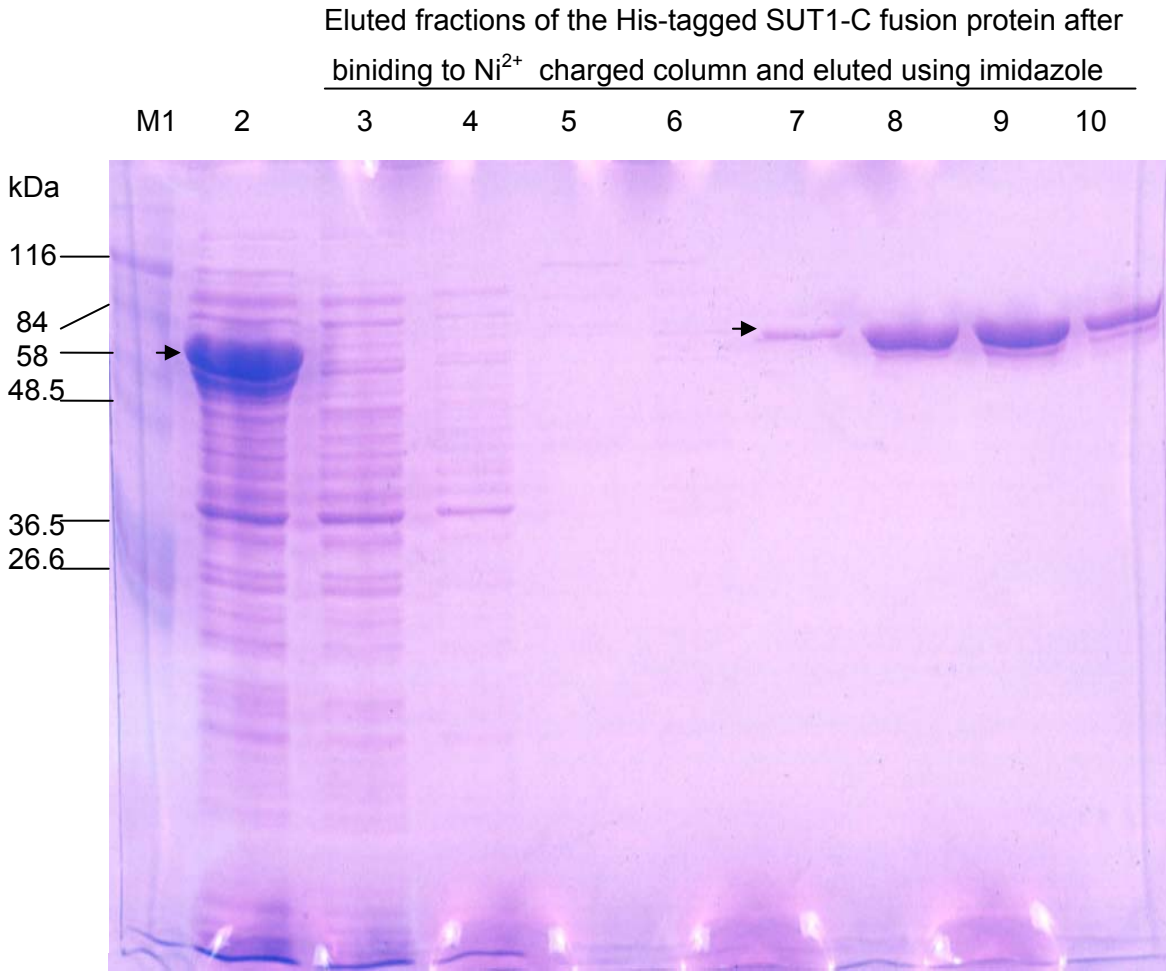


Figure 6.5: Coomassie blue stained SDS-PAGE of the protein fractions eluted during the purification of the His-Tagged SUT1-C protein encoded by the pET-43.1c(+)::SUT1-C construct. The induction of the fusion protein encoded by the pET-43.1c(+)::SUT1-C construct was achieved with the addition of 1 mM of IPTG to the *E. coli* culture and the cells were harvested 3 hrs post induction with IPTG and the His-Tagged SUT1-C fusion protein was eluted using immobilised metal affinity chromatography. Marker 1 (M1) is SDS-7B (Sigma), Lane 2 represents the protein expression pattern of the pET-43.1c(+)::SUT1-C construct 3 hrs post induction with 1 mM IPTG and the His-Tagged SUT1-C fusion protein is indicated with an arrow. Lane 3 is the protein profile of the pET-43.1c(+)::SUT1-C protein after binding to a Ni^{2+} charged His•Bind column (Novagen). Lanes 4-10 are the eluate from the column after the addition of 5, 10, 20, 30, 40, 50, and 60 mM of imidazole respectively. The majority of the His-Tagged SUT1-C protein (indicated by an arrow) was eluted with 40 and 50 mM of imidazole.

6.5.2 Extraction of membrane sugar transporter protein SUT1 from sporophores and detection of the SUT1 membrane protein using anti-sut1 polyclonal antibody

Different methods were attempted in order to extract membrane proteins from *A. bisporus* (A15) sporophores so that the SUT1 sugar transporter could be detected during sporophore developmental stages using the anti-rabbit polyclonal antibody that was raised against the SUT1-C fusion peptide. In initial experiments the detergents CHAPS and N-Octylglucoside were used to extract the membrane protein, however only the blot containing the proteins extracted with 1% CHAPS gave a weak signal when probed with the rabbit SUT1-C polyclonal antibody (Figure 6.6). There was no detection of the SUT1 protein in the blots containing proteins extracted with N-Octylglucoside (result not shown).

Therefore a subsequent experiment was carried out on two stages of sporophore development, stage 4 and 6, using only 1% CHAPS following the method as described in Section 6.4.1. The protein samples were analysed by SDS-PAGE as described in Section 2.5.1 and four identical SDS-PAGE gels were prepared containing the same samples and were transferred by Western blotting onto nitrocellulose membrane using 2 transfer buffers: Tris-Glycine and Carbonate buffer with and without SDS, therefore allowing for 4 different transfer conditions. These two buffers have a different pH, carbonate buffer has a pH 10.7 and Tris buffer has a pH 9.2 and sometimes proteins near their isoelectric point (predicted pI of SUT1 protein 9.23) at the pH of the buffer will transfer poorly. Both buffers were tested with and without SDS as SDS can increase transfer efficiency of the protein from the gel but can also reduce binding of the protein to the nitrocellulose membrane. However when the membranes were probed with polyclonal anti-rabbit SUT1-C antibody and detected using Horseradish Peroxidase conjugate, as described in Section 2.5.3 no signal could be detected (result not shown).

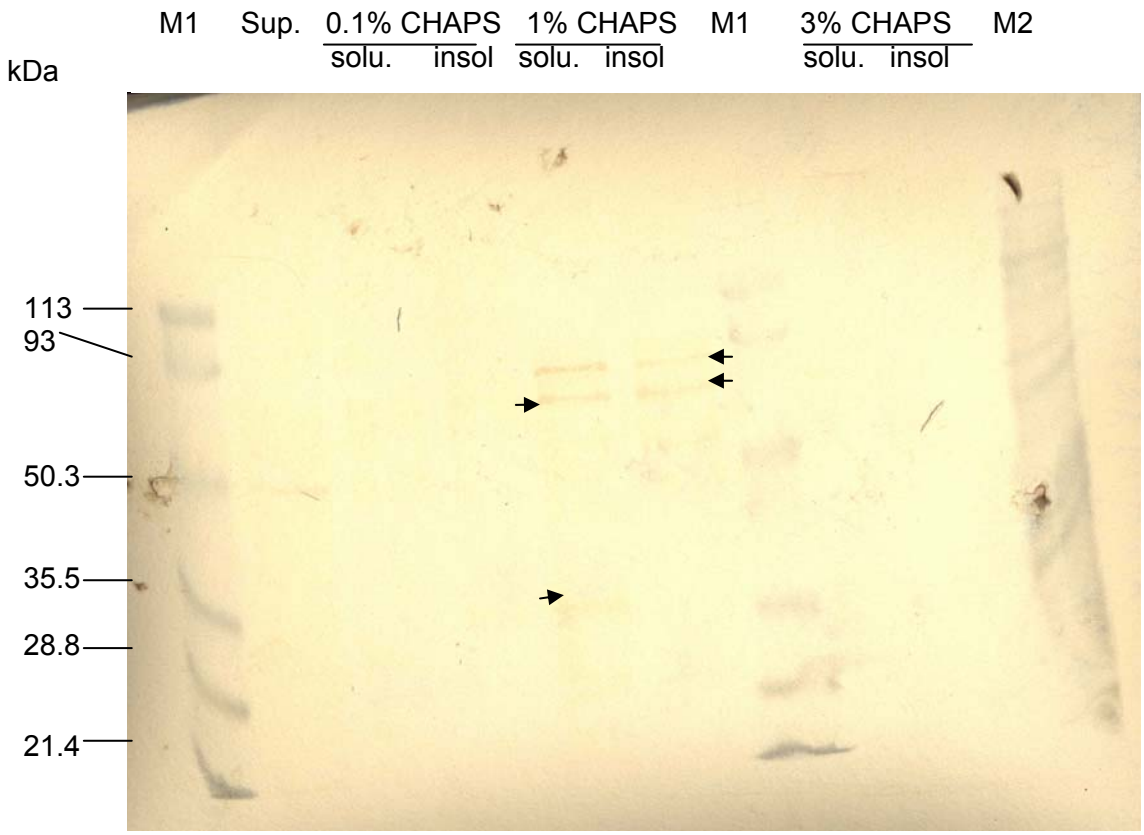


Figure 6.6: Western blot showing the detection of the SUT1 protein in both the soluble and insoluble phase of extracts from a stage 4 mushroom using different concentrations of the detergent CHAPS. Membrane proteins were extracted from a stage 4 mushroom using 0.1%, 1% and 3 % of CHAPS detergent and the proteins soluble or insoluble in CHAPS were separated by SDS-PAGE and transferred by Western blotting to nitrocellulose membrane. Marker 1 (M1) and marker 2 (M2) represent the markers Bio-Rad low range marker and SDS-7B (Sigma) respectively. The membrane was probed with the SUT1-C antibody and detected with goat anti-rabbit labelled horseradish peroxidase using the substrate chloronapthol. In the soluble and insoluble proteins extracted with 1% CHAPS detergent three bands and two bands respectively could be detected and are indicated with arrows. There were two bands between 50.3 and 93 kDa and the lower band could possibly be the SUT1 protein which has a predicted molecular weight of 61.2 kDa.

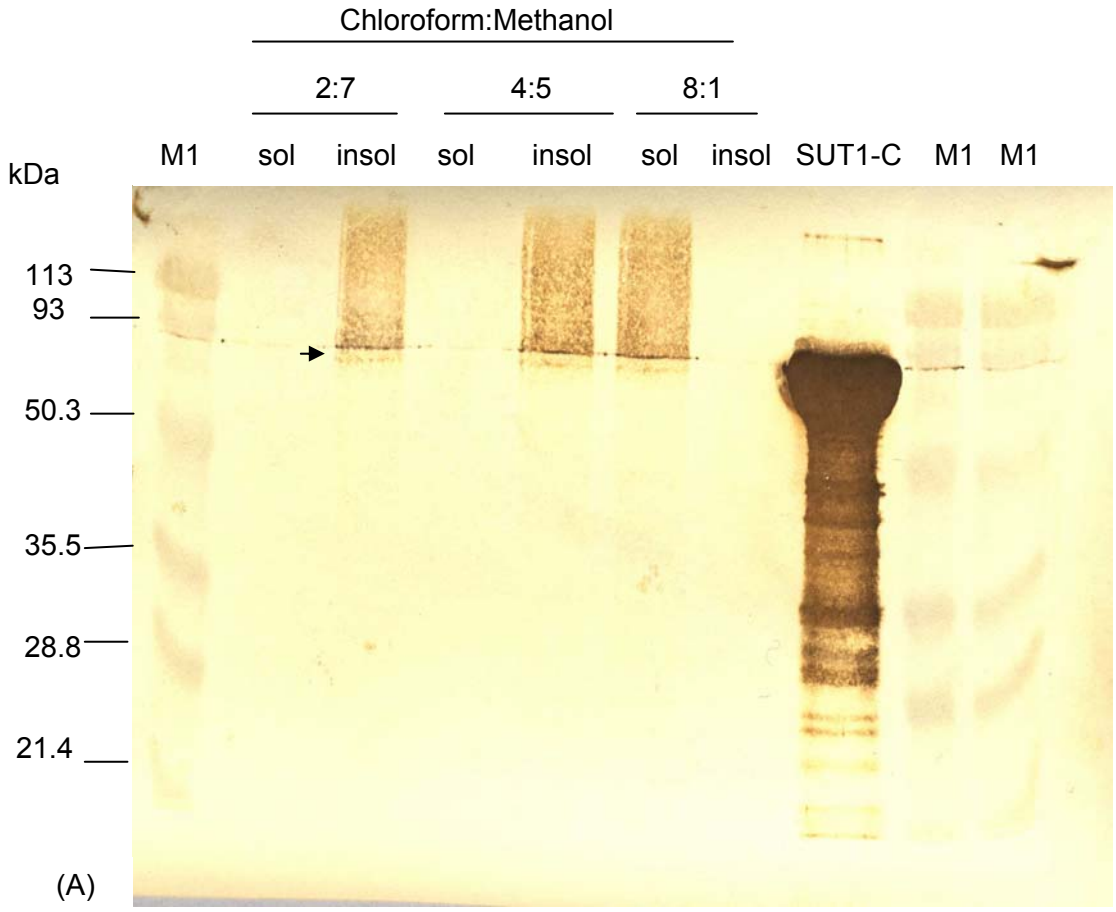
The afore mentioned detergents did not give reproducible results and were unsuccessful in solubilising the membrane bound SUT1 protein. It is possible that the amount of the detergent was insufficient to fully solubilise the lipids and fatty acid and release the protein. Therefore an alternative extraction procedure for membrane protein solubilisation was used following the method of Seigneurin-Berny *et al.* (1999) and Ferro *et al.* (2000) and this involved using different ratios of C:M solutions. Initially the following ratios of C:M solutions, 2:7, 4:5, 8:1 were used to try and extract the membrane bound SUT1 protein from sporophores. There were two identical membranes each containing the proteins extracted with these C:M ratios and one membrane were treated with glutaraldehyde and the other without. Both membranes were probed with the SUT1-C antibody and detected using goat anti-rabbit labelled horseradish peroxidase as described in Section 2.5.3. From the results shown in Figure 6.7 a protein of about 90 kDa can be detected on the blots from the insoluble pellet and soluble organic phase in C:M solutions 2:7 and 4:5 and in the soluble organic phase of C:M solution 8:1. There were no differences between the membrane treated with and without glutaraldehyde.

However extraction of the membrane protein was then carried out using a wider range of C:M ratios as performed by Ferro *et al.* (2000) and the following ratios of C:M were used: 0:9, 2:1, 2:7, 5:4, 1:8, 1:2, 8:1 and 9:0, (v/v) for the extraction of membrane protein from a stage 5 sporophore and the extraction procedure and analysis of the protein fractions was as described in Section 6.4.2 and the protein was transferred by Western blotting to nitrocellulose membrane as described in Section 2.5.2 but the membranes were not treated with glutaraldehyde. The detection of the SUT1 protein was performed using the rabbit SUT1-C polyclonal antibody and goat anti-rabbit labelled alkaline phosphatase as described in Section 2.5.3. As can be seen in Figure 6.8 the SUT1 protein could be detected from the insoluble fractions extracted using all of the C:M ratios but only in soluble fraction of the C:M ratio 0:9. The size of the protein detected was

between the MW markers 50.3 and 93 kDa which is in the size range of the predicted size of the SUT1 protein which is 61.2 kDa.

To try and improve the detection of the SUT1 protein from the insoluble phase of the C:M ratios different methods were used to try and homogenise the insoluble phase after extraction of the membrane protein with C:M. A single C:M ratio of 2:1 was chosen to extract the membrane protein from a stage 5 sporophore because this insoluble protein phase had the greatest SUT1 detection signal. The extraction procedure was as described in Section 6.4.2 except that there were 4 replicated chloroform/methanol (2:1) ratios used to extract the membrane protein and the soluble fractions and insoluble protein fractions were recovered after the solvent extraction process. The insoluble fractions were treated in 4 different ways, to try and optimise the membrane protein extraction procedure, and are described as follows: 1. the protein pellet was re-suspended using a hand-held homogeniser, 2. The protein pellet was ground using a pestle and mortar, 3. The protein pellet was homogenised using a silverson homogeniser 4. The protein pellet was boiled for 30 minutes. The soluble and insoluble fractions (treated as above) were then analysed by SDS-PAGE as described in Section 2.5.1 and the separated proteins were transferred by Western blotting to nitrocellulose membrane as described in Section 2.5.2 and the membrane was probed with the polyclonal anti-rabbit SUT1-C antibody and detected using alkaline phosphatase conjugate, as described in Section 2.5.3. The SUT1 protein could not be detected on the blot in either the soluble or insoluble phase (result not shown)

Figure 6.7: Western blot showing the detection of the SUT1 protein in extracts of a stage 4 mushroom using different ratios of chloroform:methanol (C:M). The membrane proteins were extracted from a stage 4 mushroom using different ratios of C:M and separated along with the His-Tagged SUT1-C protein, which was used as the antigen to raise the SUT1-C antibody, by SDS-PAGE and transferred by Western blotting to nitrocellulose membrane. Marker 1 (M1) and marker 2 (M2) represent the markers Bio-Rad low range and SDS-7B (Sigma) respectively. Both blots A & B are identical except that blot A was cross-linked using glutaraldehyde and blot B was left untreated and both were probed with the SUT1-C antibody and detected with goat anti-rabbit labelled horseradish peroxidase using the substrate chloronapthol. The His-Tagged SUT1-C protein was detected by the SUT1-C antibody indicating that the antibody is recognising the antigen to which it was raised against. The detection of the SUT1 protein is indicated with an arrow and was detected in the insoluble phase of C:M ratios 2:7 and 4:5 and in the soluble phase of C:M ratio 8:1 and was between the marker size 50.3 and 93 kDa and has a predicted MW of 61.2 kDa.


Chloroform:Methanol
2:7
4:5
8:1
M1
sol
insol
sol
insol
sol
insol
SUT1-C
M1
M1
kDa
113
93
50.3
35.5
28.8
21.4
(A)

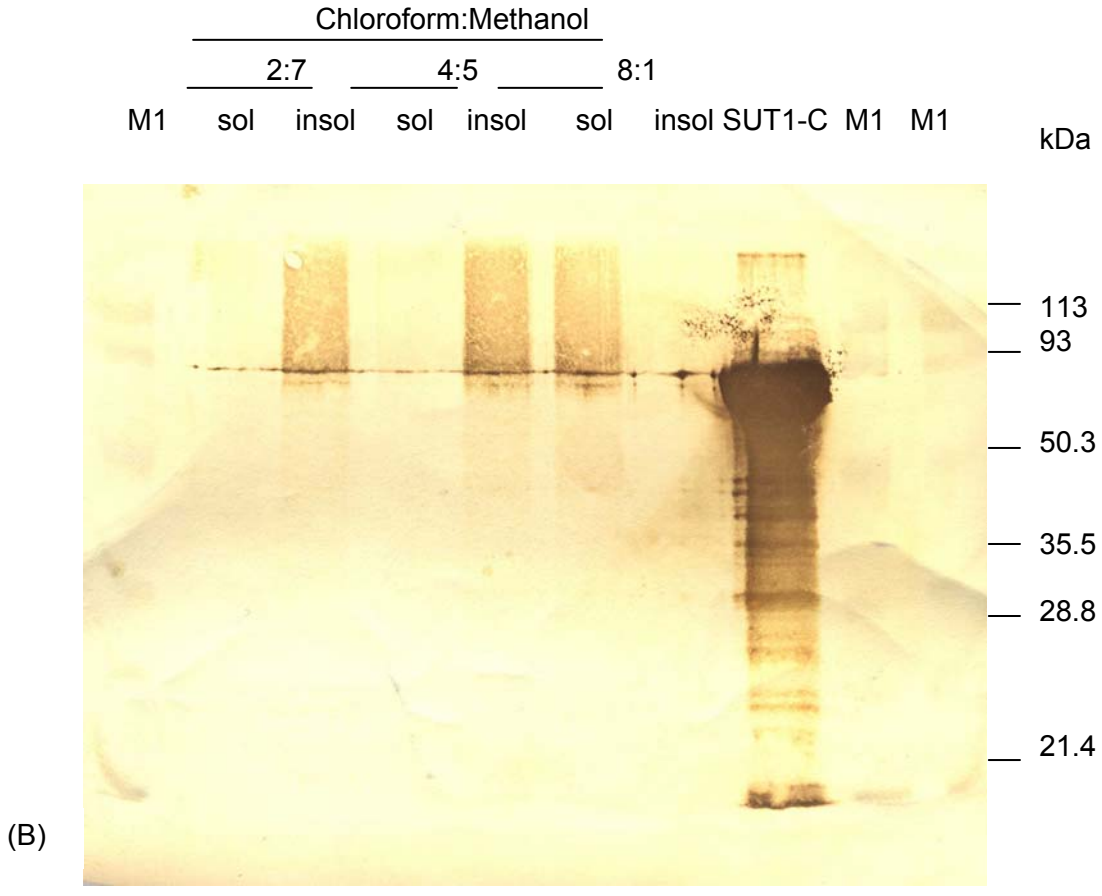
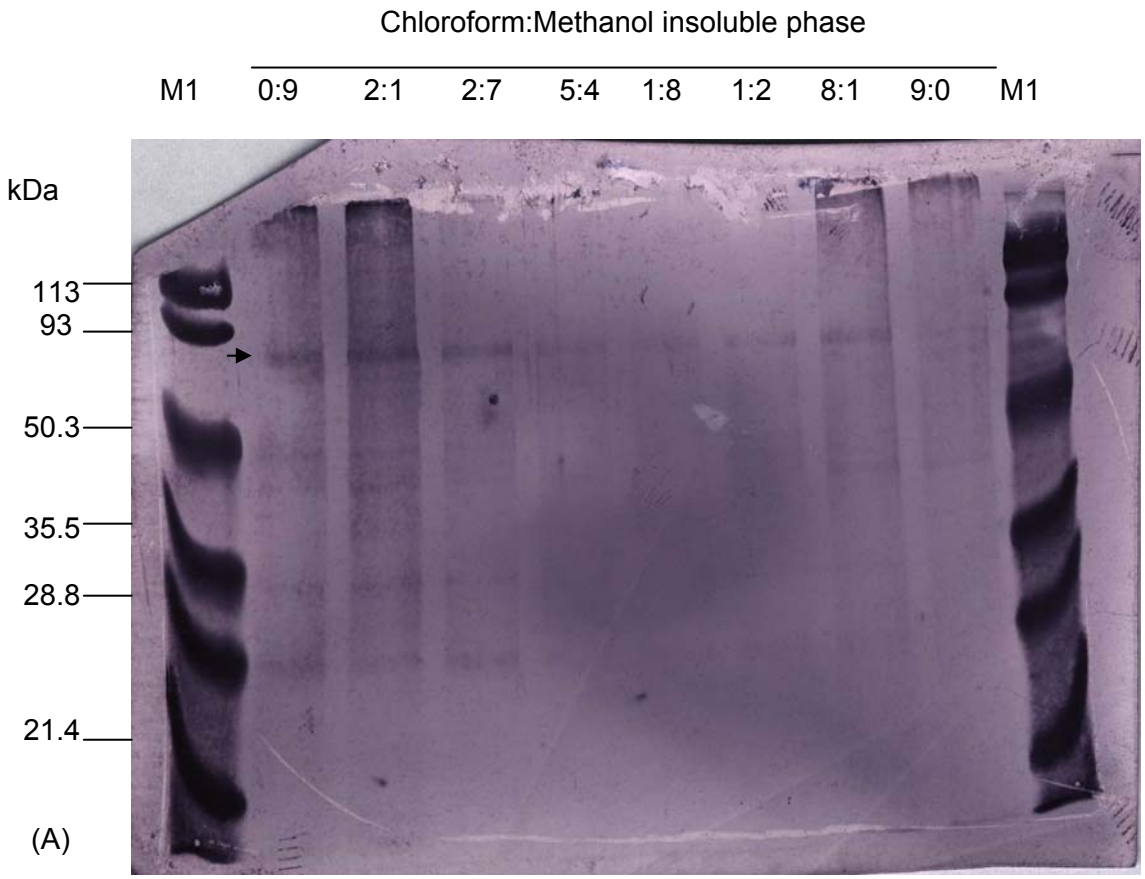
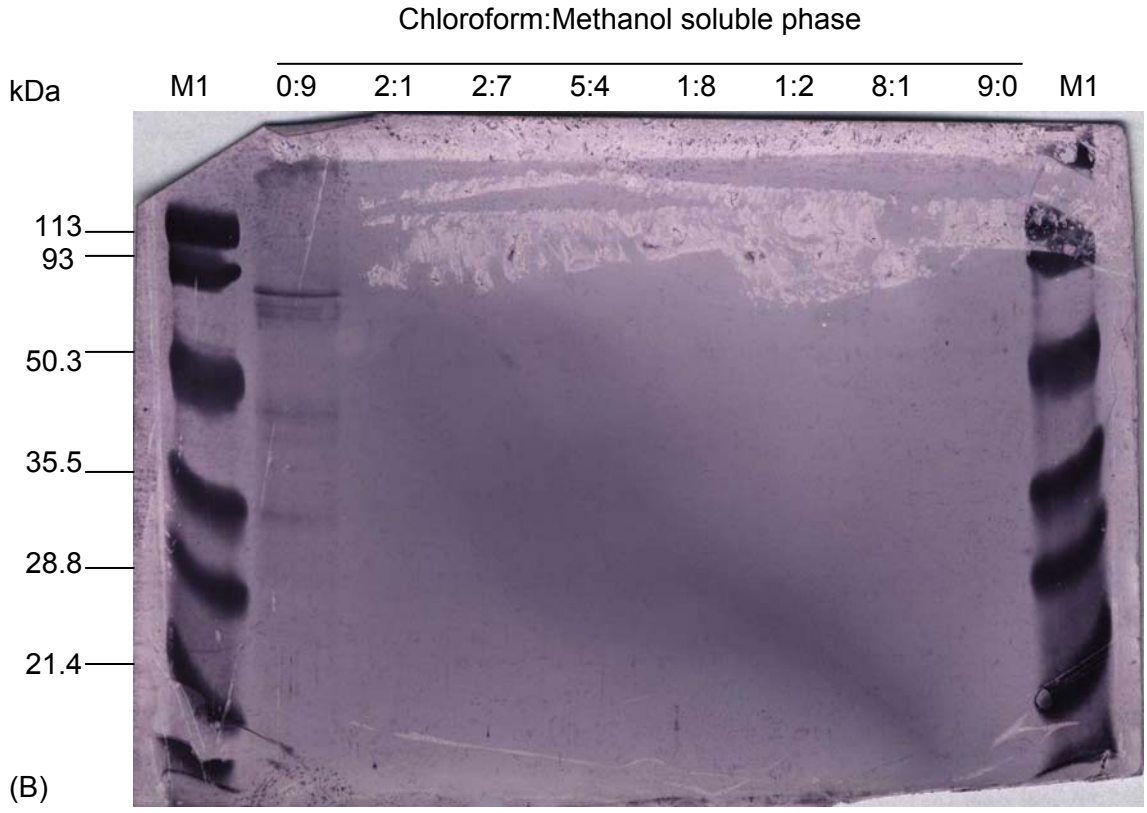

Chloroform:Methanol
2:7
4:5
8:1
M1
sol
insol
sol
insol
sol
insol
SUT1-C
M1
M1
kDa
113
93
50.3
35.5
28.8
21.4
(B)

Figure 6.8: Western blot showing the detection of the SUT1 protein in extracts of a stage 4 mushroom using different ratios of chloroform:methanol (C:M). The membrane proteins were extracted from a stage 4 mushroom using the following C:M ratios: 0:9, 2:1, 2:7, 5:4, 1:8, 1:2, 8:1, 9:0 and the soluble and insoluble phase were separated by SDS-PAGE and transferred by Western blotting to nitrocellulose membrane. Marker 1 (M1) represent the Bio-Rad low range marker. Both blots were probed with the SUT1-C antibody and detected with goat anti-rabbit labelled alkaline phosphatase using the substrate bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT). The detection of the SUT1 protein is indicated with an arrow and was detected in the insoluble phase of all the C:M ratios (blot A) and in the soluble phase of the C:M ratio 0:9 (blot B) between the markers 50.3 and 93 kDa and has a predicted MW of 61.2 kDa.


Chloroform:Methanol insoluble phase
M1
0:9
2:1
2:7
5:4
1:8
1:2
8:1
9:0
M1
kDa
113
93
50.3
35.5
28.8
21.4
(A)


Chloroform:Methanol soluble phase
kDa
M1
0:9
2:1
2:7
5:4
1:8
1:2
8:1
9:0
M1
113
93
50.3
35.5
28.8
21.4
(B)

6.6 DISCUSSION

The main aim of the experiments described in this chapter was to be able to detect the SUT1 protein and to compare it to the *SUT1* gene transcript (Chapter 5) during sporophore development and in sporophore tissue. In order to achieve this it was necessary to raise an antibody to the SUT1 protein and this would then be used to detect the SUT1 protein in *A. bisporus* sporophores using Western blots.

Only the C-terminal region of the SUT1 protein (highlighted in Figure 5.5) was used and expressed as part of a His-Tagged fusion protein because it could be difficult to express the full length 12 transmembrane SUT1 protein due to its highly hydrophobic nature. This C-terminal region was shown to have a high hydrophilicity value which indicates an antigenic region (Hopp & Woods, 1981). The method of expressing only the highly gene specific C-terminal region of a sugar transporter was carried out previously with a 12 transmembrane hexose transporter form the rust fungus *Uromyces fabae* (Voegele *et al.*, 2001).

The SUT1-C protein was successfully expressed as part of a Nus/His/S•Tag fusion protein (69.8 kDa) as can be seen in Figure 6.2 and was encoded by the pET-43.1c(+)::SUT1-C recombinant vector. The N-terminal His-Tag allows for the purification of the fusion protein using immobilised metal affinity chromatography (IMAC) and the purified His-tagged SUT1 fusion protein (Figure 6.5) was used as an antigen to produce a polyclonal antibody in a rabbit.

After successfully producing a polyclonal antibody to the SUT1 protein it was then necessary to try and extract the sugar transporter SUT1 membrane protein from *A. bisporus* sporophores. Two detergents CHAPS and N-Octylglucoside as recommended by Hjelmeland & Chrambach (1984) for the extraction of difficult to solubilise membrane proteins were used to try and solubilise the 12 transmembrane SUT1 protein from sporophore extracts. The solubilisation of membrane proteins by detergents is achieved

by the incorporation of the detergent molecules into the hydrophobic regions of the lipid bilayer and when the bilayer become saturated with detergent it disintegrates and mixed micelles are formed consisting of large heterogenous complexes of lipids, detergents and protein (Hjelmeland & Chrambach, 1984; Bhairi, 2000). Increasing detergent leads to the formation of protein-detergent micelles and micelles consisting of lipids and detergent (Hjelmeland & Chrambach, 1984; Bhairi, 2000).

No SUT1 protein could be detected in protein extracts from stage 4 sporophores using ratios 0.1, 1 and 3% of the detergent N-Octylglucoside (result not shown). The SUT1 protein was detected in the protein extract from stage 4 sporophores using 1% CHAPS detergent and could not be detected in protein extracts using the following ratios 0.1% and 3%of CHAPS and the size of the protein was approximately 85 kDa which is larger than the predicted size of the SUT1 peptide which is 61.2 kDa (Figure 6.7) and this discrepancy in size could be due to the protein detergent complex increasing the apparent molecular weight of the SUT1 protein. A subsequent experiment using only 1% CHAPS and two different stages of sporophore development: 4 and 6 yielded no detection of the SUT1 protein (result not shown) when the membrane was probed with the SUT1 polyclonal antibody. To understand why CHAPS was more successful in solubilising the SUT1 protein compared to N-Octylglucoside it is necessary to look at the characteristics of these two detergents. N-Octylglucoside is a non-ionic detergent and contains un-charged hydrophilic head groups and CHAPS is a zwitteronic detergent and does not have a net charge but is efficient at breaking protein-protein interactions which are sometimes formed between membrane proteins, which are highly hydrophobic, and other non-specific proteins (Bhairi, 2000). However N-Octylglucoside cannot overcome these non-specific interactions. CHAPS is less denaturing than other detergents due to its rigid steroid structure and can overcome artificial aggregation of proteins and has a lower CMC (this is the lowest concentration at which detergents form micelles) than N-Octylglucoside. It is therefore possible that due to CHAPS structure and lower CMC,

requiring lower concentration of CHAPS to achieve the CMC compared to N-Octylglucoside, that it was better at solubilising the SUT1 protein thus detection of SUT1 was only achieved with CHAPS detergent. However the detection of the SUT1 protein obtained in sporophore protein extracted with 1% CHAPS was irreproducible.

For optimal solubilisation the detergent to protein ratio is a critical parameter in achieving solubilisation of membrane proteins and it is necessary to use several different detergent concentrations. It is possible that the ratio of detergent to protein was too low in the experiments trying to solubilise the SUT1 protein from mushroom extracts. Therefore to try and achieve solubilisation of SUT1 a number of other detergents would need to be examined e.g. Triton X-100, digitonin, BIGCHAP. This would involve examining a long list of detergents and trying to optimise the correct protein to detergent ratio for solubilising the membrane protein therefore the protein concentration of the mushroom supernatant and pellet phase would have to be determined to so that the correct protein to detergent ratio could be used. This would be difficult as the pellet phase contains insoluble sporophore tissue and the concentration of the protein would be difficult to determine. Therefore a different method was attempted which involved using organic solvents to solubilise membrane proteins.

The extraction of hydrophobic proteins using a mixture of chloroform and methanol was used based on methods from Seigneurin-Berny *et al.* (1999) and Ferro *et al.* (2000). Seigneurin-Berny *et al.* (1999) extracted hydrophobic chloroplast membrane proteins from chloroplast envelope membranes using a range of ratios of C:M and identified a number of hydrophobic proteins with varying numbers of transmembrane domains which included a 12 transmembrane transporter: 2-oxoglutarate/malate translocator. Initial experiments to extract the SUT1 protein using the following C:M ratios: 2:7, 4:5, 8:1 were attempted because these were shown to be the most effective in solubilising the 2-oxoglutarate/malate membrane transporter from chloroplast (Seigneurin-Berny *et al.*, 1999). This extraction procedure using these ratios to extract the

SUT1 protein gave a weak positive signal when the membrane was probed with the SUT1 polyclonal antibody (Figure 6.7). The membrane also contained the His-Tagged SUT1-C purified protein (used as the antigen) which gave a very strong positive signal when probed with the SUT1 antibody indicating that the antibody was raised to the antigen and identifying the antigen. The full range of C:M solutions (0:9, 2:1, 2:7, 5:4, 1:8, 1:2, 8:1, 9:0) suggested by Seigneurin-Berny *et al.* (1999) were then used to try and solubilise the SUT1 protein and two Westerns were produced one containing the soluble and the other the insoluble protein fractions. When the two membranes were probed with the SUT1 polyclonal antibody (Figure 6.8) only the lane containing the soluble protein fraction extracted with 0:9 C:M gave a positive signal while all of the insoluble protein fractions extracted with the full range of C:M solutions gave positive signals. It was difficult to predict the exact size of the protein detected with the SUT1-C antibody but it was between the markers 50.3 and 93 kDa and the predicted MW of the SUT1 protein is 61.2 kDa, indicating it was in the correct size range.

Although the detection of the SUT1 protein was achieved with a range of C:M ratios it was only detected in the insoluble fraction (with the exception of the soluble protein in C:M ratio 0:9) where the protein was not completely solubilised and attempts to try and solubilise the protein pellet were not very successful.

The low level of detection of the SUT1 protein could be due to there being very low amounts of the SUT1 protein in sporophores in spite of the high levels of the mRNA detected. Pradet-Balade *et al.* (2001) found that in yeast the abundance of the corresponding protein for a given mRNA level can vary by up to 30 fold. Another possible reason for the poor detection of the SUT1 protein could be due to the specificity of the SUT1-C antibody not being optimal and could be improved by removing the undesired antibodies raised against the fusion Nus/His/S•Tag proteins which were part of the pET-43.1c(+)::SUT1-C recombinant vector. Voegele *et al.* (2001) described a method by which they removed the undesired antibody by negative adsorption. Their antibody was similar

to the SUT1-C polyclonal antibody as it was raised against the C-terminal region of a 12 TMD transporter which was expressed as a His-tagged fusion protein encoded by the pET-32a(+) vector and was used to produce a polyclonal antibody. This negative adsorption would involve immobilising the undesired fusion Nus/His/S•Tag protein expressed by the non-recombinant pET-43.1c(+) vector onto a column and applying the SUT1-C antibody and the antibodies raised to the Nus/His/S•Tag protein would bind and the SUT1-C specific region of the antibody could be eluted. This may have provided an antibody which was more specific to the SUT1 protein.

One of the major problems in detecting the SUT1 protein was the extraction process where it is generally accepted that the extraction of membrane proteins is notoriously difficult due to their highly hydrophobic nature and this would explain why only 30 of the 3-D structures of membrane proteins have been resolved to date even though they constitute 30% of sequenced genes (Ward *et al.*, 1999; Werten *et al.*, 2002).

Although the localisation of the SUT1 protein was not achievable using Westerns in *A. bisporus* sporophores it could be localised using immunolocalisation at the cellular level using the SUT1 polyclonal antibody.

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CHAPTER 7

GENERAL DISCUSSION

7.1 OVERVIEW OF WORK

The growth of the sporophore for a significant distance from its nutrient source is an example of the ability of the mycelium to translocate solutes and water over significant distances. The overall aim of this project is to understand the mechanism of translocation of solutes and water from the compost into the mycelium and then through the stipe into the cap. Previous studies have suggested that solute and water translocation occurs by osmotically derived pressure driven mass flow which would explain water and solute transport from the mycelium to the growing sporophore (Kalberer, 1990). This pressure driven mass flow would allow for a constant influx of water which is required to increase the turgor pressure which is thought to be responsible for hyphal growth. In *A. bisporus* the main osmolyte that has been shown to accumulate in mycelium and sporophores under osmotic stress (using NaCl) is the polyol mannitol (Awad & Nair, 1989; Kalberer, 1990; Stoop & Mooibroek, 1998). Beecher (1999) and Noble *et al*. (1999) showed that the sporophore is more sensitive to matric than osmotic potential in terms of production and water status of sporophores and mycelial growth rate. While the polyol levels have been shown to increase in the sporophore during development it is unlikely that they are translocated from the mycelium to the growing sporophore as this would take place against a concentration gradient therefore suggesting that there is a constant carbon source required for mannitol production. A number of studies have been carried out identifying enzymes and sugars involved in carbohydrate metabolism in *A. bisporus* sporophores and a schematic carbohydrate routing pathway has been produced (Figure 1.5) but how the carbohydrates are transported i.e. via active primary or secondary transporters from the mycelium to the sporophore and what sugars are transported remains unknown.

The first part of this study (Chapter 3) was to understand how the mycelium grows and colonises the compost and casing soil and if this mechanism was by

osmotically derived, by the accumulation of the polyol mannitol, pressure driven mass flow as postulated by Kalberer (1990). Although the concentration of mannitol has been shown to increase in the sporophore and mycelium under osmotic stress, in normal growing conditions the mycelium and sporophore are under matric stresses due to the interaction of the water with the physical and chemical components of the compost and casing soil (Nobel *et al*., 1999). The sporophore and mycelium has been shown to be more sensitive to changes in matric than osmotic potential (Magan *et al.*, 1995; Noble *et al*., 1999; Beecher, 1999). In Chapter 3 I also demonstrated that the mycelium was in fact more sensitive to matric than osmotic stresses (Figure 3.5) indicated by the slower mycelial growth rates on matrically than on osmotically modified media and there were differences in the accumulation of osmolytes and the osmotic potential of the mycelium when grown on matrically modified malt extract medium compared to compost extract medium. The mycelium had a higher growth rate at both temperatures when grown on compost extract media at all water stresses than on malt extract media. The type of media which the mycelium was grown on influenced the growth rate and polyol accumulation and osmotic potential. The notable differences were under matric stresses where the mycelium could withstand much lower water stresses on compost extract media than on malt extract media. The mycelium under matric stresses on compost extract medium accumulated lower levels of mannitol compared with mycelium grown on malt extract medium and also had a higher water potential than the external medium. The results for the osmotic potential and solute accumulation were similar for the mycelium grown under osmotic stresses on both media but with higher growth rates on compost extract medium. Therefore it can be presumed that the mycelium water potential and polyol concentration under matric stresses is influenced by the compost and casing soil. Noble *et al*. (1999) showed that the sporophore could withstand decreases in the soil matric potential below -1.0 MPa without it having an adverse effect on the development of the sporophore and that this relates to the physical and chemical components of the compost and casing soil.

It is likely that the chemical components of the compost and casing soil encourage mycelial growth and allow the mycelium to withstand matric stresses without having to create an osmotic gradient. However it is not known how the mycelium and sporophore extracts water from the compost and casing soil under matric stresses without creating an osmotic gradient by accumulating the osmolyte mannitol and how this relates to the physical and chemical components of the compost and casing soil is also unknown. At matric potentials of -0.48 and -0.98 MPa the mycelium grown on compost extract media had higher water potential than the media and accumulated the lowest concentration of mannitol compared with mycelium grown on osmotically modified media, but Nobel *et al.* (1999) found that the lowest matric potential of the casing soil was -1.06 MPa during a flush without any adverse effect on the developing sporophore. It is possible that the mycelium has adapted to decreases in matric potential of the compost and casing soil and can extract water without having to create an osmotic potential up to a certain matric potential. Therefore at these lower matric potentials up to -1.06 MPa the mycelium and sporophore doesn't produce or has very low levels of mannitol (as shown in Table 3.5 and 3.6). At matrically induced water stress up to -1.06 MPa we would expect very low levels of sugars transported into the mycelium as only low levels of mannitol is produced and a large carbon source would not be required.

The concentration of the polyols and mono- and disaccharides were measured in stage1-6 sporophore tissue (cap, gills, skin, upper and lower stipe). The polyols arabitol and erythritol were detected which had previously not been reported in *A. bisporus* sporophores where only low levels of erythritol were detected and not in all stages of sporophore development while arabitol was detected at higher levels in all stages of development but these represent the minor polyols in the sporophore. Growth under water stress due to osmotic or matric potential effects requires the maintenance of the cell turgor for cell functioning and growth. To enable cell functioning fungi accumulate solutes and polyols to create an osmotic gradient which aids translocation of nutrients,

protein synthesis, protects essential enzymes and increases cell turgor (Magan, 1997). The two major polyols glycerol and mannitol were detected in the sporophore and mannitol was the major polyol with the highest concentration during all stages of sporophore development while glycerol was the secondary polyol. Glycerol is known to act as a compatible solute in many fungi and is a low molecular weight polyol and allows structural integrity under low water potential and is the major polyol of xerophilic and xerotolerent fungi and yeast (Magan, 1997). Glycerol was found during all stages of sporophore development and in all tissues and there were significant differences between stages 1 and 5 and 1 and 6 of sporophore development. The major polyol detected was mannitol which is produced in *A. bisporus* by the reduction of fructose by MtDH using NADPH as the co-enzyme (Figure 1.5). Previously mannitol has been reported to increase from 85-302 mM between stages 1 and 5 (Wannet *et al*., 2000) and rises from 30 to 50% dry weight between stages 2 and 7 (Hammond & Nichols, 1976). The increase in mannitol levels in sporophores reported by Wannet *et al*. (2000) and Hammond & Nichols (1976) during stages of development is in contrast to the levels reported in Figure 4.5 where there were no significant differences in mannitol between or within the stages. There could be a number of reasons for these differences in mannitol concentration and it could be attributed to the sporophore growing conditions. The sporophore extracts 54-83% and 17-46% of their water from the compost and casing soil respectively (Kalberer, 1990) and under osmotic and matric stresses creates an osmotic gradient by accumulating polyols which in turn drives a net influx of water into the cells and increases turgor. However the components of the casing soil have changed in recent years and here at the HRI mushroom unit, of which the major change is the use of bulk dug peat which has a greater water holding capacity than previously used dried peat. The sporophores therefore are not under increasing matric stresses due to the increased water holding capacity of the casing soil and can extract water more easily and therefore do not have to constantly adjust the osmotic potential by increasing the mannitol

concentration. As was shown in Chapter 3 the *A. bisporus* mycelium when grown under matric stresses on compost derived medium can withstand matric stresses up to -0.98 MPa without creating an osmotic gradient. Noble *et al*., (1999) showed that *A. bisporus* sporophores could withstand decreases in matric potential of the casing soil to below -0.1 MPa without an adverse effect on the sporophores and that the mushroom is more sensitive to changes in the matric potential of the casing soil in terms of production and water status of the sporophore. Therefore it can be concluded that the levels of mannitol observed during sporophore development are intrinsically linked to the casing soil and its water holding capacity and the matric potential.

The mono- and disaccharides glucose and trehalose, respectively, were measured in all the tissues during stages 1-6 of sporophore of development. Trehalose was detected at very low levels in all stages of sporophore development and was not detected in all tissues (see Figure 4.6). Wannet *et al*. (2000) also found low levels of trehalose during sporophore development and in concomitant with this Wannet *et al*.(1999) found the that the degradative activity of trehalose phosphorylase increased in axenic fruit bodies during development. Trehalose is degraded by TP and releases glucose and glucose–1-phosphate. Glucose was detected in all sporophore tissues during stages 1-6 and there were significant differences between stages 1 and 6 and 1 and 5 with lower glucose levels at stage 1 but overall the levels of glucose remained steady. Wannet *et al*. (1999) also found that the levels of glucose remained steady in axenic fruiting bodies during fruit body development. Wannet *et al*. (2000) showed that both glucose and glucose-1-phosphate were produced at the expense of trehalose by trehalose phosphorylase from *A. bisporus*. It is not unusual to see low levels of trehalose in sporophores during developmental stages as it is likely to be degraded by trehalose phosphorylase whose degradative activity increased in axenic fruit bodies during development (Wannet *et al*., 1999).

The cDNA STIIE2 that encoded a sugar transporter SUT1 in *A. bisporus* was the first full cDNA of a sugar transporter identified in *A. bisporus* by Sreenivasaprasad *et al*. (2000b) as previously only a partial cDNA of a sugar transporter had been reported by Morales & Thurston (2003) which showed homology to a hexose transporter from *A. parasiticus*. The SUT1 cDNA STIIE2 was one of 20 cDNAs which encoded the sugar transporter, SUT1, out of 31 upregulated cDNAs and this represents *ca*. 0.6% of the transcripts which suggests a strong upregulation of SUT1 in *A. bisporus* (Sreenivasaprasad *et al*., 2000b). The full *SUT1* cDNA and genomic sequence were completed (Chapter 5) and are indicated in Figure 5.4 and 5.6 respectively. However the precise identification of the sugar transported by SUT1 was not apparent from the sequence searches as SUT1 predicted peptide sequence only showed low level of homology (28-33% identity and 46-50% similarity) to other sugar transporters and they all belonged to the sugar transporter family of the MFS. From topology prediction of SUT1 using HMM (Tusnady & Simon, 1998) it contained 12 TMDs and a number of signature sequences (Figure 5.5) which place it in the sugar transporter family of the MFS. The *SUT1* transcript increased during sporophore developmental stages (Figure 5.10) with the greatest level of transcript detected between stages 5-7 where there is rapid growth and cell expansion taking place indicating that SUT1 is involved in transporting sugars during the period of sporophore rapid expansion and the sugar could supply a readily metabolisable carbon source for mannitol production. An increase in mannitol production could in turn create an osmotic gradient under increasing water stress. There was no *SUT1* transcript detected in mycelium supporting stage 2 and 4 sporophores and this indicates that the SUT1 transporter is not actively transporting sugar from the mycelium to the growing sporophore at this stage of sporophore development. There was a higher *SUT1* transcript level in the stipe compared to the cap tissue indicating that SUT1 is active in long distance transport through the stipe to the cap tissue where rapid cell expansion takes place (Figure 5.12).

It is likely that the SUT1 transporter is active in supplying a readily metabolisable carbon source to the growing sporophore and is routed into the carbohydrate pathway (Figure 1.5). The SUT1 peptide sequence showed only 28% identity over 305 amino acids to the genome sequence of *C. cinereus* located at: http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/ but this is not surprising as they have different metabolic routes and therefore probably transport different carbohydrates. In *A. bisporus* the synthesis of mannitol is by the reduction of fructose by NADPH dependant MtDH and the NADPH is obtained through the PPP pathway (Edmundowicz & Wriston, 1963). In *C. cinereus* the glycolysis pathway represents the major carbohydrate catabolic route and the PPP pathway plays only a minor role (Tan & Moore, 1994). A second sugar transporter SUT2 has been identified in *A. bisporus* and has 65% identity to a hexose transporter MST1 from *A. muscaria* and 45% identity (over 163 amino acids) to the *C. cinereus* genome. This level of homology found between the putative hexose SUT2 transporter and *C. cinereus* genome is not surprising as *C. cinereus* fruit body contains large quantities of glycogen which are degraded by glycogen synthase and translocated from the stipe to the cap (Ji & Moore, 1993), therefore indicating the role of a possible glucose transporter in the fruit body.

The SUT1 transporter has an added importance whereby it is being used in a commercial venture at HRI in the production of pharmaceutical and therapeutic compounds in *A. bisporus* sporophores. *A. bisporus* is being investigated as a system which could be used for production of recombinant proteins at HRI and the promoter region of *SUT1* is being used to drive transgene expression using *Agrobacterium tumefaciens* mediated transformation (Foster *et al*., 2004) and has been submitted for a patent (Burton *et al*., 2003). This recombinant protein production using *A. bisporus* is as a result of the increasing demand for the production of pharmaceutical and therapeutic products and the examination of alternatives to fermenter based technologies using

plants, animals and mushrooms as bioreactors for recombinant proteins (Stoger *et al*., 2002).

The sugar transported by SUT1 could not be determined from the database searches as SUT1 predicted peptide sequence showed only low level homology (28-33% and 46-50% similarity) to a number of fungal sugar transporters belonging to the MFS. As SUT1 had such low homology level to other known sugar transporters this in turn prevented the identity of the sugar of transport by functional complementation using mutant yeast strains such as *S. cerevisiae* and *S. pombe* which have been engineered with deficiencies in specific transport pathways. But from what is known about carbohydrate routing and metabolism in *A. bisporus* it is possible to postulate that SUT1 could be a trehalose transporter. This disaccharide has been suggested as the major sugar translocated in *A. bisporus* from the mycelium to the sporophore (Hammond & Nichols, 1976; Hammond & Nichols, 1979; Wells *et al.*, 1987; Wannet *et al.*, 1999). If SUT1 were a trehalose transporter then we might expect to see an increase in the concentration of this disaccharide in the fruit body during development in concomitant with the increase in the transcript of *SUT1*. However the level of trehalose detected in the tissue of the fruit bodies was very low (Figure 4.6) but this could be explained by the increase in the degradative activity of the enzyme trehalose phosphorylase reported by Wannet *et al*. (1999) in *A. bisporus* aggregates during fruiting. Wannet *et al*. (2000) found that there were increased levels of trehalose between fruiting in the mycelium and that these trehalose levels decreased in the mycelium at the onset of fruiting which indicates that trehalose is possibly translocated from the mycelium to the growing sporophore and is synthesised during the interflush period. Because no *SUT1* transcript could be detected in the mycelium supporting stage 2 and 4 sporophores it is possible that the SUT1 transporter may not be active during these stages in the mycelium or there is another sugar transporter transporting trehalose from the mycelium into the sporophore and that

SUT1 is only involved in long distance transport of trehalose within the sporophore from stipe to the cap tissue.

Although we have determined that the *SUT1* gene is important in sporophore development due to the large increase in its transcript, the mRNA levels are not always an accurate indicator of the corresponding protein levels. Therefore to determine the levels of the SUT1 protein an antibody was raised to the SUT1-C region and fused to a Nus/His/S•Tag fusion protein encoded by the pET-43.1-C::SUT1-C recombinant vector. The purified SUT1-C fusion protein is shown in Figure 6.5 and was 69.8 kDa. To detect the SUT1 protein in Westerns using the SUT1-C fusion antibody it was necessary to be able to extract the SUT1 membrane protein from the sporophore tissue and due to the fact that SUT1 was a membrane bound protein this posed difficulties in extracting it from sporophore tissue. A number of methods were used to try and solubilise the SUT1 membrane protein and these included using detergents and organic solvents. The detergents CHAPS and N-Octylglucoside which are zwitterionic and non-ionic detergents respectively were used to solubilise the SUT1 protein and are suggested as detergents to use with difficult to solubilise proteins (Hjelmeland & Chrambach, 1984). Detergents solubilise membrane proteins by partitioning into the lipid bilayer and when the bilayer is saturated with the detergents they disintegrate and form micelles with the detergent consisting of lipid-detergent micelles and protein-detergent micelles (Bhairi, 2001). Extraction of the membrane protein was attempted using a range of concentrations (0.1%, 1% and 3% (w/v) of the detergents CHAPS and N-Octylglucoside (Hjelmeland & Chrambach, 1984). The SUT1 protein was only detected in protein fractions extracted using 1% CHAPS (Figure 6.6) and no SUT1 protein was detected in protein fractions extracted with N-Octylglucoside (result not shown). A subsequent experiment to improve the detection of the SUT1 protein extracted using 1% CHAPS was unsuccessful in detecting the SUT1 protein. There could be a number of reasons why the membrane protein extraction was not successful with the detergents used and why only CHAPS

gave a weak signal but was irreproducible. It is possible that the concentration of the detergent was too low and below the CMC for the two detergents, although CHAPS has a lower CMC and therefore will form micelles at a lower concentration than N-Octylglucoside which has a higher CMC. Also N-Octylglucoside is a non-ionic detergent and unable to break protein-protein interactions which sometimes form between membrane proteins and other non-specific proteins but CHAPS is able to break these protein-protein interactions (Bhairi, 2001). The protein concentration of the supernatant and insoluble phase needs to be determined to optimise the protein to detergent ratio which will cause complete solubilisation of the membrane protein. However it would be difficult to determine the protein concentration of the insoluble pellet phase of the sporophore which contains insoluble sporophore tissue. It is possible that the concentration of the detergent was too low to completely solubilise the protein and form micelles as SUT1 contains 12 transmembrane spanning regions embedded in a lipid bilayer. However there are a number of other detergents which could be used and these include digitonin, Triton X-100, sodium cholate and BIGCHAP (Hjelmeland & Chrambach, 1984). Instead of attempting to solubilise the SUT1 membrane protein using a range of alternative detergents organic solvents were used.

The method of extracting membrane proteins using different ratios of chloroform:methanol as described by Seigneurin-Berny *et al* (1999) and Ferro *et al.* (2000), who used this method to extract membrane proteins from chloroplasts, was used to try and solubilise the SUT1 protein from sporophore tissue. This method resulted in the detection of SUT1 protein in the insoluble phase of the following C:M ratios 0:9, 2:1, 2:7, 5:4, 1:8, 1:2, 8:1 and 9:0, (v/v) and in the soluble phase of C:M 0:9. The SUT1 predicted peptide is 61.2 kDa and the size of the protein detected by the SUT1-C antibody in the C:M protein extracts was between 50.3 and 93 kDa (Figure 6.8). Further work to try and solubilise the protein in the insoluble pellet phase after C:M (2:1) extraction using a pestle

and mortar, a hand held homogeniser, a Silverson homogeniser and boiling the pellet resulted in no detection of the SUT1 protein in these fractions(result not shown).

There are a number of possible reasons why the SUT1 protein could not be detected and as previously discussed it could be due to the extraction procedure whereby it was not successfully solubilised by the extraction methods described above. However it could be that although there are large increases in the SUT1 transcript during sporophore development (Figure 5.10) there might be low levels of the SUT1 protein in the sporophore as has been shown in yeast whereby the abundance of the corresponding protein for a given mRNA expression level could vary by up to 30 fold Pradet-Balade *et al*. (2001). The antibody used to detect the SUT1 protein was raised from C-terminus of the SUT1 protein which was shown to be a highly antigenic region when analysed by the hydrophilicity scale devised by Hopps & Wood (1981) and as a result was produced as a recombinant fusion protein using the pET-43.1c(+) vector. It is possible that the protein undergoes post-translational modification which could result in a change in the antigenicity of this region, therefore the antibody might not be very effective in detecting the SUT1 protein or be very specific to SUT1.

The results of the experiments described in Chapter 6 to try and detect the SUT1 protein reinforce previous reports on the difficulties with their purification due to their highly hydrophobic nature.

7.2 OVERALL CONCLUSION

The matric potential of the compost and casing soil is more closely related to the water requirement of the mycelium and sporophores than the osmotic potential. This was demonstrated in *A. bisporus* mycelium where they are more sensitive to changes in matric than osmotic potential but there were important differences between the responses of the mycelium under matric stresses on the two different media: malt extract and compost extract. The mycelium could tolerate matric stresses on compost media without having to

create an osmotic potential gradient and had higher water potential than the external media under matric induced water stresses as low as -0.98 MPa. The components of the compost are intimately linked to the matric potential and the extraction of water by the mycelium. This has also been demonstrated by Nobel *et al*. (1999) who showed that the sporophores could withstand decreases in the casing soil matric potential to -1.06 MPa without it having an adverse effect on the development of sporophores. The water held by the compost and casing soil would be by matric forces rather than osmotic and therefore it is more likely that the mycelium and sporophore would be more sensitive to matric than osmotic stresses. Most of the previous work carried out was investigating the sensitivity of the mycelium and sporophore to osmotic stresses but not to matric stresses. Under increasing matric stresses, below -0.98 MPa, the mycelium accumulated the polyol mannitol and created an osmotic potential and had a lower water potential than the external medium indicating that under increasing stresses the mycelium must create an osmotic gradient to allow for an influx of water from the medium but surprisingly it can extract water up to a matric potential of -0.98 MPa without creating an osmotic gradient. However to create an osmotic gradient the mycelium has been shown to accumulate the polyol, mannitol and which is formed from the reduction of fructose by the enzyme MtDH.

This thesis characterised a sugar transporter SUT1 which was shown to be developmentally regulated and the transcript increased during sporophore development indicating that there is an increasing supply of a carbon source linked to sporophore development. This was the first sugar transporter which was characterised in *A. bisporus* and provides us with an insight into how carbohydrates are transported into the sporophore as previously only the carbohydrate metabolism pathway within the sporophore had been examined. The SUT1 transporter was a secondary transporter and the sugar of transport could not be discerned from its sequence although it could possibly be a trehalose transporter. Although the SUT1 transcript was not detected in the mycelium

supporting stages 2 and 4 of sporophore development it is involved in long distance transport within the sporophore, through the stipe to the cap.

The transcript of SUT1 during sporophore development was determined by Northern analysis but the SUT1 protein could not be measured due to the difficulties in extracting the membrane protein from sporophore tissue. Therefore the levels of the SUT1 protein could not be determined.

When the second sugar transporter, SUT2, identified in *A. bisporus* which is a putative glucose transporter is characterised and its sugar of transport identified using a yeast mutant transporter this will help to give an overall picture of the carbohydrate transport system in *A. bisporus*.

This project has determined a number of important features of water and solute translocation in *A. bisporus* mycelium and sporophores but importantly it has led to the identification of areas which require further study (Section 7.3) to address the link between the matric stresses and the extraction of water by the mycelium and sporophore and sugar transport.

7.3 FUTURE WORK

1) The water potential of the casing soil could be reduced osmotically and matrically (as carried out by Noble *et al*. (1999) and the effects on the yield of sporophores and their water, osmotic and turgor potential and the endogenous sugars and polyols would be measured. This would determine if the effect of osmotic and matric stresses as seen on *A. bisporus* mycelium (Chapter 3) were similar in the sporophore and if the levels of polyols accumulated under matric and osmotic stresses were different.

2) Because there was mycelial growth at all water stresses, between -0.48 and-2.48 MPa, at 25°C on CE/CYM media the level of tolerance of *A. bisporus* mycelium to both matric and osmotic stresses on both MEA/MEB and CE/CYM media

could be examined by extending the water potential stresses beyond -2.48 MPa and examining smaller decreases in the water potential. The mycelial growth, water potential and sugars/polyols at all water stresses would be measured unlike in Chapter 3 where only the sugars and polyols were measured at the two extreme water stresses which were -0.48 and -2.48 MPa and this would allow us to see the pattern of changes in sugars and polyols in response to matric and osmotic stresses.

3) RNA interference (RNAi) could be used to silence the SUT1 gene and examine the effect on sporophore growth using *A. tumefaciens* mediated transformation of *A. bisporus*. RNAi technology is currently being explored in *A. bisporus* at HRI with a number of genes involved in postharvest quality with the aim of improving postharvest quality (Eastwood *et al*., 2004).

4) Examine the activity of trehalose phosphorylase enzyme (following the assay method of Wannet *et al*. (1999) and the levels of trehalose in the mycelium and during fruit body developmental stages during the flushing cycle of a mushroom crop. This would determine if there is an increase in the degradative activity of TP during sporophore developmental stages and if this was a possible reason for the low levels of trehalose detected as shown in Figure 4.6 in the fruit bodies. Previously the activity of TP was only measured during axenic fruiting by Wannet *et al*. (1999) and not in *A. bisporus* sporophores grown commercially.

5) The SUT1 antibody could be used to localise the sugar transporter in the sporophore using fluorescent labelled antibody and confocal microscopy.

6) Examine the *SUT1* gene transcript in fruit bodies which have been subjected to matric and osmotic water stresses and quantify the sugar and polyol content of the sporophores to determine if there is an increase in the polyol content under both stresses and how this correlates with the *SUT1* transcript level in these sporophores. Stoop & Mooibroek (1998) found that the MtDH transcript increased in salt-stressed sporophores (grown under osmotic stresses by the addition of 150 mM NaCl to the casing soil) and this would be as a result of the increase in mannitol concentration in salt stressed sporophores to create an osmotic potential gradient. However it is likely that the *SUT1* transcript would increase in sporophores grown under osmotic stress but possibly show lower levels in sporophores grown under matric stresses.

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APPENDIX 1

COMPOSITION OF BUFFERS AND MEDIA

All chemicals used were supplied by Merck BDH unless otherwise stated. Water used in media and buffer preparation was provided by the Elga Maxima ultra pure water system.

A1.1 MEDIA COMPOSITION

LB medium

Composition	Amount per litre
Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g

Compost Extract (CE)

Phase II mushroom compost was baked in an oven at 150/80°C for 3-4 hours and then ground to a fine powder and 690 g was added to five litres of boiling ultrapure water and allowed to simmer for 1 hour. The boiled compost was then allowed to cool and strained through Miracloth (Calbiochem Co.) into 2 litre jugs. It was then centrifuged at 5,000 g for 20 minutes at 5°C and the supernatant was placed in 400 ml beakers and kept at -20°C until required.

5X Complete Yeast Extract Media (CYM)

Composition	Amount per litre
Mycological Peptone (Oxoid)	10 g
Yeast extract (Oxoid)	10 g
D-Glucose	100 g
$MgSO_4$-$7H_2O$ (Sigma)	2.5 g
KH_2PO_4	2.3 g
K_2HPO_4	5 g

The above reagents were dissolved in ultra pure water and 40 ml aliquots were placed into 50 ml Greiner sterile tubes and stored at -20°C.

Malt yeast peptone glucose (MYPG) broth

Preparation for 1 litre

Composition	Amount per litre
Malt extract (Oxoid)	10 g
Yeast extract (Oxoid)	4 g
Mycological Peptone (Oxoid)	4 g
D-glucose	4 g
$MgSO_4$-$7H_2O$ (Sigma)	0.5 g
KH_2PO_4	0.46 g
K_2HPO_4	1 g

A1.2 BUFFERS AND SOLUTIONS

All buffers and solutions were dissolved in ultrapure water and where stated were sterilised by autoclaving at 121°C and 15 psi for 15 minutes.

5X Tris-borate EDTA (TBE)

Composition	Amount per litre
Tris base (Invitrogen)	54 g
Boric acid (Sigma)	27.5 g
0.5 M EDTA pH8	20 ml

All the reagents were dissolved in ultrapure water and sterilised by autoclaving.

5X DNA loading dye

Composition	Amount per 100 ml
0.25% (w/v) Bromophenol blue (Fisons)	0.25 g
50% (w/v)Glycerol (Sigma)	50 ml

All the above reagents were dissolved in ultrapure water and stored at room temperature.

20X SSC

Composition	Amount per litre
3 M NaCl	175.3 g
0.3 M Tri-sodium citrate	88.2 g

All the reagents were dissolved in ultrapure water and the pH was adjusted to 7 using concentrated HCl and then autoclaved.

50X Denhardts

1% (w/v) Ficoll (Type 400, Pharmacia), 1% polyvinylpyrrolidine (PVP) (Sigma) and 1% (w/v) bovine serum albumin (Fraction V) (Sigma).

Southern pre-hybridisation solution

Stock concentration	per 30 ml/final concentration
20X SSC	9 ml/(6X)
50X Denhardts	3 ml/(10X)
10% (w/v) SDS	1.5 ml/(0.1%)
herring sperm DNA (10mgml^{-1}) (denatured at 100°C for 5 minutes and snap cooled on ice for 5 minutes)	300 μl/(100 μgml^{-1})
sterile distilled water	16.2 ml

Diethylpyrocarbonate (DEPC) treated water

DEPC (Sigma) 0.1% (v/v) was added to ultrapure water and was incubated overnight and then autoclaved and incubated at 55°C for 3 hours to remove all traces of DEPC and high temperatures reduce DEPC to ethanol and CO_2.

RNA extraction buffer

Stock concentration	Amount per 100 ml/final concentration
2 M sodium acetate (NaAc)	10 ml/(200 mM)
0.5 M EDTA	2 ml/(10 mM)
10% SDS	10 ml/(1%)
0.5% (v/v) β-mercaptoethanol	0.5 ml
DEPC water	78 ml

10X 3-N-morpholinopropanesulphonic acid (MOPS)

Composition	Amount per 100 ml
0.4 M MOPS	8.4 g
2 M NaAc	5 ml
0.5 M EDTA	2 ml

The volume was adjusted to 90 ml using DEPC water and the pH to 7 using 1M NaOH

5X RNA loading dye

Composition	Amount per 50 ml
50% (v/v) glycerol (Sigma)	25 ml
0.5 M EDTA	0.1 ml (1 mM)
0.4% (w/v) bromophenol blue (Fisons)	0.2 g

All of the above reagents were dissolved in DEPC water and stored at room temperature.

20X SSPE

Composition	Amount per litre
3.6 M NaCl	58.44 g
0.2 M NaH_2PO_4	28.4 g
0.2 M EDTA	7.44 g

All the reagents were dissolved in DEPC water and the pH was adjusted to 7.4 using 1m NaOH and then autoclaved.

Northern pre-hybridisation solution

Composition	Amount per 30 ml/final concentration
20X SSPE	7.5 ml/5X
formamide	15 ml
50X Denhardts	3 ml/5X
10% SDS	1.5 ml/0.5%
herring sperm DNA ($10mgml^{-1}$) (denatured at $100^{\circ}C$ for 5 minutes and snap cooled on ice for 5 minutes)	0.3 ml/100 μgml^{-1}
DEPC water	2.7 ml

A1.3 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Polyacrylamide gels were prepared using Bio-Rad Mini Protean II assembly apparatus (see manufactures instructions for gel assembly protocol).

Volumes necessary for two 12 % SDS polyacrylamide gels of 0.75 mm in thickness are detailed below.

12% Separating gel

To prepare 10 ml of monomer

Composition	Amount per 10 ml
Distilled water	3.35 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10% (w/v) SDS	100 μl
Acrylamide/Bisacrylamide (37.5:1), 30% stock (Sigma)	4 ml
10% ammonium persulphate (Sigma)	50 μl (0.05%)
Tetramethylethylenediamine (TEMED) (Sigma)	5 μl (0.05%)

Ammonium persulphate 10% solution was freshly prepared by dissolving 0.1 g in 1 ml of distilled water.

4% Stacking gel

Composition	Amount per 10 ml
Distilled water	6.1 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml
10% (w/v) SDS	100 µl
Acrylamide/Bisacrylamide (37.5:1), 30% stock (Sigma)	1.3 ml
10% ammonium persulphate (Sigma)	50 µl (0.05%)
(TEMED) (Sigma)	10 µl (0.1%)

5X Tris-Glycine

Composition	Amount per litre
Tris base (Invitrogen)	15 g
Glycine	72 g

The reagents were dissolved in 850 ml of distilled water and the pH was adjusted to 8.3 using 1 M HCl and the volume adjusted to 1 litre with distilled water and the buffer was stored at 4°C. To prepare a 1X solution of electrophoresis buffer for SDS-PAGE the stock solution was diluted with distilled water and SDS was added from a 10% stock solution to give a final concentration of 0.1%.

10X Tris buffered saline (TBS)

Composition	Amount per litre
NaCl	80 g
KCl	2 g
Tris base (Invitrogen)	30 g

All the reagents were dissolved in 800 ml of ultrapure water and the pH was adjusted to 8 using 5N HCL and then the volume was adjusted to 1L with ultrapure water and autoclaved. 10X TBS was diluted 1:10 with ultrapure water to prepare a 1X working solution.

TBS-Tween

To prepare 1L of TBS-Tween to 1X TBS 5 ml of 10% (v/v) Tween 20 (Sigma) was added to give a final concentration of 0.5% (v/v) Tween 20.

A1.4 WESTERN TRANSFER BUFFERS

Tris glycine pH 9.2

Composition	Amount per litre
48 mM Tris base (Invitrogen)	5.82 g
39 mM glycine (Sigma)	2.93 g
1.3 mM SDS	3.75 ml of 10% (w/v) SDS.
20% (v/v) Methanol	200 ml

All the reagents were dissolved in distilled water and the volume adjusted to 1 litre with distilled water and the buffer was pre-chilled at 4°C prior to use.

Carbonate buffer pH 10.7

Composition	Amount per litre
10 mM $NaHCO_3$	0.84 g
3 mM $NaCO_3$	0.318 g
20% (v/v) MeOH	200 ml
10% (w/v) SDS	10 ml

All the reagents were dissolved in distilled water and the volume adjusted to 1 litre with distilled water and the buffer was pre-chilled at 4°C prior to use.

A1.5 HIS-BIND RESIN CHROMATOGRAPHY BUFFERS

All His-Bind protein purification buffers were stored at 4°C and prepared as either 8X or 4X stock solutions in sterile water and diluted using ice cold sterile water to give 1X concentrations for use in protein purification.

8X Charge buffer

400 mM $NiSO_4 \cdot 6H_20$ (Fischer)

The appropriate amount of $NiSO_4$ was dissolved in sterile water and stored at 4°C.

8X Binding buffer

Composition	Amount per 500 ml
40 mM imidazole (Sigma)	1.36 g
4 M NaCl	116.88 g
160 mM Tris-HCl pH 7.9	80 ml 1M Tris-HCl pH 7.9

All the reagents were dissolved in sterile water and the volume adjusted to 500 ml.

4X Strip buffer

Composition	Amount per 200 ml
400 mM EDTA	29.78 g
2 m NaCl	23.4 g
80 mM Tris-HCl pH 7.9	16 ml 1M Tris-HCl pH 7.9

8X Wash buffer

A series of 8X wash buffers were prepared containing an imidazole gradient from 480 mM, 400 mM, 320 mM 240 mM, 160 mM, 80 mM.

Composition	Amount per 100 ml
480 mM imidazole	3.27 g
4 M NaCl	23.38 g
160 mM Tris-HCl pH 7.9	16 ml 1M Tris-HCl pH 7.9

All 8X wash buffers were prepared as 100 ml stock solutions in sterile water.

4X Elute buffer

Composition	Amount per 100 ml
4 M imidazole	27.32 g
2 m NaCl	11.69 g
80 mM Tris-HCl pH 7.9	8 ml 1M Tris-HCl pH 7.9

Dialysis tubing

Dialysis tubing contains chemical contaminants and needs to be pre-treated prior to use. These were removed as follows: The dialysis tubing 25.4 mm (Medicell International) was cut into the required strips and boiled in 10 mM $NaHCO_3$ (Sigma) and 1 mM EDTA for 45 minutes after which the dialysis tubing was rinsed thoroughly in distilled water. It was then stored in a beaker covered with distilled water containing 1 mM EDTA at 4°C.

APPENDIX 2

PRIMER SEQUENCES

All primers were synthesised by Sigma Genosys

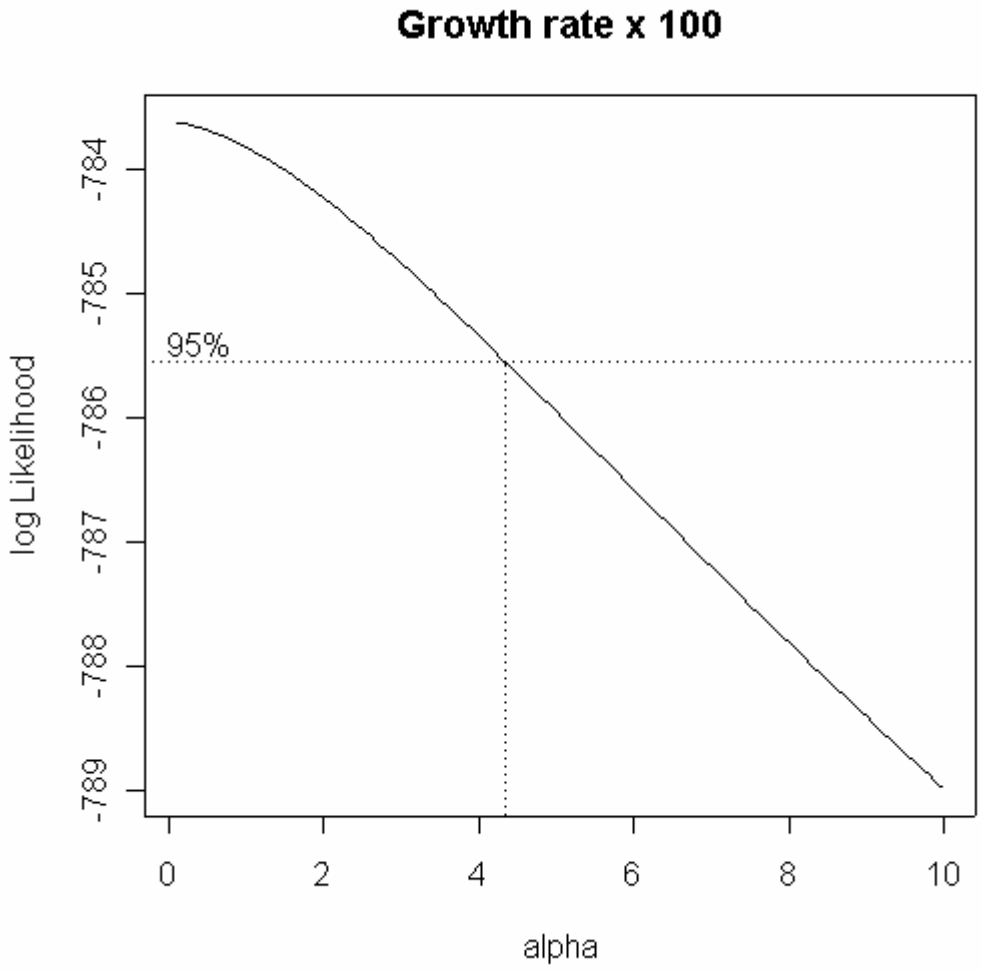
Primer title	Sequence 5'- 3'	Description of use
ST1P1	F:5' GGCAGGTTCATTTCTGGTATCG 3' R:5' GGAGTGTTCCAGTACAGCGC 3'	PCR a 0.11 kb non-specific region (highlighted in Figure 5.5) of SUT1.
ST1P2	F:5' GGAGTGTTCCAGTACAGCGC 3' R:5' CAAACGGATGAAGTTTCCTTC 3'	PCR a hydrophilic C-terminus region (highlighted in Figure 5.5) which is highly specific to SUT1.
ID4PINT	F:5'GCAAGTATCCTTTTGACGCTGGCTCATCCCTTGTTTGCTGATGGTTAATTTGTAG 3' R:5'CTACAAATTAACCATCAGCAAACAAGGGATGAGCCAGCGTCAAAAGGATACTTGC 3'	Oligos of 55 bp intron (highlighted in Figure 5.1) from STID4 cDNA clone and used as a probe in Southern and Northern hybridisations.
SUT1-C	F:5'CATGGATCCGAGAGCGAGACGTCATCATTCGT 3' R:5'AGACTCGAGTCAAACGGATGAAGTTTCCTT 3'	To PCR *SUT1*-C terminus to include *Bam*HI and *Xho*I restriction sites for cloning into pET-43.1c(+) vector

pET-43.1c(+)	F:5’ CGGTGACGAAGCGACTAGTG 3’	Sequence and positively identify a recombinant pET-43.1c(+)::*SUT1*-C
20A11 INTF	F:5’ GGATGTTGATCATCCTAGTG 3’	For PCR amplification
20A11 INTR	F:5’ ATGGTTAGGTGCAACCTCTC 3’	and sequencing of an internal region of SUT1 cosmid 20A11 to fill a gap in sequence assembly.
Vector primer T3(forward)	5’AATTAACCCTCACTAAAGGG 3’	Sequencing of SUT1 cDNA STIIE2
Vector primer T7(reverse)	5’GTAATACGACTCACTATAGGGC 3’	
ID4F1	5’GGTTCATTTCTGGTATCGGTG 3’	
ID4R1	5’CGGTCCAACATAATTGGCGT 3’	
ID4F2	5’CGGGTACAAATGCCATCAACC 3’	
ID4R2	5’GGAAGAACATCACTAGCGGTC3’	
ID4F3	5’GGACCGTATCAAATATGGC 3’	
ID4R3	5’ GCTATGATGAACAAGAGGC 3’	
STGF	5’ AGGCCTTATAGGAGGCATACTCTCGC 3’	Sequencing of *SUT1*
SPGF1	5’ GCGACTAACAGTGGTCTGATGC 3’	cosmid
SPGF2	5’ GCGGCAAGGATGTTGATCATCC 3’	20A11
SPGF3	5’ CCGCTCTTGAACAGACCAA 3’	
STGF4	5’ GGTTCATTTCTGGTATCGGTG 3’	
STGF5	5’ CGGGTACAAATGCCATCAACC 3’	

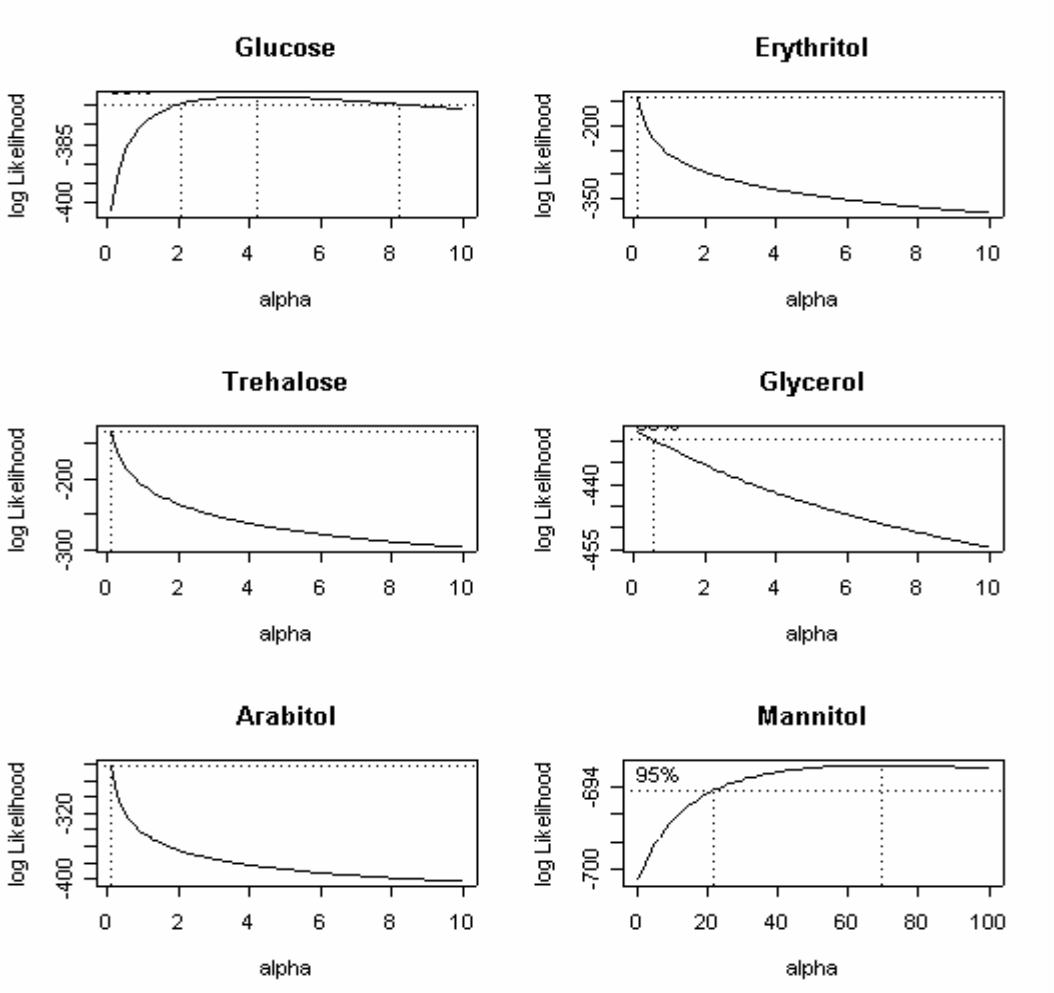
STGF6	5’ CAGTTGTTGGATGAGCAAAG 3’
STGF7	5’ GGCTATCGTATCTTCCGATC 3’
STGR	5’ ATAGTCCTAGGGACACACTC 3’
STGR1	5’ CGGTCCAACATAATTGGCGTC 3’
STGR2	5’ GGAAGAACATCACTAGCGGTG 3’
STGR3	5’ CCAGCATCAGACCACTGTTAG 3’
STGR4	5’ TGACCGTAGCGAGAGTATGC 3’
STGR5	5’ CACTAGTGTCGACCTGCAGG 3’
STGR6	5’ CCTCAGATATGGGAATCCGA 3’
STGR7	5’ CTTTGCTCATCCAACAACTG 3’

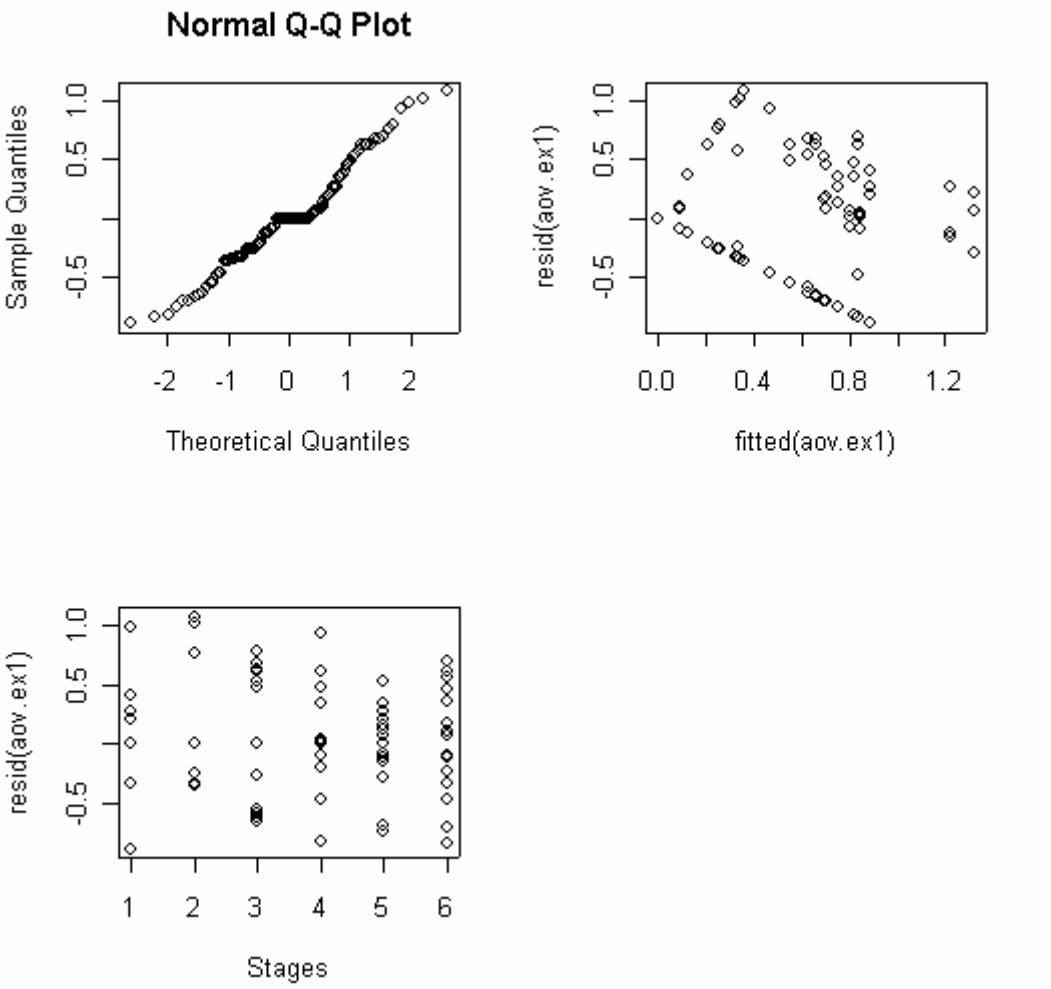
APPENDIX 3

A3.1 Alpha programme for the best value of alpha to use.

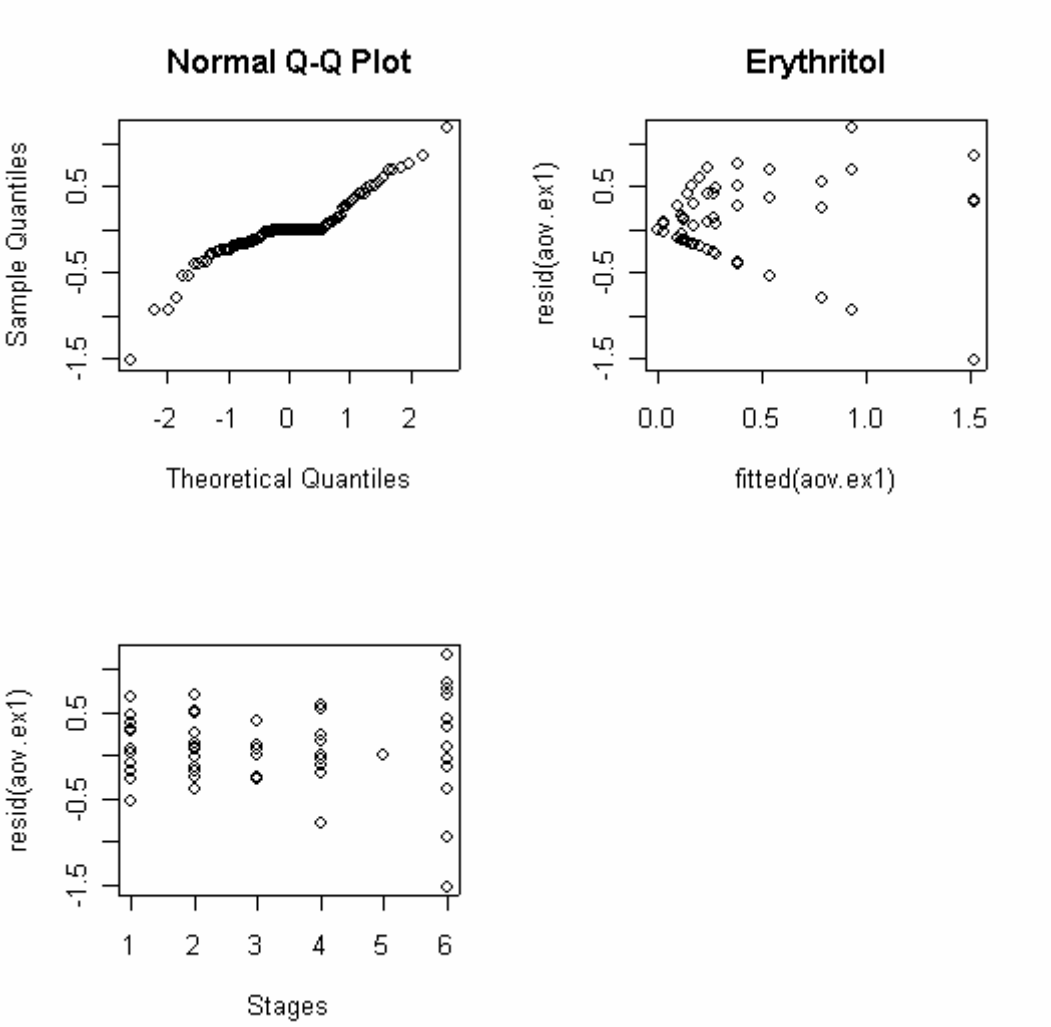


A3.1: The log transformation model for each of the following sugars: trehalose and glucose and polyols: glycerol, arabitol, erythritol, arabitol and mannitol.

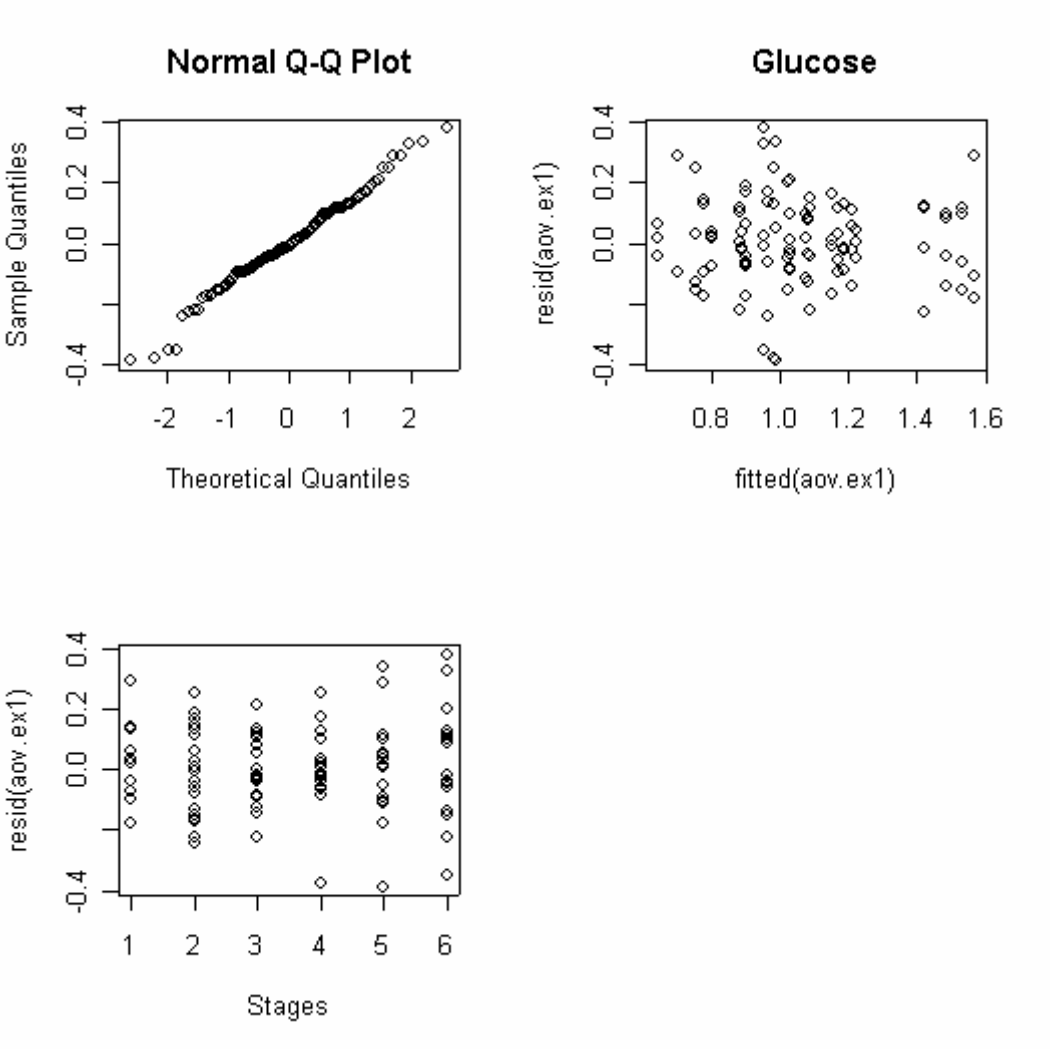




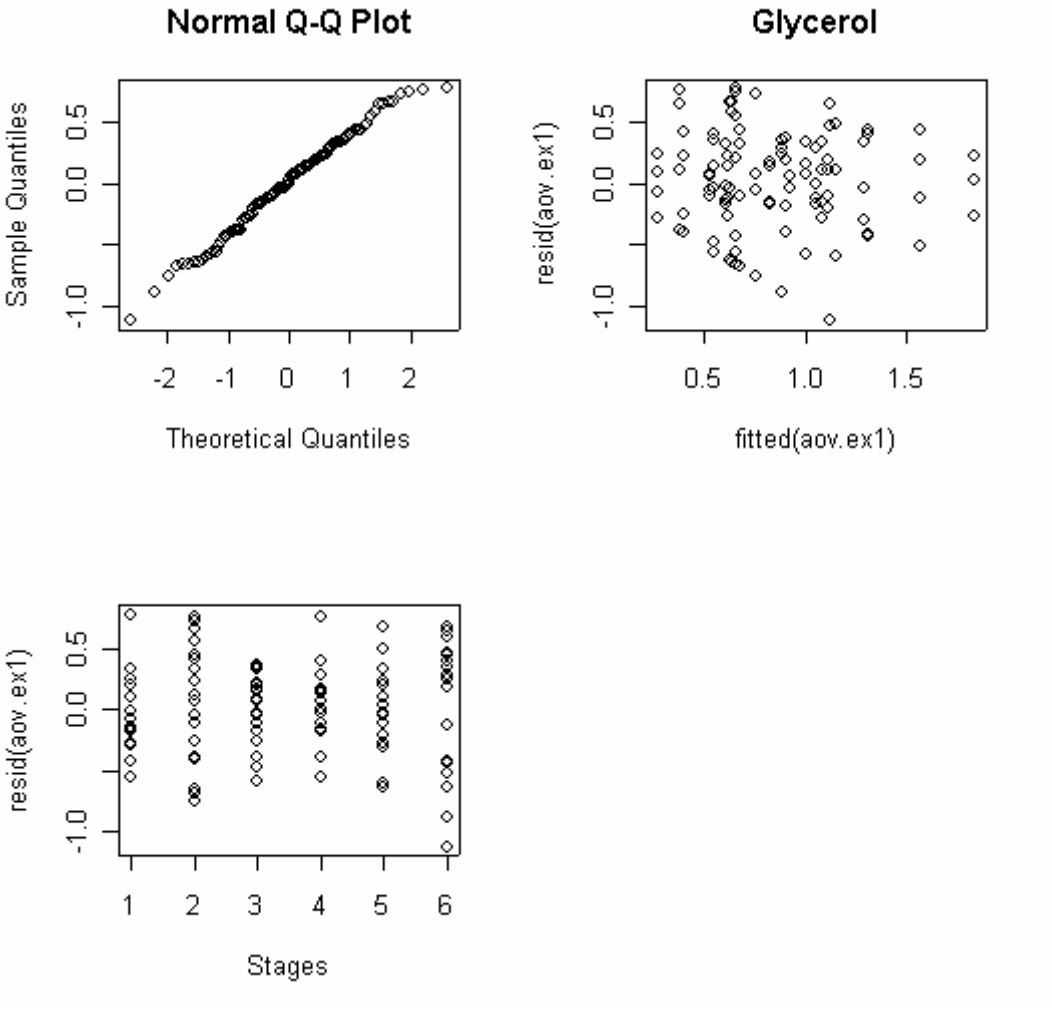
A3.2 Arabitol residual plot.



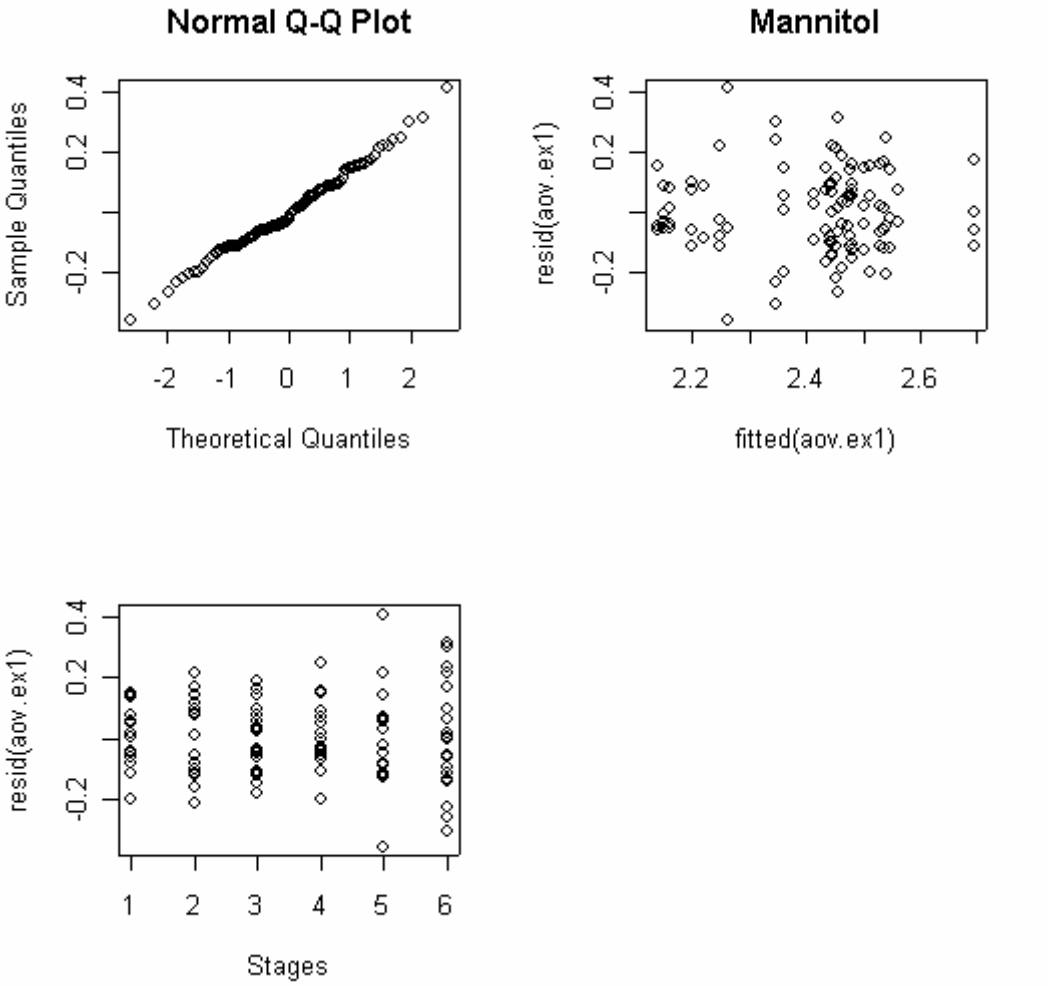
A3.3 Erythritol Residual plot.


Normal Q-Q Plot
Sample Quantiles
Theoretical Quantiles
-2
-1
0
1
2
-0.4
0.0
0.2
0.4
Glucose
resid(aov.ex1)
fitted(aov.ex1)
0.8
1.0
1.2
1.4
1.6
resid(aov.ex1)
Stages
1
2
3
4
5
6

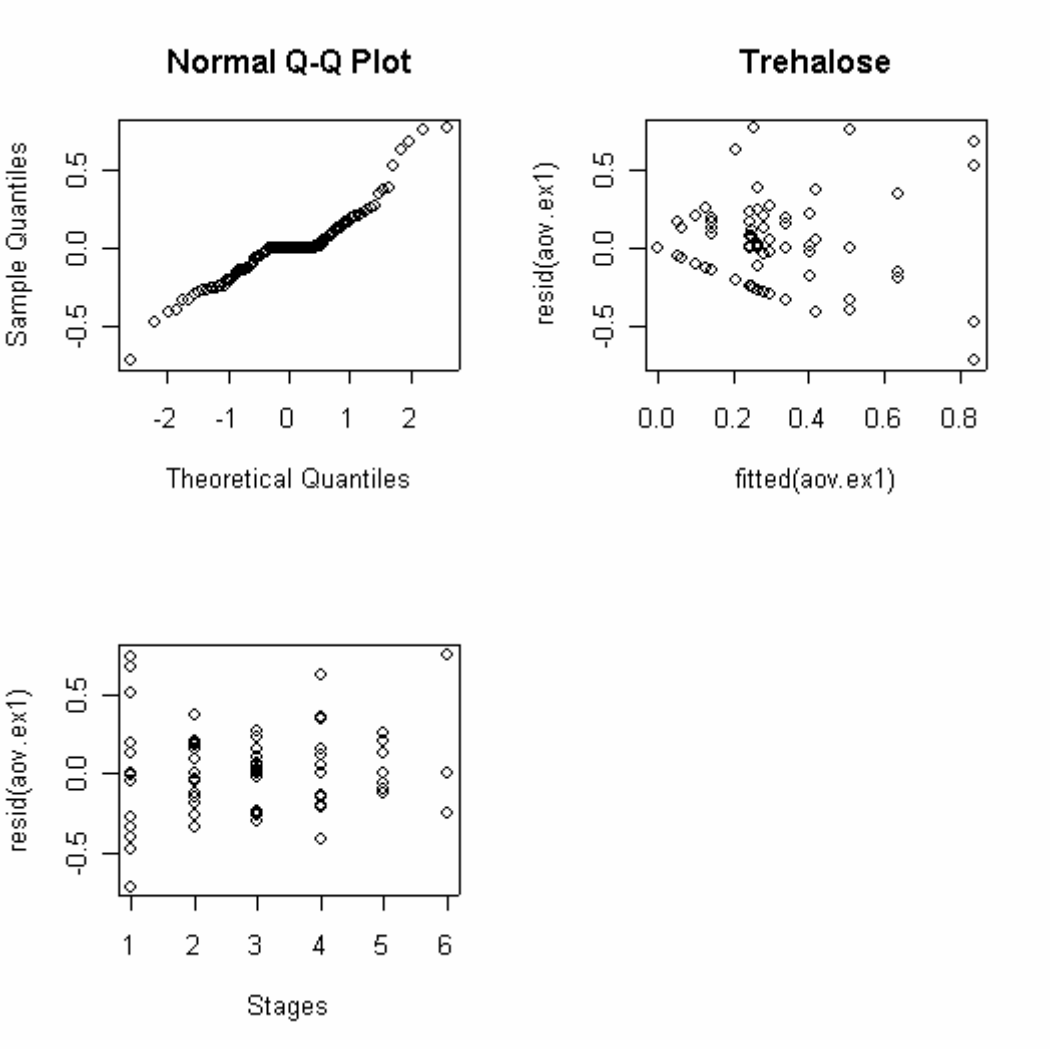
A3.4 Glucose



A3.5 Glycerol



A3.5 Mannitol



A3.6 trehalose