CRANFIELD UNIVERSITY

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ECOPHYSIOLOGICAL APPROACHES TO PRODUCTION AND FORMULATION OF THE BIOCONTROL YEAST *PICHIA ANOMALA*

Institute of Bioscience and Technology

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Ithaca

When you set out on your journey to Ithaca,
pray that the road is long,
full of adventure, full of knowledge […]

Always keep Ithaca in your mind.
Arriving there is what you're destined for […]

Ithaca gave you the marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.
And if you find her poor, Ithaca won't have deceived you.
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithacas mean.

K. Kavafis (1911)

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ABSTRACT

To produce commercial biocontrol agents (BCAs) successfully, it is important that cheap and economic substrates are used which support high numbers of good quality inoculum. Production of formulations conserving ecological competence and shelf-life should also be ensured. With this in mind, studies focusing on yeast ecophysiology were conducted to produce and formulate ecologically competent *P. anomala* cells for controlling spoilage of moist cereal grain. The liquid culture systems used were synthetic, nutrient yeast dextrose broth (NYDB), and a complex (industry byproduct, cane molasses) media. Manipulation of cultural conditions by means of imposing water-stress with several solute additions to the media had an impact on yield, cell water potentials (Ψ_c), viability and endogenous sugar/polyol accumulation. Glucose addition resulted in higher yeast yield (6.15 and 3.4 mg cell ml^{-1} medium for NYDB and molasses, respectively). Water activity (a_w) modifications of the media resulted in modification of Ψ_c so that $\Psi_c \leq \Psi_w$ (medium water potential). The change in yeast Ψ_c was attributed to the intracellular accumulation/synthesis of polyols, mostly glycerol and arabitol and sugars, mostly trehalose. In molasses-based medium cells accumulated/synthesized trehalose [32 mg g^{-1} fresh weight (f.w.) yeast cell]. Higher amounts of endogenous trehalose (up to 140 mg g^{-1} f.w. yeast cell) were retained intracellularly when modified yeast cells were isotonically washed compared to those subjected to hypo-osmotic shock by washing with water. The pattern was similar for endogenous arabitol. Trehalose retention doubled and quadrupled, and arabitol increased by 65 and 100% in proline and NaCl treatments respectively. The molasses control medium gave high [>10^{10} colony forming units (CFU) ml^{-1}] cell viability, which was further increased by addition of NaCl and proline ($\approx$ 3x10^{10}CFU ml^{-1}).

Fluidised bed drying of yeast cells showed that drying at 50°C for 20 min resulted in high cell viability (67%) and low moisture content (7%). Osmoprotection and several carriers and adjuvants affected viability and moisture content. Cotton seed flour (CSF) + 10% skimmed milk (SM) resulted in the highest cell protection (74%) during the drying process, with a final moisture content of about 5% and this was easy to resuspend. Storage stability of the formulation was 50% at 4°C and ambienta temperatures for up to 150 days. *P. anomala* cells grown in NaCl

modified molasses-media, when osmoprotected, retained four times more trehalose and resulted in significantly increased survival after drying and storage stability for 150 days. When SM + sucrose at 10% (w/v) was used as a protective solution, *P. anomala* cells were highly resistant to freezing, thawing and freeze drying processes. Storage stability at 4°C of freeze dried *P. anomala* cells was particularly high (>86%) over a period of 150 days while storage at 22°C resulted in a rapid decrease in cell viability to <35% over a period of 30 days. Osmoprotection using post-harvest isotonic washing treatment had no effect on storage stability. *P. anomala* produced high β-(1-3)-glucanase (>2.2 activity units), low chitinase (<0.9 activity units) and β-glucosidase (<3 nmol 4-nitrophenol min^{-1} μg^{-1} protein of specific enzyme activity) amounts. The role of the first hydrolase in biocontrol activity is possibly important while that of the other two is not clear for *P. anomala*. In lab-scale bioassays using wheat grain under aerobic conditions, populations of *P. verrucosum* 22625 were significantly reduced by formulated *P. anomala* cells at both 0.93 and 0.95 a_w levels while OTA production was significantly reduced at 0.93 a_w only.

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1 Chapter 1 LITERATURE REVIEW

1.1 BIOLOGICAL CONTROL IN GENERAL

Biological control is the practice in which, or process whereby, the undesirable effects of an organism are reduced through the agency of another organism that is not the host plant, pathogen or man (Deacon, 1983). The first attempt to use micro-organisms to control plant diseases (Hartley, 1921) used antagonistic fungi to control damping-off of pine seedlings. Since then, biological control using microbial antagonists has attracted much interest as an alternative to/or combined with chemical methods of horticultural crops (Janisiewicz, 1988a; Wilson & Chalutz, 1989; Wilson & Wisniewski, 1989; Buck, 2004; Qin & Tian, 2004). Although much research on biological control has been carried out in the last two decades, very few products based on antagonistic micro-organisms to control plant diseases have been commercialised. There are several technical, socio-economical and political reasons, for the paucity of Biological Control Agents (BCAs); the most important of them are:

- ➢ Availability of cheap and effective fungicides which can easily be applied;
- ➢ BCAs' efficacy is not always consistent;
- ➢ BCAs control a narrow spectrum of diseases; thus small niche-markets exist for individual BCAs each having specific requirements;
- ➢ Ecological constrains in plant parts/surfaces do not favour survival and activity of introduced BCAs;
- ➢ Formulation and distribution of BCAs are regarded as far more difficult than those for chemicals;
- ➢ Absence of a universally accepted biopesticide registration procedure;
- ➢ Need for education of growers and extension workers;
- ➢ Problems in cost-effectively producing BCAs.

However, fungal BCAs have a good perspective of use as some chemical pesticides are banned (e.g. organochlorines) or will be withdrawn (methyl bromide, by 2010) and as cases of microbial resistance to chemicals occur worldwide (Butt *et al.,* 2001). Nowadays, there is an increasing public concern about environmental matters and healthier food products; as a result, several Acts such as the Food Quality Protection

Act (FQPA) and Federal Food Drug and Cosmetic Act (FFDCA) (Whipps & Lumsden, 2001) have already been signed. Some European Union Members (Netherlands, Denmark, Sweden) have decided to reduce chemical inputs into agriculture by 50%, while in U.K. and U.S.A., there are organic farming certification standards ensuring minimal chemical use. These low chemical input products are interestingly sold at premium prices (Whipps & Lumsden, 2001). Moreover, Integrated Pest Management (IPM), which is an approach of crop protection that combines multiple crop protection practices, low chemical input, careful monitoring of pest and pathogens and the use of their natural enemies, is gradually being adopted by farmers. BCAs can be a key component of IPM (Whipps & Lumsden, 2001, Karabulut & Baykal, 2004).

The procedure for developing a BCA includes (Butt *et al.,* 2001):

➢ Isolation-*in vitro* screening. However, inhibition *in vitro* does not always result in inhibition *in vivo* (Pusey & Wilson, 1984)

➢ Studies on the ecology, physiology, taxonomy of a BCA to meet registration requirements.

➢ Laboratory and field bioassays. Ecological fitness is of great importance as it determines the niche where the BCA will be able to survive, reproduce and exert biological control.

➢ Economic mass production.

➢ Formulation and shelf-life.

➢ Application strategies.

➢ Risk assessment (very important for registration.)

➢ Training processes.

1.2   MODES OF ACTION OF BCAs

Understanding the modes of action of effective antagonists is important for improving their performance through the development of formulations enhancing the expression of useful traits, establishing screening criteria for searching for such traits in the selection of new potential BCAs and for registering the antagonists for commercial use (Droby & Chalutz, 1994). Mechanisms that play a significant role in biocontrol of these antagonistic mechanisms are (Wilson & Wisniewski, 1989; Droby *et al*., 1991; Droby & Chalutz, 1994; Castoria *et al.,* 1997):

➢ Competition for space and nutrients. In order to demonstrate that nutrient competition is a mechanism of antagonism, it is essential to show that the plant pathogen involved requires an external source of nutrients to penetrate the living host.

➢ Antibiosis; production of diffusible substances with fungicidal of fungistatic action.

➢ Activation of host defences; stimulation of phytoalexins (scoparone and scopoletin) production when *C. famata* was inoculated in orange wounds (Arras, 1996).

➢ Mycoparasitism; destruction or alteration of the hyphae of the pathogen, involving physical contact and predation, followed by enzymatic lysis. Extracellular glucanases and chitinases possess antifungal activity and are involved in mycoparasitism (Castoria *et al.,* 1997; Jijakli & Lepoivre, 1998).

Despite the efforts devoted to elucidating the mode of action of BCAs, no one major mechanism is predominant in their biocontrol activity. It is likely that there are multiple interactions between antagonist, plant, pathogen and other components of natural microflora (Droby & Chalutz, 1994).

1.3 ECOLOGICAL CONSTRAINS IN THE PHYLLOSPHERE

The phyllosphere, which refers to the aerial surfaces of the plants and leaves in particular, is a heterogeneous and dynamic environment where conditions (including temperature, water availability, nutrient availability and radiation) vary both temporally and spatially. Water availability in the phyllopshere is of great importance for a BCA as it affects antagonist-pathogen interactions (Whipps & Magan, 1987). However, conditions alternate between a surfeit of free water and drought (Dickinson, 1986) resulting in intermittent growth of micro-organisms and thereby posing problems of survival during dry periods (Blakeman & Fokkema, 1982). Therefore, BCAs must be ecologically adapted to the environment where they are expected to work. Thus, they need to be able to survive after application, tolerate environmental stress, promptly take advantage of favourable environmental conditions and retain activity as long as required. Moreover, they should be able to overcome the action of the indigenous and competitive microflora and withstand agrochemical presence.

Members of the epiphytic microflora of the phyllosphere, e.g., filamentous fungi and yeasts, seem the most appropriate antagonists for use in the field (Blakeman & Fokkema, 1982). In any case, ecological fitness of BCAs should be taken into serious consideration when developing a BCA.

1.4   POST-HARVEST BIOLOGICAL CONTROL

1.4.1   BCAs post-harvest

The confined environment for the storage of harvested commodities provides a great opportunity for applying BCAs since:

> ➢ Temperature and humidity are stable and controllable (Spotts & Sanderson, 1994);

> ➢ BCAs are easy to apply, they act on more concentrated plant parts;

> ➢ Value of the harvested crops is great enough to justify expensive control procedures (Ippolito & Nigro, 2000).

For these reasons, it is generally believed that biological control by means of microbial antagonists may have a greater potential for success when applied after harvest than before (Wisniewski & Wilson, 1992). However, storage conditions are designed to maintain a high quality of plant products during storage and should not be changed to accommodate the antagonists. Thus, from the beginning antagonists should be selected to be compatible with storage conditions. However, the factors affecting the BCA can be manipulated (Janisiewicz & Bors, 1995).

Desirable characteristics of a BCA for control of post-harvest diseases are (Droby *et al.,* 1991):

> ➢ Absence of antibiotic production.

> ➢ Effectiveness against a wide range of post-harvest pathogens.

> ➢ Compatibility with commonly used fungicides and waxes for the control of major post-harvest diseases.

> ➢ Survival and high efficacy under storage conditions (low temperature and high relative humidity).

> ➢ Non-pathogenicity to the host.

> ➢ Ease of multiplication and handling.

> ➢ Indigenousness to the plant environment (ability to colonise the plant surface for long periods of time under various environmental conditions and ability to use nutrients and proliferate rapidly).
>
> ➢ Genetic stability.

1.4.2 Yeasts as BCAs post-harvest

At present, yeasts appear to be promising BCAs, either providing alternatives or combined with chemical fungicides in the post-harvest storage of plant products (Arras, 1996; Chand-Goyal & Spotts, 1996; Piano *et al.*, 1997; Teixidó *et al.*, 1998c; Fan & Tian, 2001, Qin & Tian, 2004). This potential benefit can be attributed to several properties of yeasts:

> ➢ Yeasts are tolerant to extreme environmental conditions of storage (temperature close to 0^oC, high relative humidity) and are adapted to high sugar concentrations, high osmotic pressure; they are also tolerant of low pH (Janisiewicz, 1991) and of low partial pressures of O_2. Large amounts of extracellular polysaccharides contribute to their survival (Janisiewicz, 1988a). Extracellular polysaccharides also offer protection against UV radiation by forming cell capsules or accumulating around the colony as slime **(Dickinson, 1986).** Production of the slime on the phylloplane appears to be a mechanism by which *A. pullulans* adheres to leaves **(Andrews *et al.*, 1992).**
>
> ➢ Yeasts do not produce mycotoxins or allergenic spores (Janisiewicz, 1988a).
>
> ➢ Yeasts have a favourable surface-to-volume ratio (in contrast to mycelial fungi) and can utilize available nutrients, increase in number and colonise the surface (particularly at the wound site) very rapidly (Janisiewicz, 1988a).
>
> ➢ Pesticide impact on yeasts is minimal; moreover they are able to fill a niche after "negative alternation" (niche created after destroying non-target microflora by pesticides; Janisiewicz, 1991).
>
> ➢ Their genetics are relatively well known and thus they can be manipulated through genetic engineering (Janisiewicz, 1991).
>
> ➢ The main modes of action appear to be nutrient competition, direct interaction with the pathogen and induction of host defence (Droby *et al.,* 1994); since production of antibiotics seems not to be involved.

> ➢ Yeast activity may be enhanced by nutritional manipulation e.g. nisin (El-Neshawy & Wilson, 1997); L-proline (Nunes *et al.*, 2001); sodium salts (Ippolito & Nigro, 2000); calcium salts (Nunes *et al.,* 2001); 2-deoxy-D-glucose (El Ghaouth *et al.*, 2000).

> ➢ Finally, the vast majority are not pathogenic to humans or other animals; thus public acceptance of yeasts as organisms applied directly to consumed commodities assures them of a better chance than other micro-organisms of more questionable ingestive properties (Janisiewicz, 1988a).

Control of *B. cinerea* and *P. expansum* on apples by *Cryptococcus albidus* (Saito) Skinner alone was directly related to the population of antagonist **(**Fan & Tian, 2001**)** and agreed with previous reports **(**Janisiewicz, 1988b; Björnberg & Schnürer 1993). Moreover, the use of the BCA in their study was found to be compatible with several post-harvest practices including cold storage, fungicide and Ca^{+2} treatment, thus making feasible an integrated control strategy under commercial conditions **(**Fan & Tian, 2001).

In 1995, *Candida oleophila* Montrocher was registered for post-harvest biocontrol as Aspire^{TM} biofungicide by the Environmental Protection Agency in the United States (El-Neshawy & Wilson, 1997). The product efficiently inhibits growth of *Penicillium.* and *Botrytis* spp. on citrus and pome fruits. *C. albidus* is commercially produced under the name Yield*Plus*® (Whipps & Lumsden, 2001). The recent product Shemer ®, registered, in Israel is based on a newly identified yeast *Metscnikowia fructicola*, (Kurtzman & Droby, 2001) and is claimed to be effective against a wide range of pathogens of grape, strawberry, and sweet potato (Ädel-Druvefors, 2004).

1.5   MASS PRODUCTION OF INOCULA

1.5.1   Solid-substrate fermentation (SSF)

Both solid and liquid fermentation systems have been used for the mass production of biocontrol agents (Lewis & Papavyzas, 1991). Solid substrate fermentation (SSF) is the oldest method used (Hesseltine, 1977) and it involves the growth of microorganisms on moist solid substrate in the absence of free-flowing water. The solid substrates act as a heterogeneous source of carbon, nitrogen, and minerals as well as growth factors, and they have a capacity to absorb water (Murthy *et al.*, 1993). SSF offers the greatest possibilities when fungi are used as their growth habit in nature is to colonise solid substrates such as pieces of wood, seed, stems, roots, leaves of plants and drier part of animals (Hesseltine, 1977). SSF has been used for the production of some BCAs, such as *Talaromyces flavus* (Fravel *et al.*, 1985). It offers advantages for small scale production by easier sterility maintenance and by the fact that the production of a formulation may be incorporated into the growth phase (Graham-Weiss *et al.*, 1987). It does, however, present great difficulty in scale up especially when the organism requires the addition of nutrients during the growth stage (Powell, 1992).

1.5.2   Liquid fermentation

In liquid fermentation the microorganism grows in submerged culture in a medium that supplies nutrients for growth. Liquid fermentation offers the advantages of a sophisticated and longstanding technology developed originally for production of microbial products (drugs, organic acids, enzymes) rather than the microorganisms. It is used for the production of bacteria (Costa *et al.,* 2001), yeasts (Abadias *et al.,* 2001b) and for certain fungi such as *Trichoderma* and *Gliocladium* (Lewis & Papavyzas, 1991). It is generally agreed that liquid fermentation is preferred to solid fermentation. Indeed, it is much easier to establish as a homogeneous system, mixing is accomplished by the design of the system, addition of nutrients can be done simply, heat can be easily removed and environmental conditions changed at will (Powell, 1992). Lappa (1979) compared solid culture with surface liquid culture and submerged fermentation of *Beauvaria* and *Metarhizium* spp., and found that

submerged culture is by far the most economical in time, productivity and cost of consumables.

1.5.3   Media used in liquid fermentation

Media used in liquid fermentation varies from synthetic defined media to complex media based on industrial by-products. On a large scale, media should allow a maximum concentration of biomass and a high quality culture to be produced at a low price (Lewis, 1991). In some cases, the medium needs to have a specific nutrient composition to meet the above criteria. However, from an industrial point of view it is important to produce microbial biomass using inexpensive means. A suitable medium should consist of inexpensive, readily available agricultural by-products, such as molasses, brewer's yeast, corn steep liquor and sulphite waste liquor (Lewis & Papavyzas, 1991). However, by-products are not as standardised as purified products and they may contain impurities that will need to be removed before fermentation. Moreover, their composition may vary according to the season and origin (Costa *et al.*, 2001).

Molasses has been widely used in the production of microorganims (Abadias *et al.*, 2000; Abadias *et al.*, 2001b; Costa *et al.,* 2001). The low cost of molasses allows its concentration to be increased in media without reducing its suitability for commercial use. However, high concentration of the substrate (40 g l $^{-1}$) appears to be toxic for BCAs (Costa *et al.*, 2001).

1.6   MODIFICATION AND QUALITY OF INOCULA

1.6.1   Concept of water activity and water potential

Microorganisms all require a source of water to enable cellular functioning to occur effectively. They all have a semi-permeable cell membrane, which allows water molecules to enter the cell through osmosis to come to equilibrium with its environment. However, under water scarcity conditions, either due to the presence of a high concentration of salts or in intermediate moisture agricultural products, cells must physiologically be able to adjust to such osmotic stress to be able to grow and reproduce (Magan, 1997).

Scott (1957) was the first to identify the importance of water availability and try to relate this to the total water content of substrates. He suggested that water activity (a_w) would best describe the water availability for microbial activity. 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. Thus:

$$a_w = P/P_o$$

The water activity of pure water is 1. A substrate containing no free water has a smaller vapour pressure than pure water and its a_w is consequently less. An alternative measure to a_w is that of water potential Ψ_w (in Pascals, Pa). Ψ_w is defined as the amount of work that must be done per unit quantity of pure water in order to transport reversibly and isothermally an infinitesimal quantity of water from a pool of pure water at atmospheric pressure to a point in a system under consideration at the same point elevation (Griffin, 1981). Ψ_w is the sum of the pressure potential (Ψ_p, the potential of water per unit volume as affected by external pressures; in filamentous fungi it represents the turgor potential of the protoplasm created by plasma membranes and cell walls), solute (or osmotic) potential (Ψ_s, the potential of water per unit volume as affected by the presence of solutes), and matric potential (Ψ_m, the potential of water per unit volume as affected by the presence of a solid matrix). Thus,

$$\Psi_w = \Psi_p + \Psi_s + \Psi_m$$

Ψ_w is related directly to a_w by the following formula:

$$\Psi_w = (RT/V)\log_n a_w$$

where R is the ideal gas content, T the absolute temperature and V_w is the volume of 1 mole of water. The advantage of Ψ_w is that it is possible to partition osmotic and matric components and their influence on growth and physiological functioning of microbes. Soil microbiology studies use water potential, while for solid substrates, where solute potential is the major force, a_w is commonly used. The relationship between a_w and Ψ_w is shown in Table 1.1.

Table 1.1. Water activity, equilibrium relative humidity and water potentials at 25°C (Magan, 1997).

Water activity	E.R.H. (%)	Water potential (-MPa)
1.00	100	0
0.99	99	1.38
0.98	98	2.78
0.97	97	4.19
0.96	96	5.62
0.95	95	7.06
0.90	90	14.50
0.85	85	22.40
0.80	80	30.70
0.75	75	39.60
0.70	70	40.10
0.65	65	59.30
0.60	60	70.30

The microorganisms that are able to tolerate and actively grow under conditions of water stress have been described by the terms halophilic, osmophilic, osmotolerant, xerotolerant or xerophilic. The two most appropriate terms for fungi are probably osmophilic, which describes specialised groups of yeast which are able to grow in high salt environment, and xerophilic (Magan, 1997). Pitt (1975) defined a xerophile as a fungus that is able to grow in some phase of its life cycle at –22.4 MPa (0.85 a_w), and this has now become generally accepted. Anand & Brown (1968) suggested that osmotolerant yeast species generally had slower growth rates than non-osmotolerant ones, with the former having a broad a_w range for growth, and the latter having a very sharp narrower optima.

Experimentally, the water potential has been simulated by modifications of media using ionic or non-ionic solutes: NaCl, KCl, glucose, glycerol, sorbitol and proline for the osmotic potential (Hallsworth & Magan, 1994a, Abadias *et al.,* 2000); polyethylene glycol (6000 – 8000 mw) for the matric potential (Beecher & Magan,

2000). Modifications of carbon concentration of media used can also have a significant influence on the water stress imposed on the fungus. This close interaction has seldom been recognised and in most cases, it has been ignored (Hallsworth & Magan, 1994b).

1.6.2 Osmoregulation and compatible solutes

Growth under water stress due to osmotic or matric potential effect requires the maintenance of cell turgor for cell functioning, growth and reproduction to occur. A shift to a high osmotic/water potential affects nutrient uptake, protein biosynthesis and a number of enzyme activities. In order to respond, fungi need to osmoregulate. They need to adjust their internal Ψ to a level lower than that of the immediate environment. Thus, they produce organic solutes often polyhydric alcohols (polyols), organic acids or sugars that make an important contribution to the protoplasmic osmotic potential, but at the same time have minimal effect on the confirmation of proteins. These solutes are called compatible solutes and accumulate in protoplasmic and vacuolar spaces in fungal propagules (Brown & Simpson, 1972). Uptake and accumulation of external stress solutes represent an alternative option to osmoregulation, in particular when the external osmoticum has the characteristics of a compatible solute (Blomberg & Adler, 1992).

1.6.3 Polyols and the importance of glycerol

The polyols include glycerol, arabitol, erythritol and mannitol but not all of them may occur in one species. In several filamentous fungi that produce a spectrum of polyols, the total polyol concentration appears to be constant for any one low osmotic potential. However, the ratios of the various polyols can vary within the total (Jennings, 1995). Ratios of polyols are found to be affected by culture age, temperature, pH (Hallsworth & Magan, 1996) and by the solute used to modify Ψ_w (Van Zyl & Prior, 1990; Teixidó *et al.*, 1998a, b; Abadias *et al.*, 2001).

Polyols are believed to play other roles besides that in osmoregulation in cell physiology. These are:

➢ Slow metabolism and ultimate use in respiration or conversion to higher M_r (molecular weight) compounds such as glycogen (Kiyosama, 1991)

> ➢ Alternative source of reducing power (Jennings, 1984)
>
> ➢ Co-enzyme regulation (Jennings, 1984)

Some compatible solutes may be involved in energy spillage through being involved in futile cycles. Futile cycles occur when two compounds can be interconverted by two different irreversible reactions. When the enzymes responsible for both reactions are functional there is a futile cycling in which there is energy spillage as heat. Energy spillage is important under stress conditions as cell synthesis is curtailed (Jennings, 1995). The suggested metabolic cycles that result in energy dissipation in fungi are:

> ➢ The mannitol cycle in many fungi
>
> ➢ A glycerol cycle in *D. hansenii* growing in saline conditions.

Energy spillage through futile cycles and variation of polyol ratios offer a metabolic flexibility to fungal cells (Jennings, 1995).

Van Eck *et al.* (1993) gave recognition to the ability of the lower molecular weight (M_r) polyol glycerol to increase intracellular viscosity more effectively than higher M_r polyols. Moreover, carbon chain length of polyols was found to correlate positively with inhibition of yeast NADP-specific isocitrate dehydrogenase (Brown 1976); thereby lower M_r polyols act as more compatible solutes (Chirife *et al.*, 1984). Hallsworth & Magan (1994a) found that increased water stress favoured accumulation of the lower M_r polyols and resulted in decrease of higher M_r mannitol accumulation.

However, polyols have different abilities to depress intracellular a_w and this difference may, in part, account for the accumulation of lower M_r polyols in mycelia or propagules of cultures grown at reduced a_w. Mannitol is the least soluble and a saturated solution has an a_w of 0.978 at 25 °C (Chirife *et al.*, 1984) meaning that mannitol would not be able to depress intracellular Ψ sufficiently when external potential is < 0.978 a_w. Mannitol is, however, known to act as a compatible solute (Brown, 1978; Jennings & Burke, 1990). Arabitol is freely soluble and a saturated solution has an a_w of 0.819 (Chirife *et al.*, 1984). The solubility of erythritol is limited; a saturated solution has an a_w of 0.914 (Hallsworth & Magan, 1994a). Glycerol is miscible with water and, at a given concentration, a glycerol solution has a slightly lower a_w than that of erythritol (Hallsworth & Magan, 1994a). For this reason

it seems likely that accumulation of lower M_r polyols, erythritol and glycerol, can confer greater osmotic tolerance than polyols such as mannitol (Hallsworth & Magan, 1994b).

In yeasts, polyols are the main compatible solutes and their accumulation is related to the yeast species, the growth phase of the yeast (Nobre & da Costa, 1985) and the carbon source used for growth (Van Eck *et al.*, 1989). Moreover, significant excretion of polyols has been reported during yeast growth (Van Eck *et al.*, 1993). Glycerol is the predominant polyol accumulated and arabitol the minor one (Edgley & Brown, 1978). However, different yeasts have different patterns of glycerol use as a compatible solute. These are:

➢ Occurrence of both synthesis and sretion of glycerol that results in an approximately same ratio between intra- and extracellular glycerol concentration (Edgley & Brown, 1978).

➢ Synthesis and accumulation of glycerol due to a lower permeability of the plasma membrane in the osmotolerant yeast *Zygosaccharomyces rouxii.* In general, during osmotic stress, the permeability of the yeast membrane to glycerol and other solutes may be reduced by changes in the phospholipid composition (Tunblad-Johansson & Adler, 1987)

➢ An active transport of glycerol into the yeast cell via a carrier-mediated system with a high specificity for glycerol in *Z. rouxii* and *D. hansenii*. Direct glycerol uptake has been reported in several other cases by yeast cells subjected to water stress (Blomberg & Adler, 1992; Teixidó *et al.,* 1998b; Abadias *et al.*, 2001b).

Glycerol is of particular importance as it is able to protect hydrated biopolymers and allow structural integrity under low water potential conditions (Magan, 1997). The key in the glycerol production in *S. cerevisiae* appears to be glycerol-3-phosphate dehydrogenase (GDPH). Blomberg & Adler (1992) reported that this enzyme exhibits high controlling capacity in glycerol formation. The two genes *GDP*1 and *GDP*2, encoding this enzyme activity were later cloned and characterised (Larsson *et al.*, 1993; Albertyn *et al.*, 1994), although only *GDP1* is induced by osmotic stress (Eriksson *et al.*, 1995). In the case of *S. cerevisiae* GPD1 gene expression was independent of the type of stress solute used (Eriksson *et al.,* 1995). In the yeast *C.*

sake stress caused by different solutes resulted in different levels of glycerol production (Abadias *et al*., 2001b). This may be due to differences in glycerol retention when different solutes are used to modify the osmotic potential (Van Eck *et al.,* 1993).

Glycerol accumulation varies temporally and this is in close relation with its metabolic role inside the cell. In xerotolerant fungi (*Chrysoporium fastidium* and *Penicillium chrysogenum*) glycerol accumulated rapidly during active phases in mycelial growth but depleted significantly when sporulation occurred (Hocking, 1986), partially due to the use of energy reserves for the production of conidia. However, Ramos *et al.* (1999) found that significant accumulation of glycerol occurred both in mycelium and conidia of the xerophilic spoilage fungus *Aspergillus ochraceus*, perhaps indicative of a survival response. Similar results have been obtained with *Epiccocum nigrum* (Pascual *et al.*, 1998). In yeasts, the glycerol content of *D. hansenii* was found to increase during the lag and early exponential phases and decreased again during the late exponential phase (Adler & Gustafsson, 1980). Abadias *et al*. (2001b) found that glycerol was higher when cells reached the stationary phase and then maintained or decreased slightly.

1.6.4   Sugars and the importance of trehalose

Glucose is a monosaccharide and thus a readily utilisable sugar converted to pyruvate by glycolysis during fungal metabolism. Glucose is the substrate for synthesis of the polyols mannitol and glycerol (Jennings, 1995).

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is a non-reducing disaccharide composed of two molecules of glucose linked at their 1-carbons. Although barely detectable in exponential growth of yeast on glucose, trehalose accumulates to remarkably high levels (up to 20% of the dry weight of the cell) in stationary-phase cells as well as in exponential-phase cells exposed to high temperatures (Singer & Linquist, 1998). In general, trehalose accumulates in the cells in response to a number of stress conditions, especially heat and osmotic stress (Meikle *et al.,* 1991; Parrou *et al.*, 1997). The enzymes catalysing the synthesis of trehalose (trehalose-6-phosphate

synthetase and trehalose-6-phosphatase) are localised in the yeast cytosol, where production of the sugar occurs in two steps (Blomberg, 2000).

Trehalose is an important sugar as it protects cells against high temperatures by stabilising proteins (Singer & Lindquist, 1998) and against cellular desiccation by helping to both stabilise proteins (Allison *et al.*, 1999) and to maintain membrane integrity (Crowe *et al.*, 1984). In the latter, it replaces water in dehydrated phospholipid membranes (the 'water replacement hypothesis'). This inhibits transition of the liquid crystalline phase to the gel phase and in doing so preserves the cell membranes. Trehalose concentrations of greater than 10% have been found to be critical for stress resistance to freezing and freeze-drying of the industrial yeast *S. cerevisiae* (Van Dijk *et al.*, 1995). It has been suggested that trehalose enhances desiccation tolerance of conidia of the BCA *T. harzianum* (Harman *et al.,* 1991). However, Hallswoth & Magan (1995) showed that elevated trehalose concentrations in conidia of entomopathogenic fungi did not improve stress tolerance at lower water availability, although they did prolong shelf-life.

1.6.5   Modification of inocula

Hallsworth & Magan (1994a; b) demonstrated that it is possible to physiologically manipulate growth conditions, carbon sources and carbon:nitrogen ratios to channel specific low M_r polyols (glycerol and erythritol) into spores of filamentous fungi. Studies with *Gliocladium roseum*, used for control of a range of soil-borne and foliar diseases, showed that the total sugars and glycerol accumulated in conidia does vary with temperature and level of water stress (Magan, 2001). Furthermore, by using glycerol, glucose or trehalose as the major carbon source the relative proportions of sugars and polyols varied significantly. Studies with the BCAs *Epiccocum nigrum*, *Candida sake* and *Penicillium frequentans* have all recently demonstrated that the endogenous contents of sugars and polyols can all be significantly modified using this approach (Teixidó *et al.,* 1998a; Pascual *et al.*, 1999; Pascual *et al.*, 2000; Abadias *et al.*, 2000; Abadias *et al.*, 2001). Other cases of physiological manipulation of compatible solutes include *Ulocladium atrum* and *Agaricus bisporus* (Beecher & Magan, 2000; Frey & Magan, 2001).

1.6.6   Efficiency of inocula *in vitro*

The previous modifications of the content of propagules have to be translated into improved viability *in vitro* over a wide range of environmental stress conditions and improved biological control *in vivo* in comparison to unmodified inocula. Cells of the yeast *C. sake* used for control of *Penicillium* rot of apples modified with either glucose or glycerol showed a mixture of predominantly glycerol, arabitol and trehalose with much lower concentration of mannitol and erythritol. These modified cells were significantly more tolerant over a range of water availabilities (0.95 to 0.93 a_w) than unmodified yeasts cells predominantly containing intracellular arabitol (Teixidó *et al.*, 1998b). Studies with BCAs such as *Beauvaria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus* showed that glycerol-modified inocula produced conidia containing more glycerol and erythritol than those from unmodified inocula. These conidia were associated with high germination rates both at high and reduced a_w levels (Hallsworth & Magan, 1995). Jin *et al.* (1991) reported that *T. harzianum* produced at low water potential had increased desiccation tolerance, which was correlated to higher trehalose concentration in conidia. Studies by Jackson & Bothast (1990) and by Jackson & Schisler (1992) have also pointed to variation in C:N ratios and limitation as a means of improving desiccation tolerance of mycoherbicides *(Colletotrichum truncatum)* and the entomopathogenic fungus *P. farinosus* .

In contrast, in modified conidia of BCAs such as *E. nigrum* and *Ulocladium atrum*, no improvement in germination was achieved under water stress conditions. This suggests that such larger heavily pigmented spores from harsher phyllosphere environments may already be to a large extent evolved to be tolerant of environmental stress (Magan, 2001). Moreover, modified *C. sake* cells showed significant differences in compatible solute accumulation but no significant changes in viability (Abadias *et al.,* 2001b). Consequently, the effect of ecophysiological manipulation on endogenous accumulation of reserves varies with fungal species and groups, and the type of modification needs to be appropriate for the needs.

1.6.7 **Efficiency of inocula** *in vivo*

Studies with pre-harvest spraying of modified *C. sake* cells on apples have demonstrated that low-a_w tolerant yeast cells gave better post-harvest control of *Penicillium* rot and that the low a_w-tolerant inocula could be applied at a lower concentration to obtain the same efficacy (Teixidó *et al.*, 1998b). This could be important for the development of economic production, formulation and application systems for BCAs. Application of *E. nigrum* inocula to peach twigs in the field also demonstrated that better control of brown rot could be achieved than that obtained with the unmodified inocula of *E. nigrum* or the fungicide captan (Pascual *et al.*, 1996). Studies of biological control of *Monilinia laxa* by *P. frequentans* showed that inocula modification was not effective as the initial advantage of modified inocula was quickly lost (Pascual *et al.*, 2000).

1.7 FORMULATION OF BCAs

In order for biological control products to be used as alternatives to fungicide they need to meet several requirements concerning the economical feasibility of their use. One of the major obstacles to the commercialisation of BCAs is the development of appropriately formulated products. Formulation technology must be considered at all stages from production of a BCA to its eventual action on the target. In any case it has to be understood that formulation of BCAs has special requirements that are different from conventional fungicides. In general, there are four basic functions of formulation of BCAs (Fravel *et al.*, 1998):

> ➢ Stabilisation of the organism during production, distribution and storage, so that viability is retained throughout the above process;
> ➢ Aid in handling and application of the product so that it is easily delivered to the target in the most appropriate manner and form;
> ➢ Protection of the BCA from harmful environmental factors at the target site, thereby increasing persistence;
> ➢ Enhancement of activity of the BCA at the target site by increasing activity, reproduction, contact and interaction with the target organism.

Minuto *et al.* (1995) demonstrated that the development of a suitable formulation and delivery system could greatly increase the performance of antagonistic *Fusarium* spp. against *Fusarium* wilt of cyclamen.

Formulation generally consists of the active ingredient (the BCA), a solvent or carrier (inert ingredients in the sense that they do not have pest control capabilities but can affect shelf–life and efficacy of the product; Fravel *et al.*, 1998) and adjuvants (compounds that assist or modify actions of the active ingredient; Foy, 1989). Inert carriers include fine clay, peat, vermiculite, alginate or polyacrylamide beads and should be inexpensive, easily sterilised, non-toxic and consistent in physical properties **(**Boyetchko *et al*, 1998). Tank-mix adjuvants are used world-wide and the main ways in which they enhance ultimate biological performance are by increasing the amount of active ingredient retained by the target and by promoting its uptake (Fravel *et al.*, 1998). Moreover, adjuvants increase the population and survival rate of the BCA used (Ippolito & Nigro, 2000).

The maintenance of BCAs in an active or viable form is a big technological hurdle as microorganisms are easily inactivated or killed by unfavourable conditions during the formulation process, drying being one of them (Kirshop & Doyle, 1991). However, there is the example of Bio-Save 11, a commercially available product formulated as a dry wettable powder (10% active ingredient, *Pseudomonas syringae* strain ESC-11), which was as effective as the fresh cell preparation; this indicates that full biological control potential was achieved with the commercial formulation (Janisiewicz & Jeffers, 1997).

For a biological control product to be commercialised, a shelf-life (stability in terms of viability and efficacy) of at least one year, preferably 2 or 3 years, is required (Rhodes, 1993). While specialised resting structures and spores designed for survival under adverse environmental conditions are already environmentally tolerant, other types of inocula, in particular yeast cells, require other treatments to be preserved. These can be the use of several additives (mainly protectants and carriers) and a drying process (Wraight *et al.*, 2001). Suspension in oil is another alternative as it avoids BCA respiration (Butt *et al.*, 1998).

During drying**,** surface, intercellular and some free intracellular water is removed, followed by removal of intracellular water bound to proteins, membranes, amino acids but in slow rates. In yeasts this occurs at approximately 15-20 % water content (Beker & Rapoport, 1987). Metabolic processes still occur but at a very low rate and after

rehydration of tissue, BCAs recover and resume normal activity (Aguilera & Karel, 1997). However, in some cases dehydration causes irreversible membrane damage (Lievense *et al.,* 1994), thereby resulting in very poor viability (Kirshop & Doyle, 1991). Others suggest that osmotic shock is the major contributory factor in cell death during dehydration (Harrison & Cerroni, 1956). Dehydrated cells have the advantage of not requiring cool temperatures during storage and distribution and thus make the product more economic (Costa *et al.*, 2000).

The drying temperatures have an impact on inoculum viability. In the case of *S. cerevisiae*, drying cells at 80°C in a fluidised bed dryer resulted in a 10-fold lower viability than using 30°C to reach to the same dehydration point (Bayrock & Ingledew, 1997a). Microbial cell survival during freeze-drying is dependent on microorganism concentration, protectants and the rehydration conditions (Costa *et al.,* 2000)

The role of polyhydroxy compounds (mainly trehalose and glycerol) in membane protection during dehydration is thought to be of great importance as these compounds:

> ➢ replace water in membranes during dehydration (Crowe *et al.*, 1984)
> ➢ lower the temperature at which a damaging transition in membranes occurs (Crowe & Crowe, 1986)
> ➢ act as compatible solutes and prevent unfolding and aggregation of proteins by hydrogen bonding with polar groups of proteins (Carpenter *et al.,* 1990)

The available ways of drying are:

> ➢ freeze drying (Kirshop & Doyle, 1991).
> ➢ spray drying (Dulmage *et al.*, 1990)
> ➢ fluidised bed drying (Bayrock & Ingledew, 1997a) and
> ➢ drying over silica gel (Kirshop & Doyle, 1991).

Freeze drying is the most convenient and successful method of preserving bacteria, yeasts and sporulating fungi (Berny & Hennebert, 1991). However, not all strains survive the process and, among those surviving, quantitative viability rates as low as 0.1% have been reported (Abadias *et al.,* 2001a); freeze drying affected severely the

viability of *E. nigrum* spores (Pascual *et al.,* 1999) and *C. sake* cells (Abadias, 2000). However, the use of high initial concentration (10^{10} CFUs ml^{-1}), of sucrose as a protectant and non-fat skim milk as a rehydration medium resulted in 100% viability of *P. agglomerans* to be conserved after freeze drying (Costa *et al.*, 2000).

Spray drying has the potential of becoming an alternative method for preserving microorganisms as it allows large quantities of cultures to be dried at low cost (Costa *et al.*, 2002). Several studies using the technique for practical application with lactic acid and other bacteria resulted in very poor viability. The main reasons for this appeared to be high temperatures and thermal stress that cause detrimental effects on microorganisms and non-thermotolerant enzymes (Hutter *et al.*, 1995; Costa *et al.*, 2002). However, high volume spray dryers that allow inlet temperatures to be decreased could significantly improve performance of this drying technique (Costa *et al.,* 2002).

Fluidised bed drying has been extensively used to manufacture active dry yeast (ADY) on a large scale **(**Bayrock & Ingedew, 1997). Larena *et al.,* (2003a) dried *E. nigrum* spores by using different drying methods and fluidised bed drying retained 100% viability of spores. Wraight *et al.* (2001) fluidised bed dried yeast cells treated with only one additive, an emulsifier, and showed that the temperature exceeds 50°C only when the dry-matter content was above 88% of the weight, about 10-45 min at the end of the drying process.

One of the most recent technologies for the formulation of antagonistic microorganisms is the immobilisation of wet or dry biomass within cross-linked polymers. For example, incorporation of fungal mycelium into alginate pellets has been found to be successful for the delivery of fungal BCAs (Papavyzas *et al.*, 1985; Knudsen & Bin, 1990; Knudsen *et al.*, 1990; Knudsen *et al.*, 1991). Pellets can be stored dry and then reactivated by rehydration. Cho *et al.* (1999) developed a procedure for the formulation of biocontrol fungi involving the immobilisation of wet biomass within gluten. The formulated biocontrol agents reduced the amount of biomass required, compared with non-formulated fungi. Other cases of formulation technology included the use of resistant polysaccharides such as xanthan gum (Mugnier & Jung, 1985) and fermenting BCAs on solid substrates that later become

the carrier (Paau *et al*., 1991). In the case of RootShield ^{TM} the entire biomass of *T. harzianum* is colonised on clay particles (Whipps & Lumsden, 2001).

1.8   SPOILAGE OF PERISHABLES

Living foods are biologically active to a greater or less degree, and plants or parts of plants have developed extremely effective defence mechanisms (e.g. physical and chemical barriers) against invasion by their natural predators, fungi. Fungal pathogens in turn, have sought evolutionary pathways to enable them to invade plant tissue. Post-harvest diseases develop on plant products during harvesting, grading and packing, during transportation to market and to the consumer, and while the product is in the possession of the consumer until the actual consumption.

Spoilage of living fresh foods can be divided into two categories:

> ➢ Fruit which are perishable unless rapidly processed
> ➢ Cereals which naturally tend to dry and become stable in the field or are artificially dried prior to storage and processing.

1.8.1   Spoilage of fruit

Losses of fresh fruits after harvest may reach very high values (25%, Droby *et al*., 1991**)** depending on the particular product (physiological age of the host and its defence mechanisms), on the pathogen and storage conditions (Agrios, 1997). Losses are particularly high in underdeveloped countries (Wilson & Wisniewski, 1989). Within the Italian market, losses after harvest may reach 8-10% of the harvested crop (Ippolito & Nigro, 2000). Losses can result from reduction in quality, quantity or both. Mycotoxin production is sometimes implicated (Filtenborg *et al*., 1996).

Fruits are usually quite acid, pH 2.2-5.0 (Splittsoesser, 1987) and thus quite resistant to invasion by bacteria. Microbial spoilage of fruit is almost always caused by fungi (Pitt & Hocking, 1997). Fruits have a moist-rich environment and become increasingly susceptible to fungal invasion during ripening, as the pH of the tissue increases, skin layers soften, soluble carbohydrates build up and defence barriers weaken. Changes in the nutritional status of fruit as the season progresses cause changes in microbial composition on the fruit surface (Davenport, 1976). The fruit

surface is an area of constant interaction among saprophytic and parasitic organisms and their products (Blakeman & Fokkema, 1982).

Infection of fruit by post-harvest pathogens often occurs in the field prior to harvest usually with inconspicuous symptoms, and continues to develop after harvest (Agrios, 1997). Defence mechanisms in fruit appear to be highly effective, as only a relatively small number of Ascomycetes and Mitosporic fungi and a few species of Oomycetes (*Pythium* and *Phytophthora* when the fruit is close to or in contact with the soil), two Zygomycetes (*Rhizopus* and *Mucor*) and two Basidiomycetes (*Rhizoctonia* and *Sclerotium*) are able to invade and cause serious losses. Most of them are necrotrophs that require exogenous nutrients for the initiation of the infection process (Janisiewicz & Jeffers, 1997). Depletion of these nutrients by antagonists at wound sites can lead to effective biocontrol (Droby *et al*., 1991).

Control measures include careful handling during harvest, cooling of the fruit after harvest, controlled atmosphere (O_2 <5%, CO_2 5-20%), heat treatment (Garcia *et al*., 1995) clean and disinfected storage containers and shipping cars, ventilation to ensure relative humidity control, along with disposal of infected fruit (Agrios, 1997). However, these beneficial practices are usually not sufficient to protect the product from fungal infection. Application of synthetic fungicides has been the primary means of controlling post-harvest diseases (Eckert & Ogawa, 1988). The chemicals commonly used are: diphenyl, sodium o-phenylphenate (SOPP), dichloran, vinclozolin, thiabendazole, imazalil, fluazinam, fosetyl-aluminium, chlorothalonil, thiram (dithiocarbamate), captan, carbendazim, dichlofluanid, benomyl. They are mostly applied as wash treatments, at temperatures of 28-50°C. Some are applied in paper sheets during storage and transport (Agrios, 1997). Fumigation with SO_2, hydrogen peroxide vapour, ozone and chlorine gass is also used (Ippolito & Nigro, 2000). However, pathogen resistance to fungicides (Holmes & Eckert, 1999) is a big problem. Thus, biological control provides a serious alternative (Wilson & Wisniewski, 1994), see also Stion 1.4.2.

1.8.2   Spoilage of cereal grains and mycotoxin production

Fungi are very tolerant of low water availability; thus causing serious problems in cereal grain spoilage. Damage caused can be:

➢ quantitative by loss of dry matter,

➢ qualitative by means of reduced germination and baking quality, formation of pathogenic or allergenic propagules and discoloration. However, mycotoxin production by fungi is of great importance (Lacey & Magan, 1991; Chelkowski, 1991).

Mycotoxins are sondary metabolites of filamentous fungi appearing to have no role in the normal metabolism involving growth of the fungus. Their molecules are small in size and they induce no response in the vertebrate animals immune system; thus they represent a major potential danger in the latter's diet when introduced via a natural route (Pitt, 2000). Mycotoxins have four basic kinds of toxicity, acute, chronic (so-called mycotoxicosis; Sweeney & Dobson, 1998), mutagenic and teratogenic (Pitt, 2000). The most important toxic effects are different kinds of cancers and immune suppression. More than 400 mycotoxins are known today, and some of them act synergistically. Mycotoxins are formed during growth of moulds on foods, some excreted in the foods (Filtenborg *et al*., 1996). Their role in plant pathogenesis has just started to be investigated (Desjardins & Hohn, 1997). The most important mycotoxins are shown on Table 1.2.

Even though fungal colonisation of grain may start in the field (field fungi) and can result in post-harvest losses, storage fungi are mainly responsible for grain spoilage (Pitt, 1975). In the more stable conditions of storage, water availability and temperature of grains along with the gaseous composition of the intergranular atmosphere determine which fungi will predominate. Climate at harvest influences the contamination of cereal grains with potential spoilage and mycotoxigenic moulds (Lacey & Magan, 1991; Miller, 1994). Present harvesting methods for grain are most efficient at slightly elevated moisture contents (17-20%) which means that grain needs to be effectively dried to 15% m.c. for safe storage. However, drying often takes a number of days, giving an opportunity to spoilage moulds to develop in layers of moist grain (Magan *et al.,* 2004).

The most characteristic fungi of the storage environment are species of *Aspergillus* and *Penicillium* genera (anamorphs). *Penicillium* species are in general psychrotolerant and under low ambient temperatures or through ventilation with cool air, will increase their predominance. *Penicillium* species are characterized by the so-called "*Penicillium* growth pattern" which is the complete colonization of a nutrient source with concomitant heavy sporulation (Filtenborg *et al.,* 1996). The most common species in wheat, barley and oats are *P. aurantiogriseum*, *P. chrysogenum, P. brevicompactum* and *P. crustosum* (Filtenborg *et al.,* 1996).

P. verrucosum is a specific wheat/barley pathogen in Europe and responsible for ochratoxin A (OTA) production (Sweeney & Dobson 1998). This mycotoxin appears to be a problem in north temperate growing areas particularly in Europe (Miller, 1994) and is entirely produced in storage. However, pre-harvest grain infestation by *P. verrucosum* is particularly important as it results in dissemination of conidia during combining **(**Sweeney & Dobson 1998). OTA can be produced over a wide temperature range, with an optimum at 20°C (Sweeney & Dobson 1998). In fact significant levels of OTA production can occur at 4°C and an a_w as low as 0.86. *P. verrucosum* grows from 0 to 31°C (Sweeney & Dobson 1998). It is a xerophile, capable of growth at an a_w of 0.80 (Pitt & Hocking, 1997); it grows in the pH range of 2.1 to 10, with an optimum of 6.0 to 7.0.

Penicillium roqueforti, P. carneum and *P. paneum* (previously belonging to one species, *P. roqueforti*; Boysen *et al.*, 1996) can colonise the cereal grains in malfunctioning airtight silos resulting in poor quality animal feed with potential contamination by mycotoxins (Peterssen & Schnurer, 1999). *P. roqueforti* can tolerate high CO_2 concentrations and its growth is only halved with CO_2 concentrations >15% or CO_2 concentrations <0.14%. Indeed it is reported to tolerate at least 80% CO_2 (Lacey & Magan, 1991).

Aspergillus species responsible for cereal grain losses include *A. flavus*, *A. candidus*, *A. ochraceus, A. niger, A. versicolor, A. penicillioides* (Pitt & Hocking, 1997) and, along with *Penicillium* species, require lower a_w for growth (Lacey & Magan, 1991). *A. flavus, A. parasiticus* and *A. nomius* produce aflatoxin (closely related species; Filtenborg *et al.,* 1996). *Eurotium* species, better known as members of *Aspergillus*

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glaucus group, and principally *E. chevalieri, E. repens E. amstelodami* along with *Aspergillus restrictus* are far more significant components in storage mycoflora in warmer/drier conditions since they are the most xerophilic invaders of grain (Lacey & Magan, 1991).

Predominantly field pathogens *Fusarium* spieces (*F. culmorum, F. graminearum* and *F. avenaceum*) occasionally develop in storage (wheat and rye grain) when a_w is high and temperature low, some of them producing the mycotoxins zearalenone, different trichothecenes, moniliformin and fumonisins. *F. culmorum* being predominant in cooler areas while *F. graminearum* in warmer areas (Miller, 1994). *F. moniliforme* and *F. proliferatum* are mainly field pathogens in maize causing fusarium kernel rot (Miller, 1994).

Methods to control storage moulds can be:

> ➢ High temperature drying; it employs the use of hot air and aims at reducing the relative humidity of the crop to a level at which the growth rate of moulds is very close to/or zero so that spoilage will be minimised during storage (Bruce & Ryniencki, 1991).

> ➢ Ventilation that aims at removing heat, moisture and CO_2.

> ➢ Moist grain storage. It is used in countries with a temperate climate where heated-air drying is costly and energy demanding. In this case airtight storage is used and relies on perfect sealing in combination with anaerobic conditions caused by grain and microorganisms respiration. Energy consumption is only 2% that of hot-air drying; however, imperfect sealing, daily temperature fluctuations and the frequent removal of grain contribute to air leakage to the silo atmosphere resulting in spoilage (Petersson & Schnurer, 1998; Petersson *et al.*, 1999).

> ➢ Modified atmosphere storage employing addition of gases, mainly CO_2 and N_2, useful in controlling mostly insts.

> ➢ Irradiation.

> ➢ Chemical Preservation by the use of acetic, propionic, iso-buturic and sorbic acids (Lacey & Magan, 1991); at the moment antifungal properties of several volatiles including plant extracts and essential oils are being investigated (Yin & Tsao, 1999).

Table 1.2. Spoilage fungi and production of mycotoxins (adapted by Pohland, 1993; Filtenborg *et al.*, 1996; Sweeney & Dobson, 1998)

Plant	Fungus	Mycotoxin	Way of acting	Association with disease
Cereal grain Stored conditions	*P. aurantiovirens* *P. cyclopium* *P. freii* *P. hordei* *P. verrucosum* *P. roquefortii* *P. aurantiogriseum* *P. viridicatum*	Ochratoxin A Ochratoxin A	Acute and chronic	Nephrotoxic,immunosup pressive, embryonic, urinary track cancer
	A. flavus *Eurotium* sp.	Aflatoxin	Acute and chronic	Liver damage, liver cirrhosis, carcinogenic, teratogenic, association with Hepatitis-B
Field condition	*F. culmorum* *F. graminearum* *F. avenaceum*	Deoxynivalenol (DON), nivalenol	Acute and chronic	Immunosuppression, anorexia, nausea, vomiting, headache, diarrhoea, convulsions
		Zearalenone	Chronic	Genital problems and reproductive disorders in pigs, pubertal changes in children
	Alternaria alternata *Claviceps purpurea*	Alkaloids	Chronic	Ergotism, disorders of nervous system, gangrene, gastrointestinal disorders
Citrus fruit	*P. digitatum* *P. italicum* *P. ulaiense* *Alternatia citri*	Alternaniol, Alternaniol monomethyl ester (AME),		
Pomaceous fruit	*P. expansum* *A. alternata*	Patulin, citrinin Alternaniol, AME		
Stone fruits	*P. expansum*	Patulin, citrinin		

1.8.3   Ochratoxin A (OTA)

Ochratoxin A (OTA) is the main mycotoxin in the group of Ochratoxins, and it appears to be the only one of toxicological significance. It has nephrotoxic, carcinogenic, teratogenic and immunosuppressive properties (Creppy, 1999) related to the isocoumarin moiety contained in its molecule (Figure 1.4). OTA has been classically associated, although inconclusively, to Balkan Endemic Nephropathy and has been recently related to human renal disorders in Egypt and Tunisia (Creppy, 1999).

OTA was first described as a metabolite of *A. ochraceus* (Wilhem) (van der Merwe *et al.,* 1965) and later of some closely related species from *Aspergillus* stion Circumdati (Ciegler, 1972; Varga *et al.,* 1996) and *P. verrucosum* (Pitt, 1987). More recently*, A. niger* var. niger (Abarca *et al.,* 1994) and *A. carbonarius* (Horie,1995) have also been described as OTA-producers. The origin of OTA in cool and temperate climates is generally attributed to *P. verrucosum*, whereas in warm temperate and tropical zones it is now commonly associated with *A. ochraceus* and the black aspergilli. OTA production depends on both environmental and processing conditions (climatic conditions, abnormally long storage, transportation, wet or dry milling, roasting procedures, fermentation etc.) (Anonymous, 2002). Moreover, OTA can be a very stable compound that may pass through industrial processing into consumer products such as wine and other food stuffs, without a large reduction in levels, (Krogh, 1973; Elling *et al*., 1975; Pietri, *et al.,* 2001).

Worldwide occurrence of OTA contamination of raw agricultural products has been amply documented. It occurs in a variety of plant products such as cereals and cereal-based foods, coffee beans, pulses, cocoa and spices. It has also been found in beer and in animal products such as pig meat, sausages and other meat products. OTA has also been detected in wine and grape juice (Majerus & Otteneder, 1996; Zimmerli & Dick 1996; Pietri, *et al*., 2001; Soleas *et al*., 2001; Battilani & Pietri, 2002). Human exposure is possible by direct consumption of contaminated plant products or indirectly, by consumption of meat or derived products from animals which were fed a diet containing OTA. OTA is frequently found in the blood of humans suggesting

continuous and widespread exposure to OTA (Peraica, *et al.*, 1999; Jørgenson *et al.,* 1996).

In humans, OTA is believed to be a genotoxic carcinogen so a maximum tolerable daily intake needs to be set. On the basis of OTA's nephrotoxicity Joint FAO/WHO Expert Committee on Food Additives (JEFCA) proposed a provisional tolerable weekly intake (PTWI) for OTA of 0.1 µg kg^{-1} body mass (equivalent to 14 ng kg^{-1} body mass day^{-1}) (WHO, 1996). However on the basis of carcinogenity data, the Working Group of the Nordic Council of Ministers proposed a maximum tolerable daily intake of 5 ng kg^{-1} body weight of toxin. In 1998, the Scientific Committee for Food of the European Commission suggested that it was prudent to reduce exposure to OTA as much as possible, "ensuring that exposures are towards the lower end of the range of tolerable daily intakes of 1.2-14 ng kg^{-1} bw day^{-1} which have been estimated by other bodies, e.g. below 5 ng kg bw^{-1} day^{-1}"(Anonymous, 2002).

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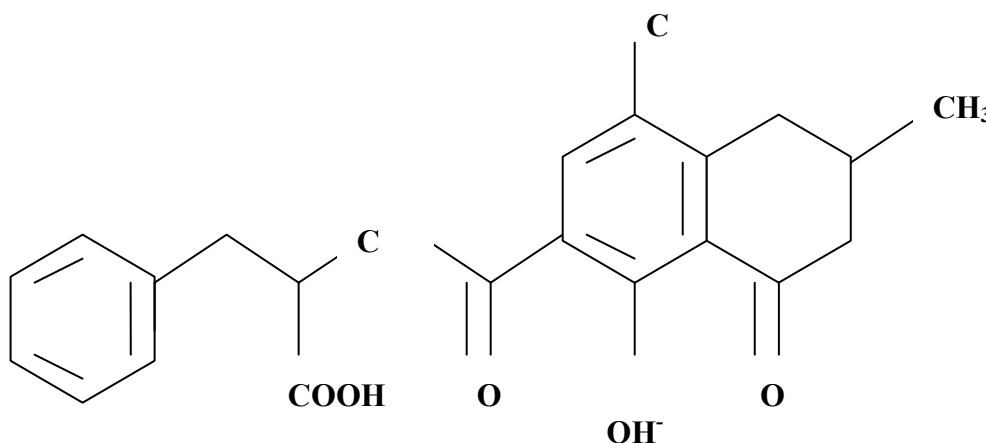
Figure 1.1. Chemical Structure of Ochratoxin A

1.9 BCA USED IN THIS STUDY: *Pichia anomala*

Anamorph: *Candida pelliculosa*

Synonym: *Hansenula anomala*

P. anomala was formerly known as *Hansenula anomala* (Kurtzman, 1984); however recent studies **(**Naumov *et al*., 2001**)** suggest that the previous genus name *Hansenula* should be used instead. *P. anomala* is found in berries, rubber, brewer's and baker's yeasts, beer, soft drinks, fruit juices, condensed beans, salt beans, molasses and can utilize a multitude of carbon sources (Barnett *et al*., 2000). In general, the yeast can grow at body temperature (Barnett *et al*., 2000) and in rare cases can be an opportunistic human pathogen (Nohinek *et al*., 1987**,** Moses *et al*., 1991**,** Yamada *et al*., 1995, Chakrabarti *et al*, 2001; Aragao *et al*., 2001). The species is xerotolerant and was shown to have protective ability against *Botrytis cinerea* on apples (strain K) with exo-beta-1,3-glucanase activity implicated and being suggested as a possible mechanism of action (Jijakli & Lepoivre, 1998) and on grape-vine *in vitro* plants (strain FY-102) causing morphological and physiological changes such as coagulation and leakage of the cytoplasm of *B. cinerea* (Masih *et al*., 2000).

P. anomala has been frequently isolated from airtight-stored grains (Lacey & Magan, 1991) and Björnberg & Schnürer (1993) first showed that strain J121 (originally isolated in 1981 from airtight-stored grains) effectively reduced growth of *P. roqueforti* and *Aspergillus candidus in vitro* in a dose-dependent manner; optimal T was 30°C for the production of viable yeast cells when a_w was 0.98 (min.4°C, max. 40°C) and optimal a_w was 0.98-0.99 when T was 25°C, with minimum being 0.86 a_w. Inhibition was more pronounced at temperatures suboptimal for growth and sporulation of the spoilage moulds, i.e. below 15°C and above 30°C. This fact indicated the potential use of the yeast as a BCA against grain-storage moulds. Subsequent studies both *in vitro* and in non-sterile high-moisture wheat grains (Petersson & Schnürer, 1995) together with studies in high moisture cereal grain in a test-tube version simulating a malfunctioning airtight storage system (Petersson & Schnürer, 1998) confirmed *P. anomala*' s ability to reduce mould growth and sporulation in cereals.

After *P. roqueforti'* s reclassification into three species, *P. roqueforti*, *P. carneum*, and *P. paneum* (Boysen *et al.,* 1996) studies on *P. anomala* biocontrol effect when three species were co-cultured in moist grain under restricted air supply, showed the yeast's fungicidal effect against all species without differences (Boysen *et al.,* 2000). Petersson *et al.* (1999) showed that the large-scale application of *P. anomala* to airtight stored high-moisture feed wheat could protect against post-harvest spoilage. *P. anomala* has also been shown to reduce ochratoxin A accumulation in co-culture with *P. verrucosum*, and toxin production, both in agar and wheat, was inhibited at levels of *P. anomala* lower than those inhibiting growth (Petersson & Schnürer, 1998).

Despite all studies, the mode of action of *P. anomala* J121 has not yet been elucidated. The yeast has been found to produce ethyl acetate (Wagener, 1973), a mould-inhibitory compound, but high concentrations are required for fungal inhibition (Petersson & Schnürer, 1998); moreover *P. roqueforti* is known to grow on high ethyl acetate substrate levels (Samson *et al.,* 1995). Volatile metabolites restricting radial growth and sporulation of moulds have been found to be produced by the yeast (Bjornberg & Schnurer, 1993). Fredlund *et al.* (2001) in her study on elucidating the yeast's mode of action, suggested that nutrient competition, is at least partly, implicated. However, Ädel-Druvefors (2004) who worked with the yeast against *P. roquefortii* in a grain mini silos system concluded that competition for nutrients does not significantly contribute to its biocontrol activity. *P. anomala* J121 produces killer toxins active against several yeast and mould species, including *P. roqueforti* (Ädel-Druvefors, 2004). However, several other yeasts that produce toxins do not exhibit any biocontrol activity. Killer toxins isolated from *P. anomala* have been reported to show considerable differences regarding amino acid composition, killer spectrum, optimum pH and temperature (Passoth & Schnürer 2003). However, killer toxins are mainly produced and active under acidic conditions and are adversely affected by pH and temperature increase; thus, the harsh environment in a mini silo rules out killer toxin production as a yeast biocontrol mode of action (Ädel-Druvefors, 2004).

Detailed studies have been carried out on the effect of water activity (a_w) and temperature of media on lag periods prior to growth, temporal growth rates and population increases for *P. anomala* J121 (S. Feretti, personal communication).

Nutrient yeast dextrose broth (NYDB) medium unmodified and modified with the ionic solute NaCl and non-ionic solute glycerol in the range 0.99 to 0.92 a_w were used. Studies showed that as a_w was decreased the temporal increase in biomass decreased. Generally, *P. anomala* was more sensitive to ionic than non-ionic solute. For example, at 0.92 a_w there was a long lag phase with very little growth observed, even after 70 h growth. At a_w levels of 0.99, 0.98 and 0.96, regardless of the solute used, no lag phase was observed. A comparison of growth rates in both ionic and non-ionic modified water stress media in relation to two temperatures, 25 and 30 ^{o}, showed that in unmodified medium growth was faster at 30 than 25 ^{o}C. Interestingly, at 30°C growth rates were faster in NaCl modified media. However, at 25°C accumulation of compatible solutes, mainly glycerol and arabitol, was much higher than at 30°C. Moreover, maximum accumulation of compatible solutes occurred in 3-d-old cultures (data not shown). The above studies were taken into account and the conclusions drawn were used as a guideline for the planning of studies in this project.

1.10 BACKGROUND TO THIS PROJECT

Biocontrol of post-harvest diseases has been a very interesting research area. However, it is apparent that a multidisciplinary approach of research must be undertaken to allow rapid progress needed for commercial implementation of this method of disease control. This is why a multidisciplinary team, including experts in different fields such as Food Technology, Plant Pathology, Mycology, Industrial processes and Molecular Marking techniques, together with industrial companies interested in biological control products, should be integrated. Under this principle, the European Union funded a project (QoL-PL1999-1065) with the title "Development of biocontrol agents for commercial application against post-harvest diseases of perishable foods". The project involved 8 work packages (main research fields) and 19 participants, and its major aim was to develop BCAs for control of key post-harvest pathogens to a stage where the BCAs could be commercially exploited. The aim was to provide the necessary data to facilitate registration of a number of microorganisms (Figure 2) that have already been demonstrated to be effective: *Pichia anomala* (Petersson *et al.*, 1999)*, Epicoccum nigrum* (Pascual *et al.*, 1996)*, Candida sake* (Teixidó *et al.*, 1998b; Viñas *et al.*, 1998) and *Pantoea agglomerans* (Spanish patent application n^o P 9900612).

The target fungi chosen to be studied in this project are the most important post-harvest diseases of moist cereals, stone fruits, pome and citrus fruits namely *P. roquefortii* and *P. verrucosum*, *M. laxa*, *M. fructigena* and *R. stolonifer, P. expansum* and *B. cinerea* and *P. digitatum* and *P. italicum*, respectively.

1.11 OBJECTIVES

In this work, the biocontrol yeast *P. anomala* was used as the model microorganism to study the effect of modification of synthetic and complex liquid media substrate on the production and quality of produced inocula. The aim of this study was to determine optimum cultural laboratory scale conditions under which high numbers of ecologically competent yeast cells are produced. Additionally, ecophysiological responses of the cells, and resistance to drying and subsequent stability in storage were also examined. To this end, the following steps were followed.

A. SYNTHETIC AND COMPLEX LIQUID MEDIA

- Production of yeast cells in liquid substrate fermentation using two different types of basal medium (synthetic and complex-industry byproduct cane molasses) modified at different a_w levels

- Measurements of yeast yield and internal water potential of cells (Ψ_c) for accurate use of isotonic solutions

- Determination of intracellular concentration of selected sugar alcohols and sugars

- Effect of osmoprotection, by using isotonic solutions, on endogenous reserve retention

- Evaluation of modified yeast cell viable counts under fully available and water-stress conditions

- Selection of best yeast cell treatments for subsequent formulation and storage stability studies

B. WET PASTE SYSTEM AND STORAGE STABILITY

- Examination of wet paste concentrates as a method of conserving viability of *P. anomala* cells when stored at 4°C and ambient temperature

C. FLUIDISED BED DRYING AND STORAGE STABILITY

- Optimisation of drying conditions
- Effect of osmoprotection and type of osmoprotectant on fluidised bed drying tolerance and final cell moisture content
- Effect of several additives on fluidised bed drying tolerance and final cell moisture content
- Determination of best fluidised bed drying formulations
- Comparison between the best cell treatments when using the best fluidised bed drying formulations and best type of osmoprotectant
- Studies on cell storage stability at 4°C and ambient temperature
- Effect of osmoprotection in cell storage stability

D. FREEZE DRYING AND STORAGE STABILITY

- Effect of protective (suspension) solutions and rehydration media on freezing, thawing and freeze drying of yeast cells
- Effect of osmoprotection on freezing, thawing and freeze drying tolerance
- Comparison between the best cell treatments using the best protective (suspension) solution, rehydration media and osmoprotection
- Studies on cell storage stability at 4°C and ambient temperature
- Effect of osmoprotection in cell storage stability

E. ENZYME PRODUCTION BY *P. anomala* CELLS

- Use of API ZYM kit for a quick indication of yeast enzyme profile
- Use of synthetic substrates and cell wall preparations (CWP) to induce extracellular β-1,3-glucanase chitinase and β-glucosidase production by *P. anomala* cells
- Effect of osmoprotection on extracellular β-1,3-glucanase chitinase and β-glucosidase activity and on endogenous protein content
- Comparison between the best cell treatment in terms of enzyme activity

F. BIOASSAY EVALUATION OF BIOCONTROL EFFICACY OF FORMULATED *P. anomala* CELLS

- Effect of fresh and formulated *P. anomala* cells on *P. verrrucosum* populations and OTA production in lab-scale bioassay using wheat grain at 2 different a_w levels under aerobic conditions
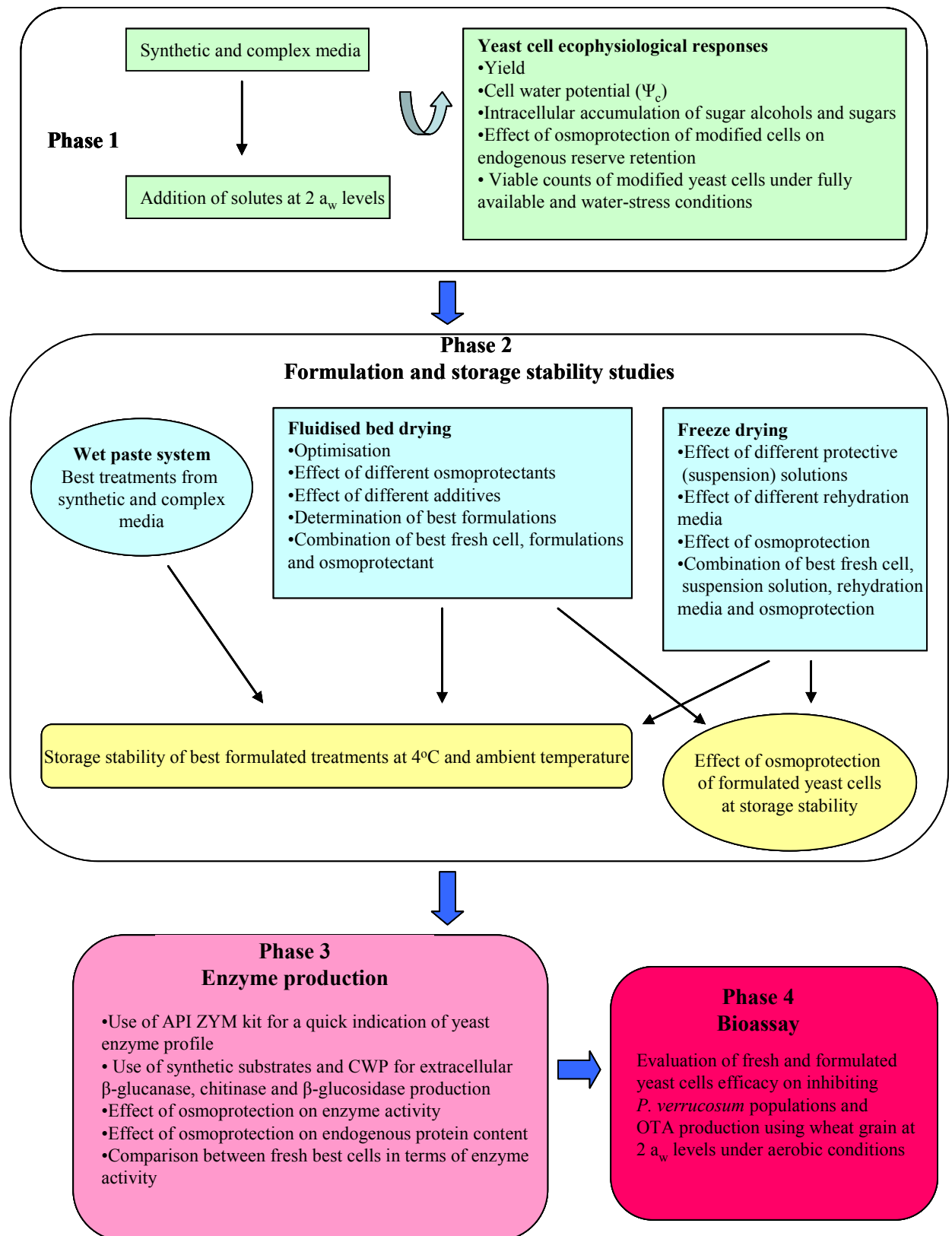
Figure 1.2. Flow diagram of the experimental work carried out in this thesis

2   Chapter 2 MATERIALS AND METHODS

2.1   BIOLOGICAL CONTROL AGENT (BCAs) AND PLANT PATHOGENS USED

The BCA used in this study was *Pichia anomala* (strain J121), originally isolated in 1981 from airtight-stored grains (Björnberg & Schnürer, 1993). Stock cultures were stored at 4°C on malt extract agar plates (MEA, MERCK, Darmstad, Germany, 48g l^{-1}) and subcultured on MEA plates as required. Plant pathogens used were *P. roqueforti* IBT 6754 (Culture collection of the Mycology Group, Technical University Denmark) and *P. verrucosum* 22625 (Swedish Food Agency, Upsalla, Sweden). Stock cultures of both fungi were stored at 4°C on MEA and subcultured on MEA plates as required.

2.2   BASIC MEDIA USED AND THEIR MODIFICATION

Semi-solid (agar) and liquid media used in experiments and modification of a_w with several solutes are described in this stion. The agar was modified with the non-ionic solute polyethylene glycol (PEG 200), while the liquid media were modified with ionic (NaCl) and non-ionic (glycerol, glucose, sorbitol, proline) solutes (Abadias *et al*., 2000).

2.2.1   Semi-solid agar media

Nutrient yeast dextrose agar (NYDA) was used in all growth and viability experiments. This contained 8 g l^{-1} nutrient broth (Oxoid Ltd., Basingstoke, England), 5 g l^{-1} yeast extract powder **(**Lab M**,** IDG Ltd, U.K.), 10 g l^{-1} D-(+)-glucose (Sigma-Aldrich, Ltd., U.K.) and 2% agar technical No3 (Oxoid Ltd., Basingstoke, England) and had a pH of 7. Unmodified a_w of media ranged from 0.992 to 0.995. NYDA medium was modified with PEG 200.The media were autoclaved for 20 min at 121 $^{\circ}$C, 103 KPa, poured into 9 cm Petri dishes and allowed to cool to 25 $^{\circ}$C. All plates were stored at 4°C in sealed polyethylene bags. All plates with the same water activity (a_w) were grouped together ensuring no fluctuation in a_w.

2.2.2 Liquid media

The following liquid media were used for liquid fermentation experiments:

1. Nutrient yeast extract broth (NYDB): this was made as NYDA without agar addition. Unmodified NYDB had a pH of 6.9-7 and a a_w 0.993-0.996. Modified NYDB media were prepared at 0.98 and 0.96 a_w by the addition of glycerol (Gly98, Gly96), glucose (Glu98, Glu96), NaCl (Na98, Na96), sorbitol (Sor98, Sor96) and proline (Pro98, Pro96) as described in Table 2.1. (Abadias *et al.*, 2000).

2. Cane molasses based medium (typical composition of cane molasses in Appendix III): this consisted of cane molasses 40 g l^{-1} and urea 1.2 g l^{-1}. Cane molasses is a commercial cane sugar industry by-product and was kindly supplied by the Post-harvest Unit, ceRTA, Centre UdL-IRTA, Lleida, Catalonia, Spain. Unmodified cane molasses medium had a a_w of 0.993-0.996 and a pH of 6.2. Modified molasses medium as described above had a pH of 5.9-6.5.

3. Czapek salt minimal medium (Fan *et al.,* 2002) containing CWP, laminarin, glucose (2 mg ml^{-1}), 1% chitin or laminarin (2 mg ml^{-1})+1% chitin and glucose (2 mg ml^{-1}) + 1% chitin as the sole carbon source . pH of the medium was adjusted from 7.6 to 6 by adding 2N HCl. This basal medium was used for *P. anomala* exoenzyme production *in vitro* (also see stion 2.11.4)

The liquid medium (50 ml) was placed in 250 ml Erlenmeyer flasks that were sealed with cotton wool and aluminium foil to avoid significant changes to the a_w during autoclaving for 20 min at 121 ^{o}C, 103 KPa. Media were then allowed to cool and inoculated immediately or after 24 h. The a_w of all media was determined with an Aqualab Series 3 (Aqualab, Labcell Ltd., Basingstoke, Hants, U.K.).

Table 2.1. Composition of modified media (g of solute to be added to 100 ml of water). Final volume was measured and then corresponding nutrient broth (8 g l^{-1}), yeast extract powder (5 g l^{-1}) and dextrose (10 g l^{-1}) were added to the solution; same procedure was followed for cane molasses based-medium with cane molasses (40 g l^{-1}) and urea (1.2 g l^{-1}) added (Abadias *et al.*, 2000)

	Water activity	
Solute	0.98	0.96
Glycerol	9.20	18.40
Glucose	18.73	39.85
NaCl	3.55	7.01
Sorbitol	19.82	30.75
Proline	10.00	20.00

2.3   STUDIES ON YIELD OF *P. anomala* IN UNSTRESSED AND WATER-STRESSED LIQUID NYDB AND MOLASSES-BASED MEDIA

Yield was measured by producing a regression line of absorbance (700 nm) against dry weight when both NYDB and molasses-based media were used. In this case, 100 ml of each medium was placed in 250 ml Erlenmeyer flasks and inoculated with 10^6 cells ml^{-1} and incubated at 25 ^{o}C on a rotatory shaker (150 rpm). After 72 h, several dilutions of the culture were made (100%, 80%, 60%, 40%, 20%, 10%, 5%, 2%) and 0.1 ml of each concentration was diluted with 0.9 ml of SDW water + Tween 20 + agar No.3 (0.01%) in a 1ml cuvette (10-fold dilution); Optical Density (O.D.) was measured with a spectrophotometer (UV-2101 PC, Shimadzu, Japan)**.** Samples were put in 50 ml sterile centrifuge tubes and centrifuged for 18 min at 3300 rpm (MSE Centaur 2) and the supernatant discarded. To obtain dry weight measurements, the tubes were left overnight at 70 ^{o}C. Three empty tubes were also dried and used as controls. This enabled absorbance to be subsequently used to measure increases in biomass (dry weight ml^{-1} of culture medium) over time (72 h)**.**

2.4 WATER POTENTIAL MEASUREMENTS

Thermocouple psychrometry was used to determine the water potential of the cells. Sixty ml of NYDB and molasses-based media (see Table 2-2) were inoculated with 10^6 *P. anomala* cells ml^{-1} and incubated on a rotatory shaker (105 rpm for NYDB and 150 rpm for cane molasses). After 24, 48 and 72 h, a 20 ml subsample of each treatment was placed in 50 ml sterile centrifuge tube and centrifuged immediately for 18 min at 3200 rpm in a MSE Centaur 2 (Norwich, U.K.). The medium was decanted and the cell paste put into the sample well. A HR-33T Dew Point Microvoltometer coupled to a C-52 chamber was used (Wescor Inc, Logan, Utah, USA). All measurements were made in the dew point mode, three measurements of each treatment were taken and the μV reading converted to Ψ by following the procedure below:

Corrected Reading = Reading/(0.325+0.027 *T)

Ψ (bar) =- Corrected reading/0.47

Ψ (MPa) = Ψ (bar)/10

Temperature (T, ^{o}C) was measured at the beginning and at the end of the measurements, and the mean was used in the calculations of Ψ $_c$.

2.5 EVALUATION OF VIABILITY IN UNSTRESSED AND STRESSED NYDB AND MOLASSES-BASED MEDIA

Unmodified and modified NYDB and molasses-based liquid media were prepared as described in Table 2-2. The a_w of all media was determined with an Aqualab Series 3 (Aqualab, Labcell Ltd., Basingstoke, Hants, U.K.). Fifty ml of medium of each treatment was incubated in 250 ml flasks and inoculated with 1 ml of 10^6 *P. anomala* cells ml^{-1}, and cultured at 25 ^{o}C on a rotatory shaker at 105 rpm (for NYDB) and at 150 rpm (for molasses-based media). Nine replicates were read per treatment and three replicates were destructively sampled after 24, 48, 72 h incubation to determine viability of *P. anomala* cells.

Viability of *P. anomala* cell treatments after 24, 48, 72 h was assessed by spread-plating on unstressed (0.995 a_w) and stressed NYDA (0.96 a_w for molasses-based, 0.94 a_w for NYDB) modified with polyethylene glycol (PEG 200, 1.25M and 1.5M

respectively). Serial dilutions were done in a solution of distilled water + Tween 80 + agar Technical No.3 (Oxoid Ltd, Basingstoke, Hampshire, England); in all cases, agar No3 was added to maintain cells in suspension for longer. Plates of the same a_w were sealed in polyethylene bags to prevent water loss and incubated at 25°C. The viability of yeast cells was counted after 48 h for unstressed and after 72 h (0.96 a_w) and 120 h (0.94 a_w) for water stressed NYDA media.

2.6   EXTRACTION AND DETECTION OF POLYOLS AND SUGARS

P. anomala was initially grown in both unmodified and modified NYDB and molasses-based media, as described previously. For each treatment, 50 ml of medium in a 250 ml conical flask was inoculated with 10^6 *P. anomala* cells ml^{-1}, and cultured at 25 °C on a rotatory shaker at 105 rpm for NYDB, and 150 rpm for molasses-based media. There were three replicates of each treatment. After 72 h, the content of each flask was placed in a 50 ml centrifuge tube and centrifuged immediately for 15 min at 2700 rpm (MSE Centaur 2, Norwich, U.K.). The yeast pellet was resuspended in 10 ml of HPLC grade water and centrifuged again for 10 min at 2700 rpm to remove any residual liquid medium (Abadias *et al.,* 2001b).

A known fresh weight (Abadias *et al*., 2001b) of *P. anomala* cells (50-100 mg) was mixed with 1 ml HPLC grade water in a 2 ml Eppendorf tube and sonicated with a 4 mm sonicator probe for 30 s at an amplitude of 26 μ m (Soniprep, Fisons). After immersion in a boiling water bath for 5 min, the samples were left to cool, and 0.67 ml of acetonitrile was added to each sample to obtain the same ratio of acetonitrile:water as the mobile phase (40:60). The Eppendorf tubes were centrifuged for 10 min at 13000 rpm (Microtube Lite Ser 98E 4000, Beckman Inc. Columbia, U.S.A.) and the supernatant was filtered through a 0.2 μm filter into an HPLC vial sealed with a plastic septum.

Solutes were analyzed and quantified by HPLC using a Hamilton HC-75 Ca^{2+} column and a Gilson RI Detector (ANACHEM Ltd., Luton, U.K.), specifically for sugar/polyol separation. The mobile phase used was a mixture of 40:60 degassed acetonitrile:water. The peak areas were integrated and compared with calibration curves constructed with standards of 100-600 ppm of each solute analyzed. Polyols,

trehalose and glucose content were calculated as mg g^{-1} fresh weight (f.w.) of *P. anomala* cells (Hallswoth & Magan, 1995; Teixidó *et al.,* 1998a; b Pascual *et al.*, 2000;).

2.7 WET PASTE STORAGE TRIALS

P. anomala cells from different NYDB and molasses-based treatments as described in Table 2-1 were retained for a long-term storage and viability experiment for a period of 6 months.

The following yeast cell treatments were assessed:

1) Control cells from NYDB liquid medium, (3-days-old culture, -0.62 MPa Ψc)

2) Control cells from molasses-based medium, (3-days-old culture, -0.52 MPa Ψc)

3) Yeast cells produced in glycerol modified NYDB liquid medium (0.98 a$_w$), (3-days-old culture, -2.60 MPa Ψc)

4) Yeast cells produced in glucose modified NYDB liquid medium (0.98 a$_w$), (3-days-old culture, -0.99 MPa Ψc)

5) Yeast cells produced in glucose modified NYDB liquid medium (0.96 a$_w$), (3-days-old culture, -3.51 MPa Ψc)

6) Yeast cells produced in sorbitol modified NYDB liquid medium (0.96 a$_w$), (3-days-old culture, -4.24 MPa Ψc)

7) Yeast cells produced in glycerol modified molasses-based liquid medium (0.98 a$_w$), (3-days-old culture, -2.59 MPa Ψc)

8) Yeast cells produced in glucose modified molasses-based liquid medium (0.98 a$_w$), (3-days-old culture, -2.34 MPa Ψc)

9) Yeast cells produced in NaCl modified molasses-based liquid medium (0.98 a$_w$), (3-days-old culture, -2.62 MPa Ψc)

10) Yeast cells produced in proline modified molasses-based liquid medium (0.96 a$_w$), (3-days-old culture, -4.41 MPa Ψ)

Yeast cells were produced by following the protocol below:

Medium (100 ml) of each treatment was placed in 250 ml conical flasks and inoculated with 1 ml of 10^6 *P. anomala* cells ml^{-1} and cultured with agitation on a rotatory shaker (150 rpm) at 25 ^{o}C. After 72 h of incubation, sub-samples of each

conical flask were distributed in 50 ml sterile centrifuge tubes and centrifuged immediately for 15 min at 3300 rpm in a MSE Centaur 2 (Norwich, U.K.) centrifuge. The supernatant was discarded and yeast cells were re-suspended with 50 ml of sterile water + Tween 20+ agar technical No.3 for control treatments, sterile water solution modified with 1M PEG 200 (-2.8 MPa Ψ), sterile water solution modified with 1.35M PEG200 (–4.2 Ψ MPa) (Sancisi-Frey, 2000) for control treatments, treatments 3,4,7,8,9 and treatments 5 and 10, respectively. Two ml of the yeast cell resuspension was dispensed into a 2 ml centrifuge microtube and centrifuged again (Microtube Lite Ser 98E 4000, Beckman Inc. Columbia, U.S.A.) at 13000 rpm for 10 min to remove any residual liquid medium. The supernatant was discarded and the resultant pellets were stored as wet pastes at ambient and 4^oC. Replicates were destructively sampled initially and then after 30, 90, 180 and 360 days storage. These were resuspended in distilled water and serial dilutions were done in a solution of sterile distilled water (SDW)+Tween 20 +agar technical No.3 (Oxoid Ltd, Basingstoke, Hampshire, England) before being plated onto unstressed (0.995 a_w) and stressed NYDA (0.96 a_w) media modified with polyethylene glycol (PEG 200, 1.25M). Plates of the same a_w were sealed in polyethylene bags to prevent water loss and incubated at 25^oC. Colonies were counted after 48 h incubation for unstressed and 84 h for stressed media, and colony forming units (CFUs) ml^{-1} were determined. There were 12 replicates of each treatment for both unstressed and stressed media.

2.8   VALIDATION OF VIABLUE STAIN

There was a need to use a quick and reliable cell viability checking method. A literature search showed that the Viablue staining method (Hutchenson *et al.*, 1998) had potential. The stain is a mixture of two stains, rose bengal (1% in 50 mM K_2HPO_4, pH 8.9) and aniline blue (1% aqueous solution); the latter needs to age for 21 days prior to mixing. The concentrated form of the stain was subsequently diluted 1 in 10 for staining yeast cells. The stain was filtered through a 0.45μm filter to remove any large particles or contaminating organisms. The validation procedure was carried out. *P. anomala* cells were grown in cane molasses-based medium for 72 h at 25^oC shaking at 150 rpm. Cell material was pooled together to obtain an homogenous mixture. Half of the material was then autoclaved for 20 min at 121 ^oC, 103 KPa to ensure that cells were dead (subsequent spread-plating of cells confirmed this), and

several mixtures of the autoclaved and fresh cells were made at 0%, 10%, 20%…100%. Viability was subsequently checked by using the stain (mixture 1 cells: 1 stain volume, left for 10 min) under a fluorescent microscope (x 40), and by spread-plating on NYDA media.

2.9   FLUIDISED BED DRYING OF *P. anomala* CELLS

2.9.1   Optimisation of fluidised bed drying procedure

P. anomala cells were grown in cane molasses-based media for 72 h at 25 $^{\circ}$C shaking at 150 rpm, washed twice in HPLC grade water and centrifuged. Cell pellets were pressed dry between filter paper and then passed through a syringe to obtain an even material. The yeast material was then introduced into a fluidised bed dryer 350s (Burkard Manufacturing Co. Ltd, Hertfordshire, UK). Drying temperatures tested were 30, 40, 50, 60 and 70 $^{\circ}$C at 2 drying times 10 and 20 min. At the end of the 10 min-drying time, cells were removed, crushed slightly with a pestel and mortar and either tested for viability or put back in the chamber for further drying. Lumps of cells were finally crushed into a powder. Viability, using the viablue stain, and % moisture content of the cells (MC%) were subsequently measured.

2.9.2   Study of effect of different isotonic solutions used prior to fluidised bed drying

Several isotonic solutions were used to wash *P. anomala* cells prior to fluidised bed drying, the main concept being to investigate if the maintenance of internal endogenous reserves resulted in any improvement in cell viability and any change in % moisture content. Cells were dried as described above at the optima conditions (50°C for 20 min). The cell treatments chosen to be dried resulted from the ecophysiological studies reported previously. The treatments were cane molasses-based media modified with NaCl 0.98 a_w, proline 0.98 a_w, glycerol 0.98 a_w, glucose 0.98 a_w and sorbitol 0.96 a_w. The different isotonic solutions used were NaCl, glucose, PEG 200 at 0.98 and 0.96 a_w levels.

2.9.3 Addition of adjuvants and carriers prior to fluidised bed drying

Several compounds were added to resulting *P. anomala* cell pellets after washing and centrifugation. Those were (i) wheat starch (Sigma-Aldrich, Ltd., U.K.), (ii) cottonseed flour (Sigma-Aldrich, Ltd., U.K.), (iii) cornmeal (Sigma-Aldrich, Ltd., U.K.), (iv) talc powder (Oxoid Ltd., Basingstoke, England) and (v) skimmed milk powder (Oxoid Ltd., Basingstoke, England). The compounds were added at 5%, 10% and 20% (w/w). Some of the compounds tested as adjuvants were subsequently tested as carriers in a proportion of 1:1 (w/w, *P. anomala* cells/carrier). Following this, the best carriers were tested with some adjuvants. Cells were dried as described above at the optima conditions (50°C for 20 min). Cell viability and % cell moisture content were subsequently tested.

2.9.4 Studies with best *P. anomala* treatments when using the best formulations and isotonic solutions prior to fluidised bed drying

The resulting best formulations were checked with the best fresh *P. anomala* treatments (NaCl 0.98 a_w, proline 0.98 a_w) and the cells were washed twice with NaCl 0.98 a_w isotonic solution and water. Cells were dried as described above at the optima conditions (50°C for 20 min). Cell viability and % moisture content were checked; formulations were also spread-plated on normal and water-stressed (0.96 a_w) NYDA media to check the CFUs mg^{-1} formulation and cells' water-stress tolerance.

2.9.5 Storage studies of *P. anomala* fluidised bed drying formulation

Samples of fluidised bed drying formulations were stored at ambient and 4°C temperatures to investigate shelf-life over a 5-month period. Viablue staining was used to check viability.

Plate 2.1. Fluidised bed dryer 350s (Burkard Manufacturing Co. Ltd, Hertfordshire, UK) that was used in the studies

2.10 FREEZE-DRYING OF *P. anomala* CELLS

2.10.1 Freeze drying procedure

P. anomala cells were grown in cane molasses-based media, unmodified and modified, for 72 h at 25°C shaking at 150 rpm, washed twice in HPLC grade water, centrifuged and then resuspended in SDW + Tween 20 + agar technical No.3 to give 10^9 CFUs ml^{-1} final concentration. Three vials were filled with 0.4 ml of yeast cell suspension and placed at -20°C for 24 h. After overnight storage in the freezer, samples were connected to a Modulyo Edwards Pirani 10 model freeze dryer operating at 2.5 Atm pressure and -60 ^{o}C for 12 h.

2.10.2 Rehydration of *P. anomala* cells after freeze drying

After freeze-drying, samples were immediately brought to their original volume (0.4 ml) with each rehydration medium at 25 ^{o}C. Then, samples were homogenized for 1 min by hand shaking and incubated at room temperature for 9 min. Viability was checked using the Viablue stain method.

2.10.3 Protectants used prior to freeze drying

Suspensions of protectants were prepared in water. The additives tested as protective agents against freeze-drying injury were: (i) monosaccharide sugar: glucose (10%, w/v) (Sigma-Aldrich, Ltd., U.K.), (ii) disaccharide sugar: sucrose (Fisher Scientific) (10%, w/v), (iii) skimmed milk powder, SM (Oxoid Ltd., Basingstoke, England) (10% and 20% w/v) and mixtures (10% + 10%, w/v) of sugars with skimmed milk powder. These protectants were selected on the basis of previous studies on other microorganisms (Costa *et al*., 2000). Protectant solutions were sterilized at 121 ^{o}C for 15 min before mixing with a volume of washed cells of antagonist to obtain an initial concentration of 10^9 CFUs ml^{-1}. The general procedure for cell preparation, freeze-drying and rehydration were described previously. After freeze drying, all samples were rehydrated with SDW + Tween 20+ agar technical No.3 to the original volume and the level of survival evaluated (Viablue staining method).

2.10.4 Rehydration assay media

In this assay, the initial concentration used was 10^9 CFUs ml^{-1} and the protectants used are described above. Freeze-dried samples were rehydrated with 10% non-fat skim milk and SDW + Tween 20 + agar technical No. 3. The general procedure of cell preparation, freeze-drying and rehydration was as described above. Viability was subsequently checked with the Viablue staining method.

2.10.5 Studies with best *P. anomala* treatments and isotonic solutions prior to freeze drying

The resulting best protectant (10% SM + 10% sucrose) and best rehydration medium (SDW + Tween 20+agar technical No. 3) were combined with best fresh *P. anomala* treatments, and the cells washed twice with NaCl 0.98 a_w isotonic solution and water. Cells were freeze dried as described above and cell viability checked.

2.10.6 Storage studies of *P. anomala* freeze drying formulations

Samples of freeze dried formulations were stored at ambient and 4°C temperatures to investigate shelf-life over a 5-month period. Viablue staining was used to check viability.

2.11 ENZYME ASSAYS

2.11.1 Total protein content determination

Fifty to 100 mg of *P. anomala* cells (fresh weight) from each treatment were put into a 2 ml microfuge tube containing 1 ml HPLC water and sonicated for 30 s at an amplitude of 28 μm using a Soniprep 150, fitted with a 3.5 mm diameter exponential probe. After sonication, samples were centrifuged for 10 min at 13000 r.p.m. in a microcentrifuge and the supernatant was used for protein determination. Total protein was determined using the Bisinchoninic Protein Assay Kit (Sigma, UK).

2.11.2 Screening for *P. anomala* extracellular enzymes using API ZYM system

The API ZYM system (API system S.A., Montalieu Vercieu, France) is a semi-quantitative micro-method that allows a rapid and simultaneous evaluation of 19 enzymatic activities using very small sample of extract quantities. For enzymatic studies, 3-day-old *P. anomala* cultures grown in molasses medium (25°C, 150 rpm incubation) were used. They were adjusted to 10^8 CFU ml^{-1} concentration and centrifuged for 10 min. The supernatant was subsequently used to determine enzyme activities by using API ZYM system This was done to assay constitutive enzymes. For inductive enzymes cells were then added to the CWP media.

Five ml of distilled water was distributed in the honey-combed wells of the tray to create a humid atmosphere and a strip was placed in it. A 65 µl sample was dispensed in each cupule. Plastic lip was placed on the tray and the strip was left to incubate for 4 h at 37°C. After incubation one drop of ZYM A reagent and one drop of ZYM B reagent were added to each cupule. Colour was left to develop for 5 min and the strip was subsequently put under a powerful light source for 10 s. The results were then recorded on result sheets.

2.11.3 Cell wall preparation (CWP)

Hyphal cell walls were prepared from 5-day-old cultures of *P. verrucosum* grown at 25°C in 50 ml of malt extract broth (35 g malt extract + 5 g Mycological peptone l^{-1}) (Difco laboratories, Detroit) and shaken on a rotatory shaker at 150 rpm min^{-1}. The mycelium was centrifuged for 10 min at 3500 rpm min^{-1}, the supernatant was discarded and the pellet was homogenized with a common mixer for 2 min at high speed. The material was subjected to five successive cycles of centrifugation, resuspension and homogenization. The final pellet of fungal material was frozen at -20°C for 24 h. The thawed pellets were sonicated twice with a probe-type sonicator for 10 min. The CWP was washed six times with distilled water and centrifuged at 3500 rpm min^{-1} before being lyophilized and stored at -20°C.

2.11.4 *P. anomala* extracellular enzyme production *in vitro*

P. anomala was cultured in Czapek salt minimal medium (Fan *et al.,* 2002) containing CWP, laminarin, glucose (2 mg ml^{-1}), 1% chitin or laminarin (2 mg ml^{-1}) + 1% chitin and glucose (2 mg ml^{-1}) + 1% chitin as the sole carbon source . The pH of the medium was adjusted from 7.6 to 6 by adding 2N HCl. Flasks (250 ml) containing 60 ml of culture medium were incubated with shaking (150 rpm min^{-1}) for various periods. Culture filtrates were harvested by centrifuging at 3500 rpm min^{-1} for 10 min. Enzyme activity was determined by use of different enzyme assays:

I. β-1,3-glucanase and chitinase activities were determined by enzyme assays using the dye-labelled substrates CM-curldlan-RBB and CM-chitin-RBV (Loewe Biochemical GmbH, D-82054Sauerlach, Mühlweg 2a, Germany) respectively.

II. β-glucosidase activity was assayed using p-nitrophenyl substrates (Sigma Chemical Co., UK).

Enzyme assays were performed in microtitre plates (Bibbily Sterilin Ltd,. Staffordshire, U.K.)

2.11.5 Dye-labelled substrates standard assay conditions

Dye-labelled substrate in aqueous solution (50 μl; 4 mg ml^{-1} for CM-curldlan-RBB and 2 mg ml^{-1} for CM-chitin-RBV) and buffer (50 μl; 0.2 sodium acetate-acetic acid buffer, pH 5) were equilibrated at 37°C for 1 h. After equilibration, 50 μl of the substrate and 50 μl of buffer were added in each well; 100 μl of sample (enzyme) were subsequently pipetted in each well (apart from the control wells). The plates were sealed with a low evaporation lid and incubated for 30 min. The reaction was terminated by the addition of HCl (50 μl; 2N and 1N, respectively) causing precipitation of the non-degraded high-polymeric substrate. Subsequently, the plates were cooled on ice (10 min) and centrifuged for 10 min at 3700 rpm. Supernatants (150 μl), containing soluble, dye-labelled degradation products were transferred to a 96-well, half-size EIA plate (175 μl cavities; Costar) and measured spectrophotometrically at 600 nm for CM-curdlan-RBB and at 530 nm for CM-chitin-RBV. One unit of enzyme activity was calculated as absorbance x 1000 x min^{-1}. Enzyme assay protocols were based on Wirth & Wolf (1992).

2.11.6 P-nitrophenyl substrates standard assay conditions

Total enzyme activity determination

B-glucosidase activity was assayed using p-nitrophenyl substrates (Sigma Chemical Co., UK). 4-Nitropenyl-β-D-glycopyranoside substrate at 2 mM concentration and 5 mM acetate buffer (pH 5.0) were used. Enzyme extract (40 μl), substrate solution (40 μl) and the appropriate acetate buffer (20 μl; BDH, U.K.) were pipetted into the wells of the microtitre plate (Bibbily Sterilin Ltd,. Staffordshire, U.K.) and incubated at 37 ^{o}C for 1h along with the appropriate controls. The control included substrate solution (40 μl) and the appropriate buffer (20 μl). The reaction was stopped by the addition of 5 μl 1M sodium carbonate solution (Sigma Chemical Co., U.K.) and left for 3 min. Enzyme extracts (40 μl) were added to the control wells. The enzyme activity was measured, using a MRX multiscan plate reader (Dynex technologies Ltd., Billinghurst, West Sussex, U.K.; Revelation software version 4.21), by the increase in optical density at 405 nm caused by the liberation of p-nitrophenol by enzymatic hydrolysis of the substrate. Total enzyme activity was calculated from the calibration curve of absorbance at 405 nm against p-nitrophenol concentration and expressed as μmol 4-nitrophenol released min^{-1}.

Calibration curves

Standard p-nitrophenol solutions (Spectrophotometric grade, Sigma Chemical Co. U.K.) of known concentrations were prepared using 25 mM acetate buffer (pH 5.0). P-nitrophenol solution (40 μl), and the appropriate buffer (60 μl) were pipetted into the wells of the microtitre plate and incubated at 37°C for 1h. Sodium carbonate 1M (5 μl) was added and left for 3 min after which, absorbance at 405 nm was measured. Calibration curves of absorbance against p-nitrophenol concentration were plotted.

Specific enzyme activity determination

Protein concentration determination was carried out using a Bicinchoninic acid protein kit (BCA-1 kit; Sigma Chemical Co., U.K.). This kit consisted of bicinchoninic acid solution, copper (II) sulphate pentahydrate 4% solution and albumin standard (containing bovine serum alnumin (BSA) at a concentration of 1.0 mg ml^{-1}). Protein reduces alkaline Cu (II) to Cu (I), which forms a purple complex with bininchoninic acid (a highly specific chromogenic reagent). The resultant

absorbance at 550 nm is directly proportional to the protein concentration. The working reagent was obtained by the addition of 1 part copper (II) sulphate solution to 50 parts bicinchoninic acid solution. The reagent is stable for one day provided it is stored in a closed container at room temperature. Samples (10 μl) of each standard of enzyme extracts were placed in the appropriate microtitre plate wells. Potassium phosphate extraction buffer 10mM pH 7.2 (10 μl) was pipetted into the blank wells. The working reagent (200 μl) was added to each well. After shaking, the plates were incubated at 37°C for 30 min. The plates were allowed to cool to room temperature (3 min) before measuring the absorbance at 550 nm using the MRX multiscan plate reader. The protein concentrations in the enzyme extracts were obtained from the calibration curve of absorbance at 550 nm against BSA concentration. These values were used to calculate the specific activity of the enzymes in nmol p-nitrphenol released min^{-1} μg^{-1} protein.

2.12 BIOASSAYS

Irradiated grain was used as the solid substrate and water activity (a_w) was modified by adding sterile distilled water (SDW). To achieve 0.95 a_w and 0.93 a_w, 1 and 0.7 ml SDW was added to 10 g of grain, respectively (calibration curve was kindly offered by colleagues). Twelve Schott-Duran bottles were used each containing 300 g of grain,with six for each water activity level; 30 g and 27 g of SDW were added to each of these to achieve 0.95 a_w and 0.93 a_w, respectively. The bottles were repeatedly shaken to obtain homogeneous water activity and left at 4°C. A_w was checked afterwards, by use of an Aqualab Series 3 (Aqualab, Labcell Ltd., Basingstoke, Hants, U.K.), and more SDW water was added where needed. The six different treatments used were:

1. *P. verrucosum* alone, served as control,

2. *P. anomala* grown in unmodified molasses medium fresh cells alone,

3. *P. anomala* grown in proline 0.98 a_w modified molasses medium and washed with NaCl 0.98 a_w isotonic solution, fresh cells with *P. verrucosum*,

4. *P. anomala* grown in unmodified molasses media molasses formulated with cottonseed flour + 10% skimmed milk and fluidised bed dried cells with *P. verrucosum*,

5. *P. anomala* grown in proline 0.98 a_w modified molasses, washed with water, formulated with cottonseed flour + 10% skimmed milk and fluidised bed dried cells with *P. verrucosum*,

6. *P. anomala* grown in proline 0.98 a_w modified molasses, washed with NaCl 0.98 a_w isotonic solution, formulated with cottonseed flour + 10% skimmed milk and fluidised bed dried cells with *P. verrucosum*.

Inoculation was done by adding 3 ml of 10^5 spores *of P. verrucosum* ml^{-1} to the grain, the water added did not change the a_w. This achieved a final concentration of 10^3 *P. verrucosum* spores grain g^{-1}. Three ml of 1×10^7 CFUs ml^{-1} of *P. anomala* were added to the grain to achieve 1×10^5 CFUs grain g^{-1} of *P. anomala*. Where both the pathogen and the BCA were used a mixture of 1.5 ml 2×10^5 spores *P. verrucosum* ml^{-1} and 1.5 ml 2×10^7 CFUs ml^{-1} of *P. anomala* (final volume 3 ml) were added to the grain. Grain (23 g) was placed into Petri dishes (3 replicates per treatment) for 4 sampling times (1, 10, 20, 30 days). Petri dishes were then placed in plastic boxes with isotonic glycerol solutions to keep the treatment unaffected during the experiment and incubated at 25^oC.

At each sampling time, 1 g of grain was removed and placed in an empty, sterile universal bottle. SDW+Tween 20+agar technical No.3 (9 ml) was added and shaken in a vortex mixing for 2 min. 0.1 ml sample of this and appropriate dilutions were spread plated on NYDA at 0.95 and 0.93 a_w levels modified with glycerol, and on MEA containing 0.1 g^{-1} chloramphenicol to prevent *P. anomala* growth, at 0.95 and 0.93 a_w levels modified with PEG 200. Growth of yeast and mould was recorded as CFU g^{-1}. The rest of the sample was either extracted as described below or kept in the freezer and extraction carried out for OTA.

2.12.1 Ochratoxin A (OTA) extraction

Ochratoxin A extraction was carried out with methanol (Abarca *et al.*, 1994). The sample was ground in a coffee grinder, and 22 and 21.8 g for 0.95 and 0.93 a_w grain treatments, respectively placed in a conical flask. Fifty ml of methanol (Fisher, HPLC grade) was then added and parafilm (Gallenkamp) was placed on top. Flasks were left on a shaker for 24 h at 25^oC. Samples were subsequently filtered through a filtration system comprised of Whatman No.1 paper placed inside a filter funnel of 1 g of celite

agent. 1 ml sample of the methanol extract were placed in a 1.5ml plastic Eppendorf tubes and centrifuged in a bench top microcentrifuge (Beckman Lite, Ser 98E 4000, Beckman Inc. Columbia, U.S.A.) at 13000 rpm for 10 minutes. The pellet produced was discarded and the supernatant put into 1ml amber HPLC vials (Fisher) and stored at -20ºC until processed.

2.12.2 OTA Quantification

OTA contents were measured using High Performance Liquid Chromatography (HPLC). A HPLC (Waters 600E System controller) with a fluorescence detector (Waters 470) and an auto-sampler (Waters 712 WISP) were used. Conditions for OTA detection were as follows.

Mobile phase	Acetonitrile (57%) : Water (41%) : Acetic acid (2%) (Fisher, HPLC grade)
Column	5μl C18 (150 x 4.6 mm) (Phenomenex Luna)
Pre column	Surity Guard provided with 4 x 3 mm cartridges (Phenomenex Luna)
Excitation λ	330 nm
Emission λ	460 nm
Flux	1 ml min^{-1}
Volume of injected sample	50μl
Toxin standards	150, 300, 600 and 1000 ng.ml^{-1} (methanol solutions) (Sigma)
Retention time	5.3-5.6 minutes
Run time	30 minutes

Under these experimental conditions, a fluorescence detection of up to 1-1.2 μg ml^{-1} OTA was possible. A calibration curve of OTA contents (μg ml^{-1}) versus peak area (mV.min) was made for every sample run. Chromatograms and toxin contents were analysed with the aid of the software program Kroma 2000.

2.13 STATISTICAL ANALYSIS

Data presented throughout this thesis were analysed using Genstat software (Genstat 7th edition). Results, where appropriate were subjected to an analysis of variance (ANOVA) or standard errors of the means (SE) were calculated. When significant differences were observed, means were compared by least significant difference (LSD) testing at the P=0.05 level. Data on percentage (%) cell viability and (%) moisture content and data on CFUs, were logit and log transformed, respectively, before statistical analysis and then backtransformed for presentation. Examples of ANOVA tables and tables of means of statistical analysis carried out in this thesis can be found in Appendix I.

3   Chapter 3 RESULTS

3.1   YIELD OF *P. anomala* IN UNSTRESSED AND WATER-STRESSED LIQUID NYDB AND MOLASSES-BASED MEDIA

3.1.1   Standard curve of absorbance at 700 nm against dry weight of *P. anomala* cells grown on NYDB liquid media

The standard curve of absorbance at 700 nm wavelength for *P. anomala* cells grown on unmodified NYDB media against dry weight expressed in mg of the yeast cells ml^{-1} media is shown on Figure 3.1; the resulting regression line (R^2=0.9902) was used to measure growth of *P. anomala* in NYDB media.
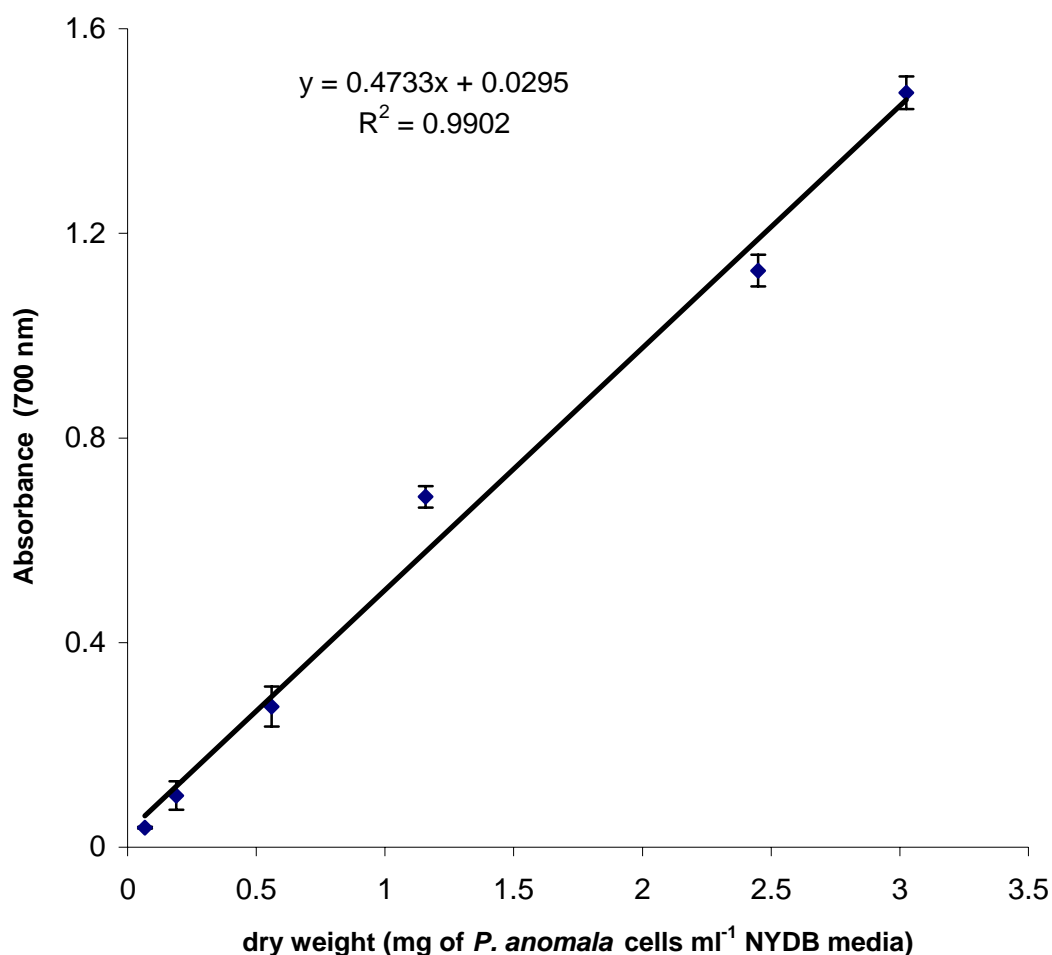
Figure 3.1. Calibration curve of absorbance at 700 nm against dry weight of *P. anomala* (expressed in mg ml^{-1} of NYDB media).

3.1.2 Standard curve of absorbance at 700 nm against dry weight of *P. anomala* cells grown on molasses-based liquid media

The standard curve of absorbance at 700 nm wavelength for *P. anomala* cells grown on unmodified molasses-based media against dry weight expressed in mg of the yeast cells ml^{-1} media is shown on Figure 3.2; the resulting regression line (R^2=0.9813) was used to measure growth of *P. anomala* in molasses-based media.
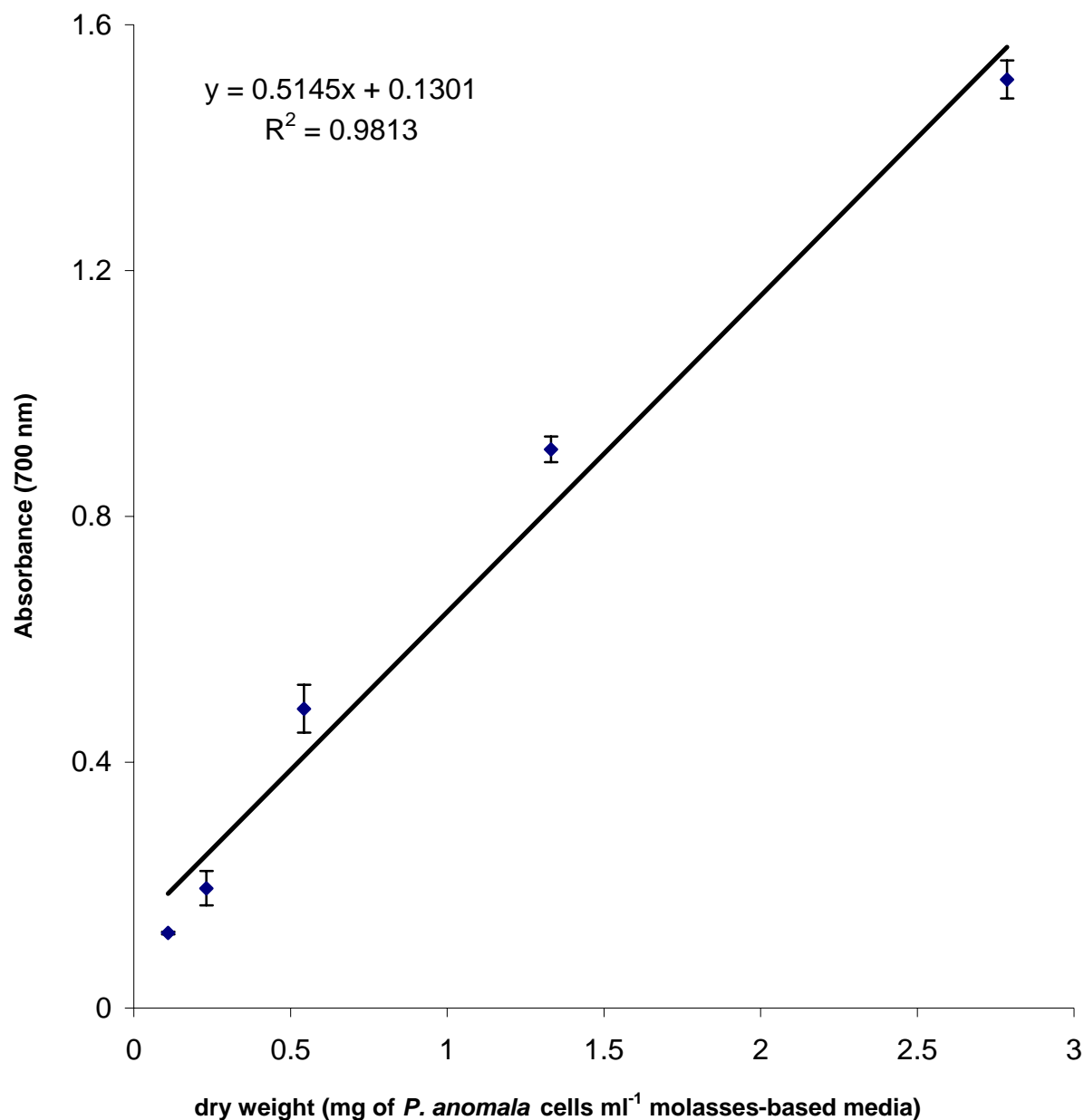
Figure 3.2. Calibration curve of absorbance at 700 nm against dry weight of *P. anomala* cells (expressed as mg ml^{-1} molasses-based media).

3.1.3   Yield of *P. anomala* in unstressed and water-stressed NYDB liquid media

Figure 3.3 shows the effect of a_w on the yield of *P. anomala* grown in NYDB liquid media. The ANOVA analysis of data showed that there was a significant ($P<0.05$) three-way interaction between the three factors (±solute, a_w, different type of solute) affecting *P. anomala* yield at 24 and 72 h incubation; two and one-way interactions were also significant. At 48 h incubation all two and one-way interactions were significant. In general, yield decreased as a_w of media decreased. However, when glucose was used to modify NYDB medium at 0.98 a_w, the yield of *P. anomala* cells was significantly higher than that of the other treatments, including cells grown in unmodified control medium. When the medium was modified with NaCl to 0.96 a_w yield was very low. Yield depended on solute used; in particular glucose, followed by glycerol, gave the best results among the solutes tested while yield in NaCl and sorbitol-modified media was the lowest. Yield increased with time up to 48 h incubation.

3.1.4   Yield of *P. anomala* cells in unstressed and water-stressed molasses-based liquid media

Figure 3.4 shows the effect of a_w on the yield of *P. anomala* grown in molasses-based liquid media. The ANOVA analysis of data showed that there was a significant ($P<0.05$) three-way interaction between the three factors (±solute, a_w, different type of solute) affecting *P. anomala* yield at three different incubation times (24, 48 and 72 h); two and one-way interactions were also significant. Similarly with NYDB medium, yield decreased as a_w of media decreased. In control molasses-based medium, yield of *P. anomala* cells was significantly higher than the other treatments after 24 and 48 h of incubation, while after 72 h, yield of yeast cells in 0.98 a_w glucose-modified medium was the highest. There were differences in yield depending on the solute used to modify water stress. In general, after 48 h of incubation, yield in glycerol, glucose and NaCl-modified media was higher; sorbitol and proline resulted in poorer *P. anomala* yield. Yield increased with time up to 48 h incubation. In the glucose treatment, yield continued to increase over incubation time, markedly so at 0.96 a_w, while with other treatments yield sligthly increased or remained the same.
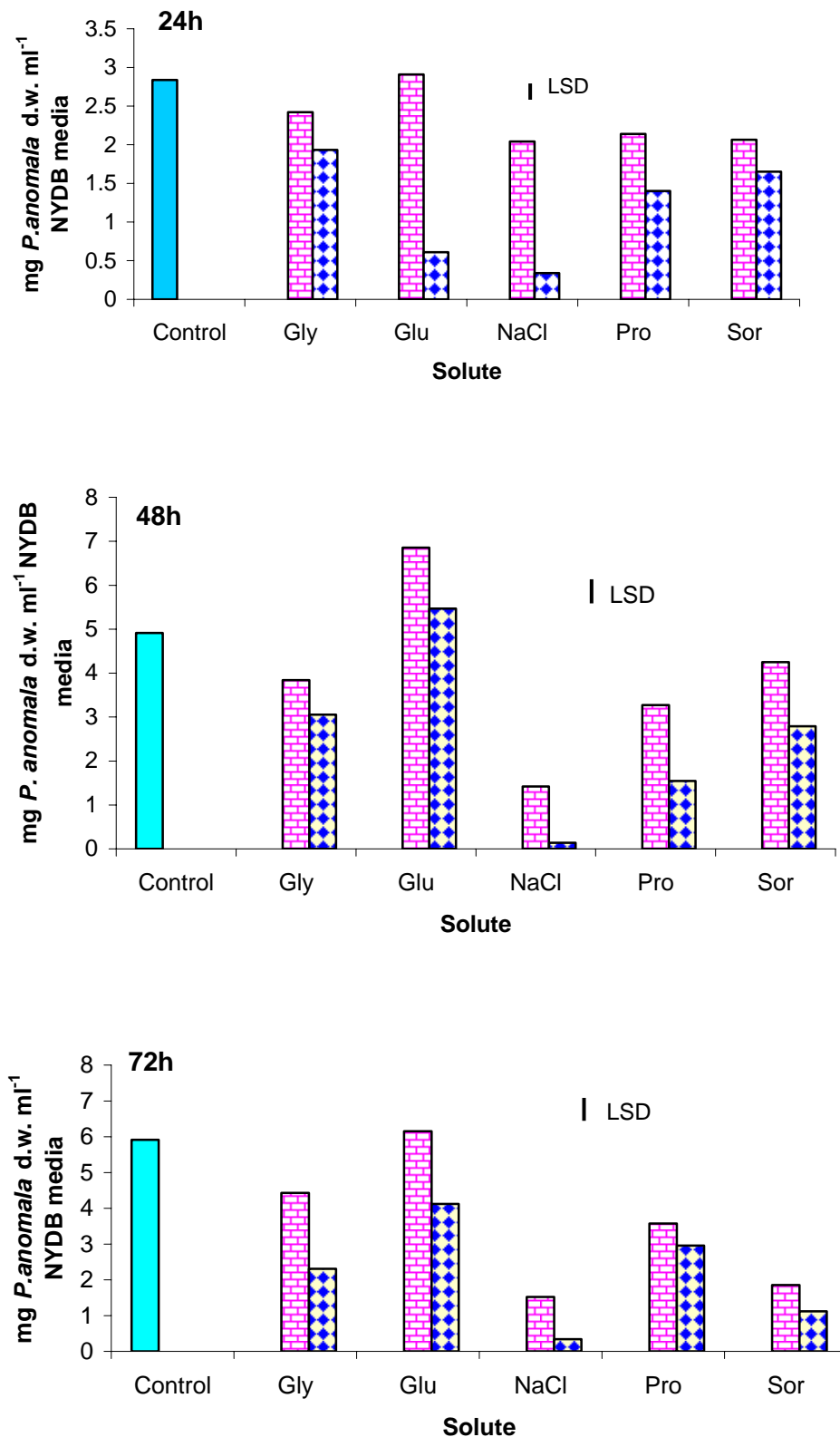
Figure 3.3. Comparison of yield (dry weight of cells ml^{-1} of NYDB media used) of *P. anomala* cells after 24, 48 and 72 h incubation at 25°C. Treatments are control (unmodified media) 0.996 aw (☐) 0.98 a$_w$ (☐) and 0.96 a$_w$ (☐). Bars=LSD (*P*<0.05).
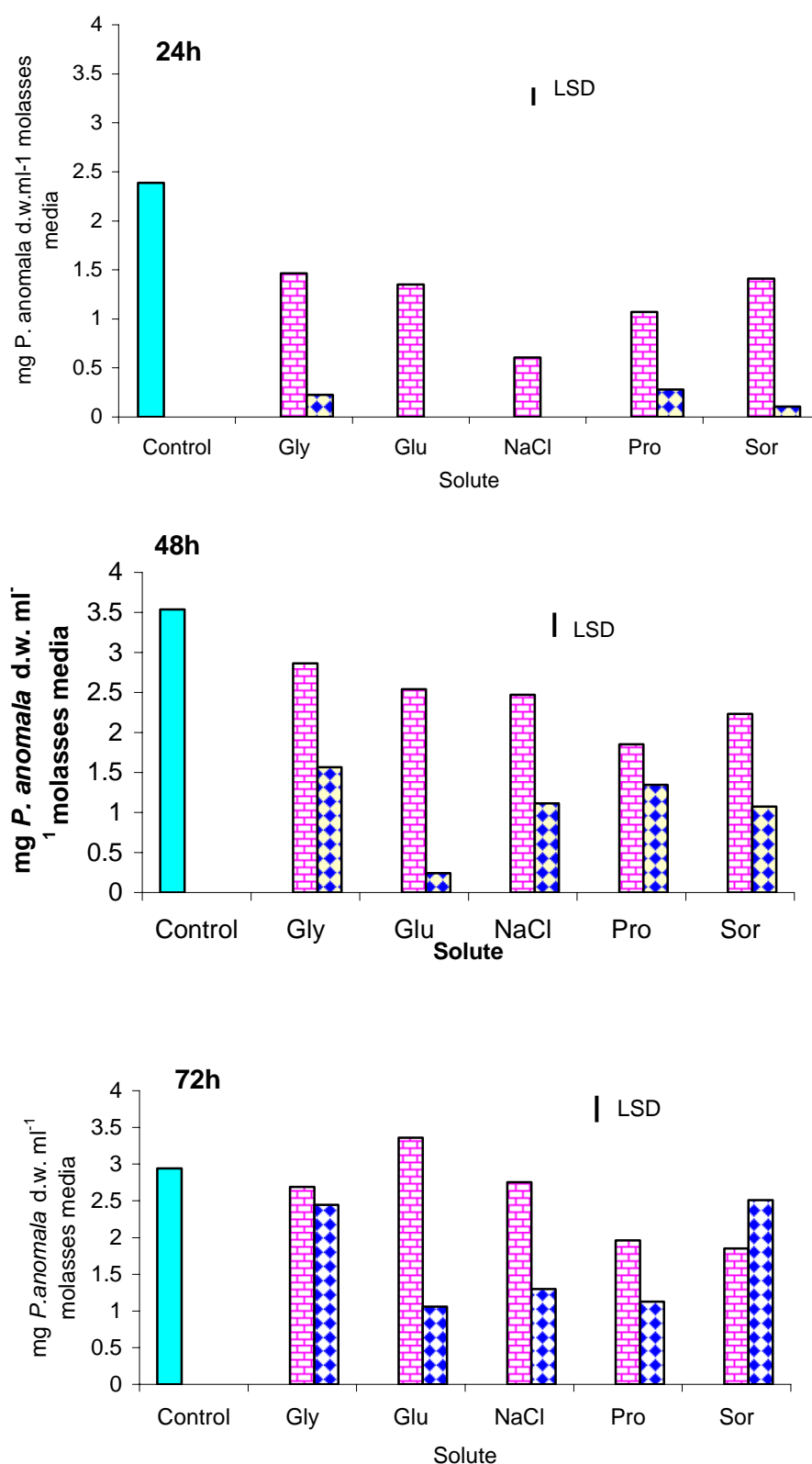
Figure 3.4. Comparison of yield (dry weight of cells ml^{-1} of molasses-based media used) of *P.anomala* cells at 24, 48 and 72 h incubation at 25°C. Treatments are control (unmodified media) 0.996 a$_w$ ( ), 0.98 a$_w$ ( ) and 0.96 a$_w$ ( ) Bars=LSD (*P*<0.05).

3.2   WATER POTENTIAL MEASUREMENTS OF *P. anomala* CELLS GROWN IN NYDB AND MOLASSES-BASED MEDIA

3.2.1   Water potential measurements of *P. anomala* cells grown in NYDB media

In order to check *P. anomala* cells response to NYDB molasses media modification with different solutes, Ψ_c measurements were taken. Results of Ψ_c measurements means are shown in Table 3.1. Statistical analysis ($P<0.05$) showed that cell water potential was affected by a three-way interaction between the three factors ($\pm$solute, a_w, different type of solute) at all time treatments (24, 48 and 72 h). For each solute tested, a decrease in a_w of the media caused a decrease in Ψ_c. Ψ_c measurements at 24 h were higher than respective measurements taken at 48 and 72 h. Yeast cells grown in unmodified NYDB medium had the highest Ψ_c value (>-0.68 MPa), while the sorbitol treatment gave the lowest value at 0.98 a_w (< -3.18 MPa). The NaCl treatment gave the lowest value at 0.96 a_w level at 72 h (- 6.04 MPa). Among the solutes tested, cells grown in the glucose-modified medium had the highest Ψ_c values followed by those grown in proline and glycerol.

3.2.2   Water potential measurements of *P. anomala* cells grown in molasses-based media

P. anomala cells response to molasses-based media modification with different solutes was investigated by taking Ψ_c measurements. Results of Ψ_c are shown in Table 3.2. Similarly with *P. anomala* cells grown in NYDB media, statistical analysis ($P<0.05$) showed that cell water potential was affected by a three-way interaction between the three factors ($\pm$solute, a_w, different type of solute) at all time treatments (24, 48 and 72 h) by solute used and water activity level of modification and that these two factors interacted for all time treatments (24, 48 and 72 h). In general, Ψ_c decreased rapidly (even after 24 h) as media a_w decreased. Yeast cells grown in unmodified molasses-based medium had the highest Ψ_c value (>-0.52 MPa), while NaCl treatments gave the lowest value at 0.98a_w (<-2.62 MPa) and at 0.96a_w (<-4.48 MPa). Of the solutes tested, cells grown in sorbitol-modified media had the highest Ψ_c value followed by proline and glucose.

Table 3.1. Means of cell potential, Ψ_c, (MPa) of *P. anomala* cells taken after incubation for 24, 48 and 72 h at 25°C in modified and unmodified NYDB media. Each value is the mean ±SE.

aw	Solute	Incubation Time (h)		
		24 h	**48 h**	**72 h**
0.996	Control	-0.53±0	-0.68±0.054	-0.62±0.040
0.98	Glycerol	-1.98±0	-2.98±0	-2.60±0
	Glucose	-0.783±0.012	-0.87±0.006	-0.99±0.013
	NaCl	-2.249±0.065	-2.25±0.065	-3.27±0
	Sorbitol	-3.61±0	-3.67±0.056	-3.18±0
	Proline	-2.97±0	-2.95±0	-2.89±0.
0.96	Glycerol	-3.004±0.032	-5.00±0.032	-4.91±0.037
	Glucose	-2.419±0	-3.42±0	-3.51±0.008
	NaCl	-3.041±0.014	-3.46±0.019	-6.04±0.082
	Sorbitol	-4.09±0	-5.02±0	-4.24±0
	Proline	-4.77±0.028	-4.73±0	-4.53±0
LSD (*P*<0.05)		**0.0709**	**0.185**	**0.797**

Table 3.2. Means of cell potential, Ψ_c, (MPa) of *P. anomala* cells taken after incubation for 24, 48 and 72 h at 25°C in modified and unmodified cane molasses media. Each value is the mean ±SE.

aw	Solute	Incubation Time (h)		
		24 h	48 h	72 h
0.996	Control	-0.48±0.007	-0.46±0.015	-0.52±0.015
0.98	Glycerol	-2.76±0.000	-2.61±0.038	-2.59±0.038
	Glucose	-2.72±0.051	-2.85±0.019	-2.34±0.000
	NaCl	-2.62±0.069	-2.87±0.000	-2.62±0.000
	Sorbitol	-2.56±0.039	-2.94±0.000	-2.66±0.040
	Proline	-2.60±0.000	-2.59±0.000	-2.10±0.020
0.96	Glycerol	-4.33±0.077	-4.14±0.000	-4.68±0.000
	Glucose	-4.44±0.077	-4.48±0.000	-4.45±0.000
	NaCl	-4.48±0.000	-5.11±0.033	-5.06±0.019
	Sorbitol	-3.78±0.000	-4.00±0.000	-4.18±0.000
	Proline	-4.45±0.039	-4.32±0.014	-4.41±0.000
LSD (*P*<0.05)		**0.132**	**0.050**	**0.056**

3.3 VIABILITY AND WATER STRESS RESISTANCE OF *P. anomala* CELLS GROWN IN NYDB AND MOLASSES-BASED MEDIA

3.3.1 Viability of *P. anomala* cells grown in NYDB media

(a)Viability of *P. anomala* on unstressed media

The viable count of *P. anomala* cells grown in the unmodified NYDB medium was high ($>6.68 \times 10^8$ CFUs ml^{-1}) even after 24 h (Figure 3.5). Viable counts of *P. anomala* cells obtained from the 0.98 a_w NYDB medium treatments with glycerol, proline and sorbitol were higher than those obtained from the 0.96 a_w treatments. However, cells obtained from 0.96 a_w NYDB medium modified with NaCl and glucose gave higher viable counts than those obtained from the 0.98 a_w treatments. The cells from the NaCl 0.98 a_w treatment had a longer lag phase than the others examined. Statistical analysis ($P<0.05$) showed that for all times (24, 48 and 72 h) there was a 3x3 significant interaction between the three factors (±solute, a_w, different type of solute) (Appendix I).

Of the 24 h-age treatments, cells from the glucose 0.98 a_w treatment had the highest number of viable cells (2.24×10^9 CFUs ml^{-1}), followed by those of glycerol (0.98 a_w, and 0.96 $a_{w,}$); the former treatments gave significantly higher viable counts than the unmodified control treatment. For each solute tested, viable counts of the cells obtained from the 0.96 a_w treatments were lower than from 0.98 a_w, except for the NaCl treatments.

After 48 h of incubation, cells from the sorbitol (0.98 and 0.96 $a_{w)}$ treatments gave viable counts of 1.31×10^{10} CFUs ml^{-1} and 1.34×10^{10} CFUs ml^{-1} respectively. There were significantly higher than the unmodified control treatment (5.43×10^9 CFUs ml^{-1}), and were followed by glucose (0.96 and 0.98 a_w treatments). Cells obtained from the NaCl 0.98 a_w treatment were the least viable of all treatments tested.

The viability of 72 h-age cells was particularly high for some of the modified treatments used and for the unmodified control treatment. Specifically, the glycerol 0.98 a_w, and glucose 0.96 a_w treatments gave the highest viable counts (7.72×10^{11}

CFUs ml^{-1}and 7.25 x 10^{11} CFUs ml^{-1} respectively), followed by those of glycerol (0.96 a$_w$) and glucose (0.98 a$_w$). All of these treatments had more viable cells than the unmodified NYDB treatment (2.49 x 10^{11} CFUs ml^{-1}). The proline (0.98 a$_w$) treatment gave the lowest viable count of all the treatments tested. Viable counts obtained from the 0.96 a$_w$ NaCl and glucose treatments were higher than those obtained from the 0.98 a$_w$ treatments.

(b) Viability of *P. anomala* cells on stressed media

No viable cell counts were obtained when *P. anomala* cells were plated on medium modified with PEG 200 to obtain different water stress treatment conditions (a$_w$ 0.935-0.945).
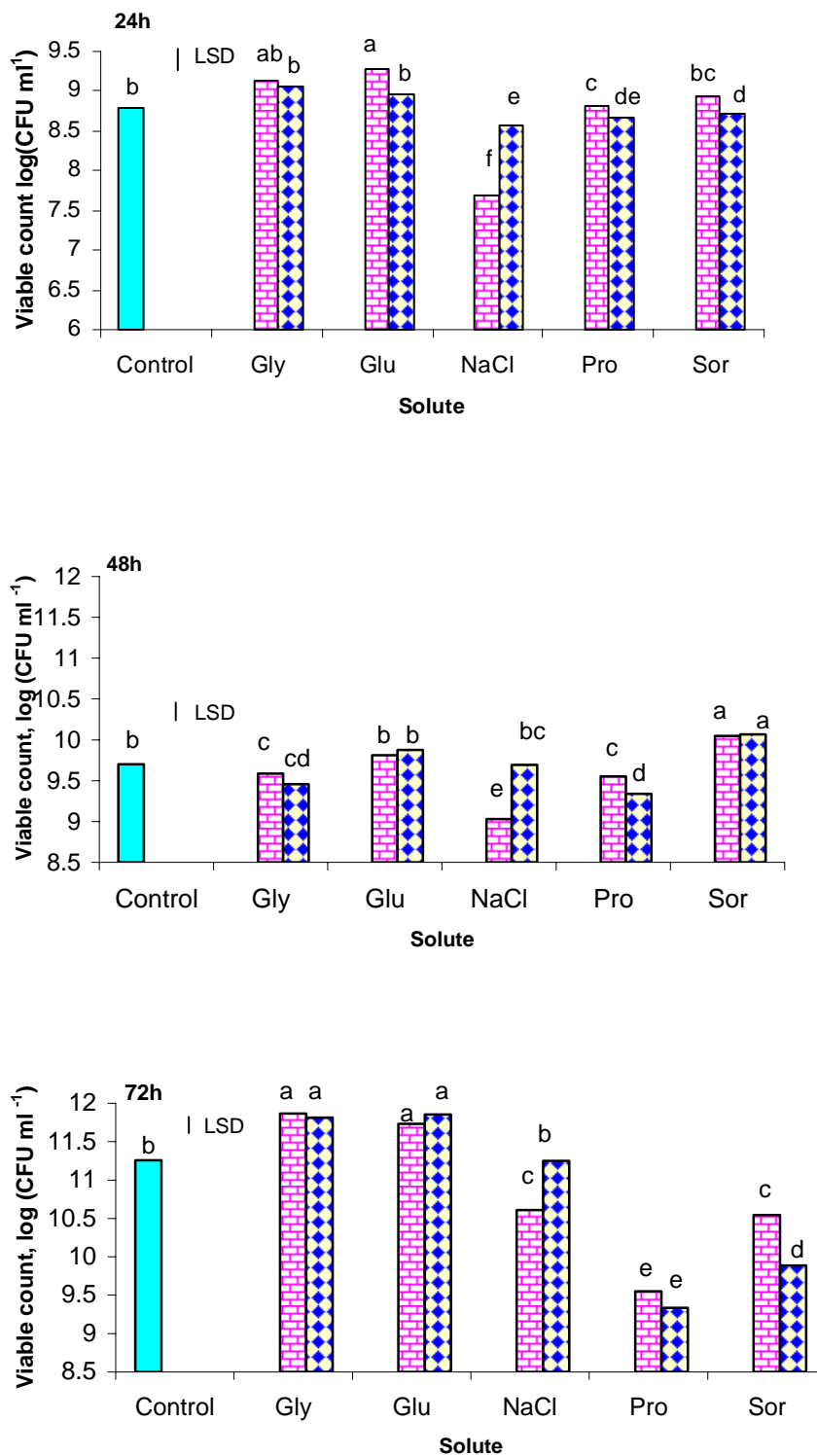
Figure 3.5. Viability of *P. anomala* cells of each NYDB treatment on unstressed (0.995 a_w) NYDA media after incubation at 25°C for 24, 48 and 72 h. Glycerol, glucose, NaCl, proline and sorbitol were used to adjust media a_w at levels 0.98 (▦) and 0.96 (▨) while unmodified media had a_w level of 0.996 (▨). Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).

3.3.2 Viability of *P. anomala* cells grown in molasses-based media

(a) Viability of *P. anomala* on unstressed media

Viable count of *P. anomala* cells obtained in the unmodified molasses-based medium was high (7.52×10^8 CFUs ml^{-1}) even after 24 h of incubation (Figure 3.6). Generally, viable counts of *P. anomala* cells obtained from the 0.98 a_w molasses-based medium treatments were higher than those from the 0.96 a_w treatments regardless of the solute used. The cells from all the 0.96 a_w NaCl, sorbitol and proline treatments had a longer lag phase than the others examined. Statistical analysis ($P<0.05$) showed that for all times (24, 48 and 72 h) there was a 3x3 significant interaction between the three factors ($\pm$solute, a_w, different type of solute) (Appendix I).

Of the 24 h-old treatments cells from the glycerol 0.98 a_w treatment had the highest number of viable cells (1.17×10^9 CFUs ml^{-1}) followed by the NaCl 0.98 a_w treatment (9.4×10^8 CFUs ml^{-1}) with both being significantly higher than those from the unmodified medium. For each solute tested, viable counts of the cells obtained from the 0.96 a_w treatments were lower than those from 0.98 a_w.

Of the 48 h-old treatments, cells from the glycerol 0.98 and 0.96 and proline 0.96 a_w treatments gave the highest viable cell counts but were not significantly better than those of the unmodified molasses control medium. Viable counts of *P. anomala* cells from the 0.96 a_w treatments of sorbitol, NaCl and proline were higher than those from the 0.98 a_w treatments. Cells obtained from the sorbitol 0.98 a_w treatment were the least viable of all the treatments tested.

For the 72 h cells, cell viability was higher than 1.21×10^{10} CFUs ml^{-1} for 0.98 a_w and 1.06×10^{10} CFU ml^{-1} for 0.96 a_w treatments, in both cases resulting from sorbitol-modified molasses media. Proline 0.96 and NaCl 0.98 a_w (2.93×10^{10} CFUs ml^{-1} and 2.87×10^{10} CFUs ml^{-1}, respectively) treatments gave the highest viable counts followed by the 0.96 a_w glycerol and NaCl ones. All of them together with 0.98 a_w glycerol, proline and glucose treatments were higher than the unmodified molasses control medium (1.28×10^{10} CFUs ml^{-1}).

(b) Viability of *P. anomala* on stressed media

In order to examine tolerance of the yeast cells to low a_w, different ages of cells were plated onto 0.96 a_w NYDA medium modified with PEG 200 (1.25M). Viable counts of yeast cells obtained in the unmodified molasses medium was high (6.88 x 10^{10} CFUs ml^{-1}) even after 24 h (Fig. 3.7). The cells from the 0.96 a_w NaCl, sorbitol and praline treatments had a longer lag phase than the others examined. Statistical analysis (*P*<0.05) showed that for 24 and 48 h, there was a 3x3 significant interaction between the three factors (±solute, a_w, different type of solute). For 72 h age cells, the two-way interaction between ±solute and different type of solute was significant (Appendix I).

Of the 24 h *P. anomala* cells, those from the glycerol 0.98 and 0.96 a_w treatments gave significantly higher viable counts than the unmodified control. For each solute used, viability of *P. anomala* cells from the 0.98 a_w treatments were higher than those from the 0.96 a_w treatments. The NaCl 0.96 a_w treatment gave the lowest viable count (1.33 x 10^7 CFUs ml^{-1}).

After 48 h incubation, cells from the 0.98 a_w NaCl, glycerol and glucose treatments and glucose 0.96 a_w one gave the highest viable counts. However, none of them was significantly higher than the one from the unmodified molasses medium (1.72 x 10^{10} CFUs ml^{-1}). The sorbitol 0.98 a_w treatment gave the lowest viable count (4.67 x 10^9 CFUs ml^{-1}).

For the 72 h cells, viability was > 9.33 x 10^9 CFUs ml^{-1}, (sorbitol 0.98 a_w treatment). All treatments except sorbitol 0.98 and 0.96 a_w and glycerol 0.98 a_w treatments resulted in significantly higher viable counts than the unmodified control; NaCl 0.98 and 0.96 a_w and proline 0.96 a_w gave the highest ones. Viable counts of *P. anomala* cells obtained from 0.96 a_w proline, glycerol and sorbitol treatments were significantly higher than those from the 0.98 a_w respective ones. In the NaCl 0.98 and 0.96, proline 0.98, glucose 0.96 and sorbitol 0.96 a_w treatments, viable counts of the yeast cells were higher when the latter were plated on stressed (0.96 a_w) than when they were plated on unstressed (0.995 a_w) NYDA medium.
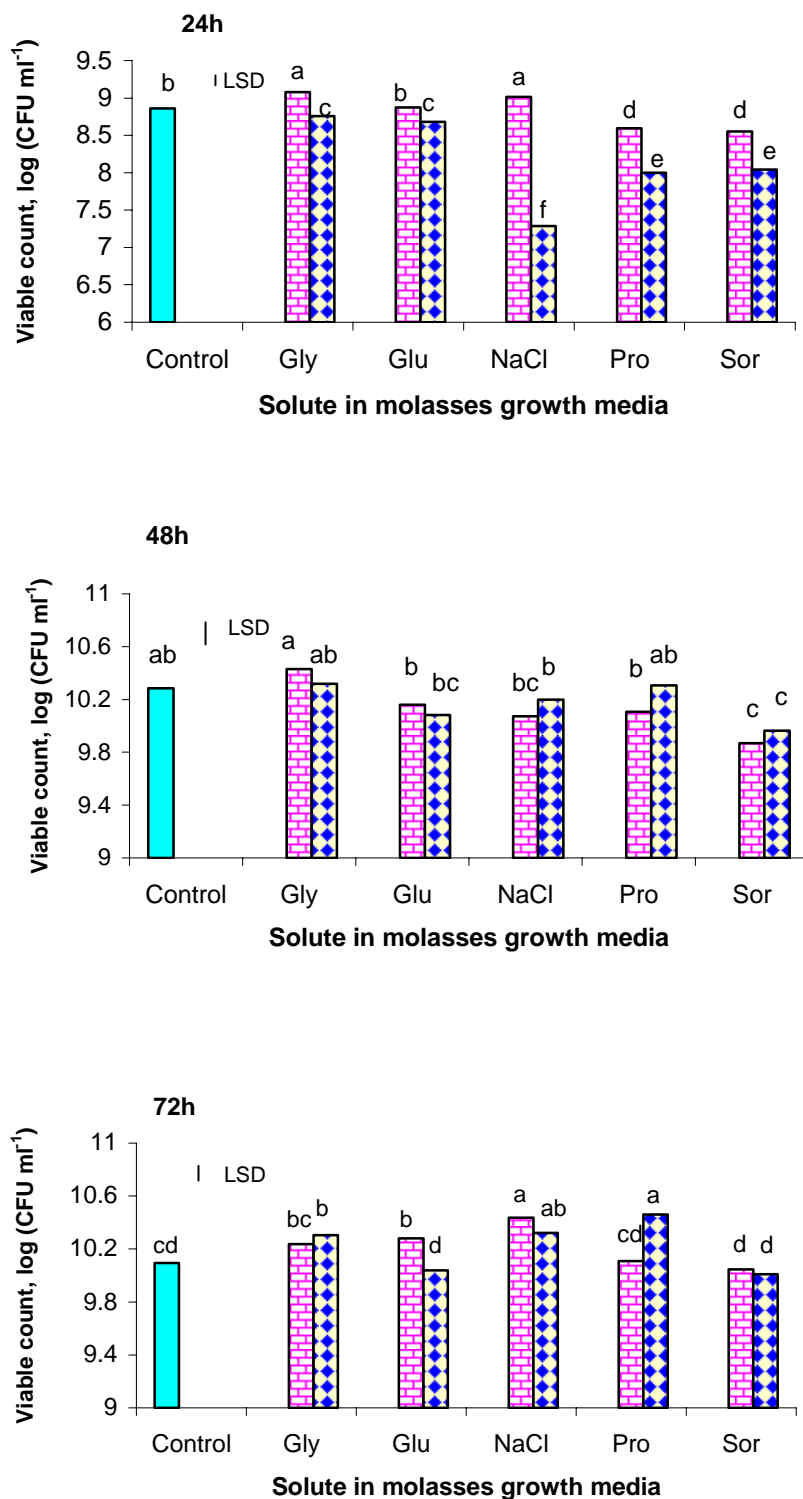
Figure 3.6. Viability of *P. anomala* cells of each (molasses-based media) treatment on unstressed (0.995 a$_w$) NYDA media. Molasses media were unmodified (control ▢) or modified at 0.98 a$_w$ (▤) and 0.96 a$_w$ (▨) Cells were incubated at 25°C for 24, 48 and 72 h. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).
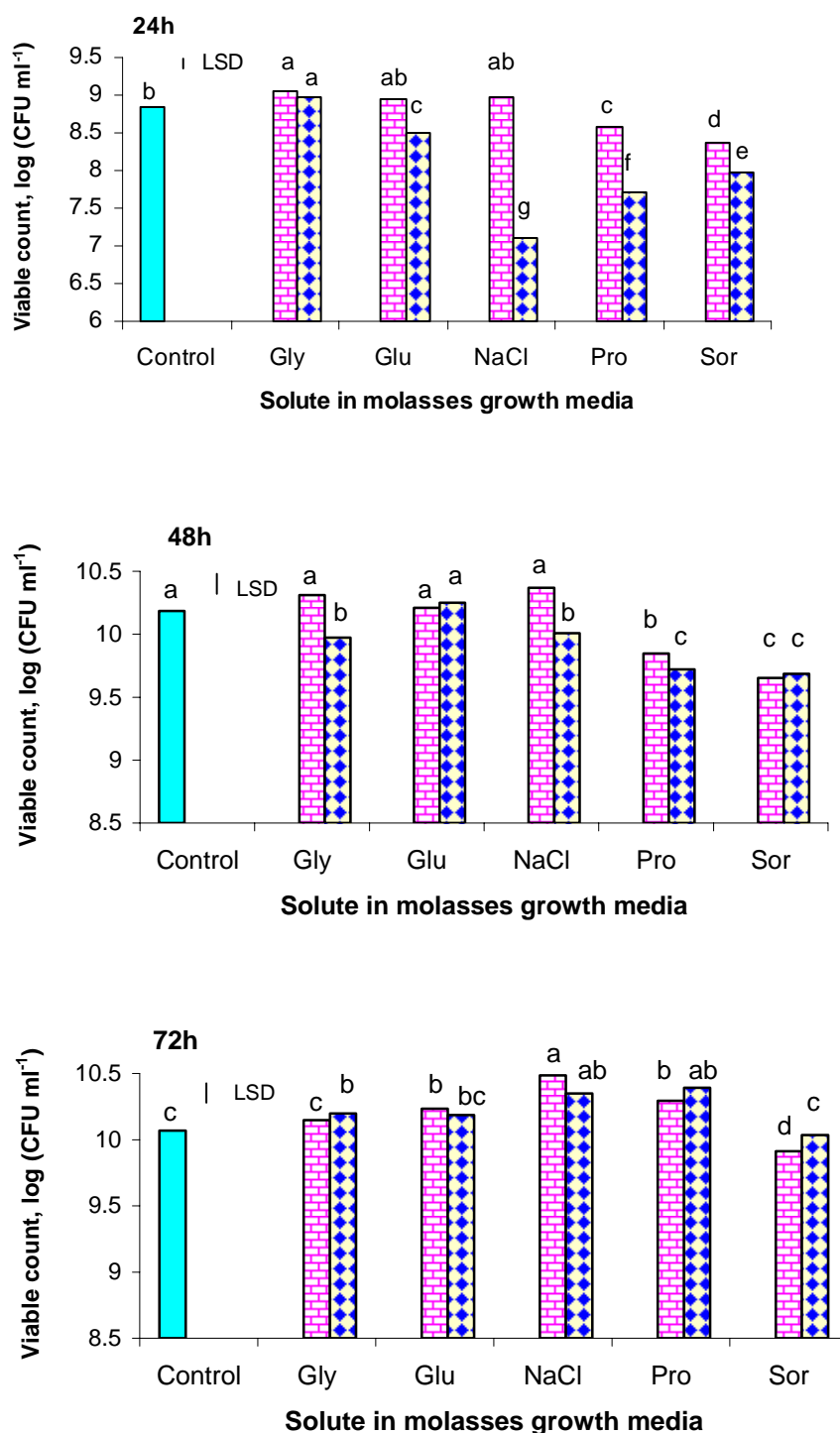
Figure 3.7. Viability of *P. anomala* cells of each (molasses-based media) treatment on water stressed (0.96 a$_w$) NYDA media. Molasses media were unmodified (control ■) or modified at 0.98 a$_w$ (▦) and 0.96 a$_w$ (◪). Cells were incubated at 25°C for 24, 48 and 72 h. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LDS (*P*<0.05).

3.4 POLYOLS AND SUGARS IN *P. anomala* CELLS GROWN IN NYDB AND MOLASSES-BASED MEDIA

3.4.1 Intracellular sugar and polyol accumulation in *P. anomala* cells grown in NYDB media

The ratios of individual sugars (trehalose and glucose) and sugar alcohols (glycerol, erythritol, arabitol and mannitol) accumulated in *P. anomala* cells after 72 h of incubation are shown in Fig. 3.8. Quantities of sugars and polyols are shown on Table 3.3. Statistical analysis ($P<0.05$) showed that, for all but mannitol, there was a 3x3 significant interaction between the three factors ($\pm$solute, a_w, different type of solute).

In the unmodified NYDB medium, small quantities of erythritol and arabitol were found to be intracellularly accumulated. In glycerol treatments, glycerol was found to be the predominant polyol accumulated; significant accumulation of arabitol also occurred. In glucose treatment, arabitol was the predominant compatible solute observed. In the ionic solute (NaCl) treatment, arabitol was found to be the predominant intracellular compatible solute followed by small quantities of glycerol in the 0.98 a_w treatment. In the 0.96 a_w treatment there was a change with more glycerol accumulation and arabitol reduced by half. For the sorbitol 0.98 a_w treatment, glycerol and arabitol were found in large quantities while in the 0.96 a_w treatment, erythritol became the predominant compatible solute followed by arabitol. Arabitol was the predominant compatible solute for both proline treatments.

In the unmodified NYDB medium trehalose was found to be the predominant intracellular compatible compound. Trehalose was detected in cells grown in proline (0.98 a_w) modified media in quantities comparable to those of cells grown in the unmodified media; smaller quantities of trehalose were detected in proline 0.96 and glucose 0.98 a_w treatments. Marked glucose accumulation was found in the glucose 0.96 a_w and NaCl 0.98 a_w treatments.

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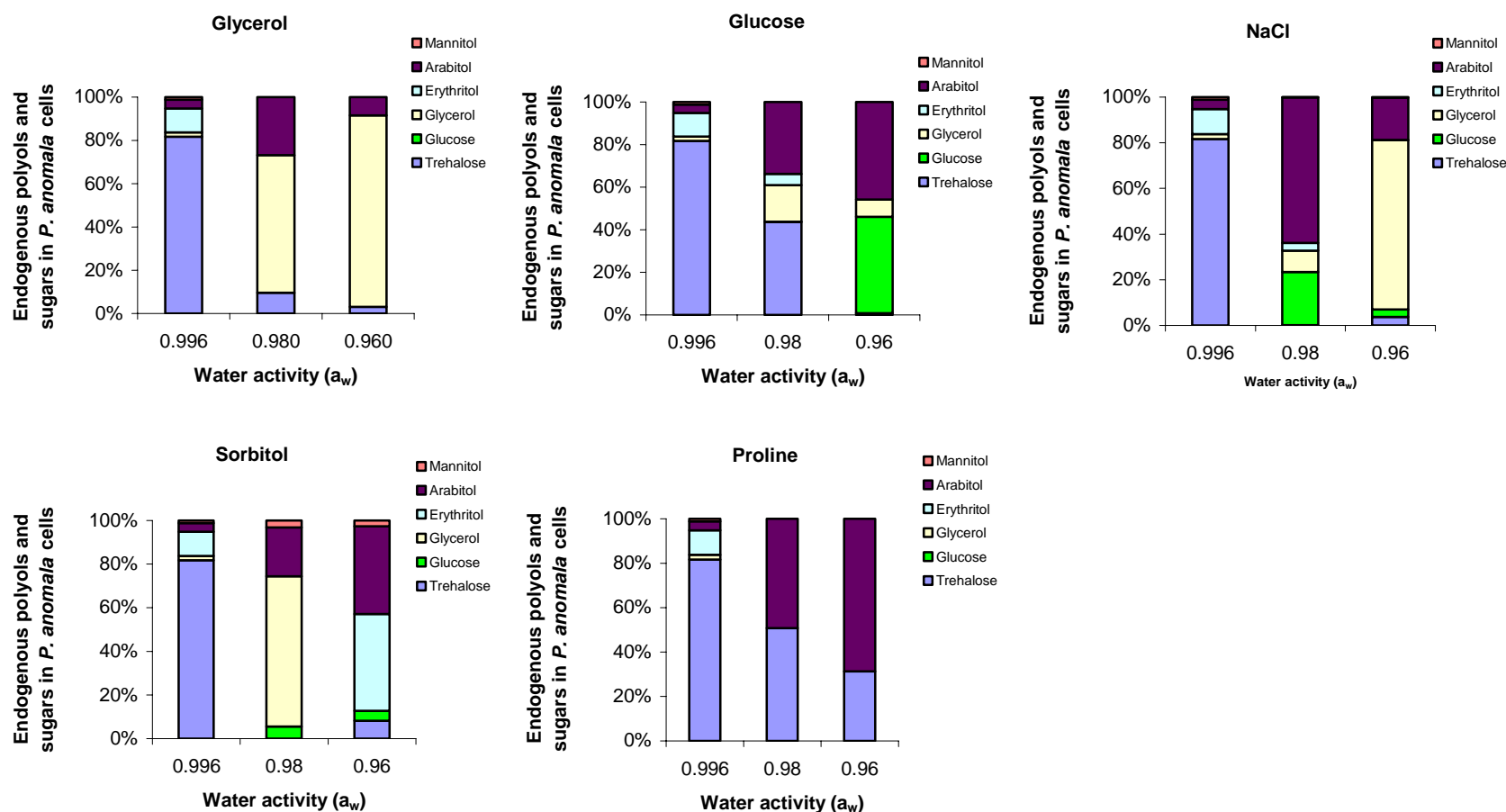
Figure 3.8. Ratio of sugars [trehalose (■) and glucose (■)] and sugar alcohols [(glycerol (□), erythritol (□) , arabitol (■) and mannitol (■)] in *P. anomala* cells grown in NYDB media unmodified (Control, 0.996 a_w) and modified with glycerol, glucose, NaCl, sorbitol and proline to achieve 0.98 and 0.96 a_w and left to incubate for 72 h. Cells were washed post-harvest with water.

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Table 3.3. Amounts of sugars and sugar alcohols (mg compatible solute g^{-1} *P. anomala* fresh weight) detected in *P. anomala* cells grown in NYDB media unmodified (Control, 0.996 a$_w$) and modified with glycerol, glucose, NaCl, sorbitol and proline to achieve 0.98 and 0.96 a$_w$ and left to incubate for 72 h. Cells were washed post-harvest with water. All data are means of three replicates per treatment. Least significant differences (LSD) (*P*<0.05) between values from different treatments are shown for each solute.

aw	Solute	trehalose	glucose	glycerol	erythritol	arabitol	mannitol
0.996	Control	33.57	0	0.845	4.532	1.652	0.482
0.98	Glycerol	11.13	0	73.74	0	31.12	0
	Glucose	18.662	0.08	7.32	2.25	14.31	0.02
	NaCl	0.03	6.01	2.4	0.89	16.47	0.03
	Sorbitol	0	1.35	16.73	0	5.42	0.76
	Proline	33.43	0	0	0	32.36	0
0.96	Glycerol	4.74	0	137.91	0	13.65	0
	Glucose	1.1	60.55	10.90	0	61.26	0
	NaCl	1.67	1.48	33.71	0.04	8.4	0.12
	Sorbitol	2.73	1.53	0	14.89	13.47	0.86
	Proline	18.62	0	0	0	40.74	0
P		**	**	**	**	**	NS
LSD (*P*<0.05)		4.564	1.871	14.06	2.525	4.955	n/a

3.4.2 Intracellular sugar and polyol accumulation in *P. anomala* cells grown in molasses-based media when washed post-harvest with water

The ratios of individual sugars and sugar alcohols accumulated in *P. anomala* cells after 72 h of incubation when washed with water are shown in Fig. 3.9-3.10a. Quantities of sugars and polyols are shown on Table 3.4. Statistical analysis ($P<0.05$) showed that, for all but mannitol, there was a 3x3 significant interaction between the three factors (±solute, a_w, different type of solute).

In the unmodified molasses-based medium, arabitol, with small quantities of erythritol and glycerol were accumulated. In glycerol treatments, glycerol was found to be the highest polyol accumulated; arabitol accumulation was also noticed In glucose treatments, glycerol was present in large quantities; arabitol was also found to be accumulated in the cells. In the NaCl treatment, arabitol was found to be the predominant intracellular compatible solute followed by small quantities of glycerol for 0.98 a_w treatment; in the 0.96 a_w treatment this changed and glycerol was found to be present in large quantities with arabitol reduced by almost half. For sorbitol 0.98 a_w treatment, arabitol and glycerol were accumulated; in the 0.96 a_w treatment, glycerol was intensely synthesized and arabitol was also in large quantities. Proline treatments also accumulated large quantities of arabitol and glycerol. Very little mannitol was accumulated and no significant differences were found.

In the unmodified molasses-based medium trehalose was found to be the predominant intracellular compatible solute. High trehalose was also found in all treatments being predominant in glucose and sorbitol 0.98 a_w ones. Most notably, trehalose was found to be significantly accumulated in proline 0.98 and 0.96 a_w treatments. In all treatments, glucose accumulation was not found.

3.4.3 Intracellular sugar and polyol accumulation in *P. anomala* cells grown in molasses-based media when washed post-harvest with isotonic solution

The ratios of individual sugars and sugar alcohols accumulated in *P. anomala* cells after 72 h of incubation when washed with PEG 200 isotonic solution are shown in Fig. 3.9-3.10b. Quantities of sugars and polyols are shown on Table 3.4. Statistical analysis ($P<0.05$) showed that, for all but mannitol, there was a 3x3 significant

interaction between the three factors (±solute, different type of solute, washing treatment).

For almost all, but glycerol 0.98 a_w, treatments, use of isotonic solution to post-harvest cell wash resulted in significantly increased glycerol retained intracellularly. The highest glycerol accumulation was found in glycerol 0.96 a_w treatments washed both with water and isotonic solution, and the lowest in NaCl 0.98 a_w washed with water and control treatment.

Regarding intracellular arabitol retained, for all but glycerol 0.98 a_w, NaCl 0.96 a_w and sorbitol 0.96 a_w treatments, use of isotonic solution resulted in significantly increased arabitol retained. The highest arabitol accumulation occurred in NaCl 0.98 a_w treatment, followed by accumulated proline 0.96 a_w, when isotonic solution was used as post-harvest treatment.

For all but, glucose 0.98 a_w, glycerol 0.98 a_w and 0.96 a_w treatments, use of isotonic solution resulted in significantly increased trehalose intracellular retention. NaCl 0.96 a_w and proline 0.98 a_w treatments retained the highest trehalose amounts followed by NaCl 0.98 a_w treatment. In the NaCl 0.98 a_w treatment, use of isotonic solution resulted in four times more trehalose retention. In proline 0.98 and 0.96 a_w treatments, the increase in trehalose retention with isotonic solution use, was two and three times more, respectively. The pattern was similar for sorbitol treatments. Only glucose 0.98 a_w, glycerol 0.98 a_w and 0.96 a_w treatments were found to retain significantly lower trehalose amounts than the control treatment.

Glycerol (a) water

Glycerol (b) ISO

Glucose (a) water

Glucose (b) ISO

NaCl (a) water

NaCl (b) ISO
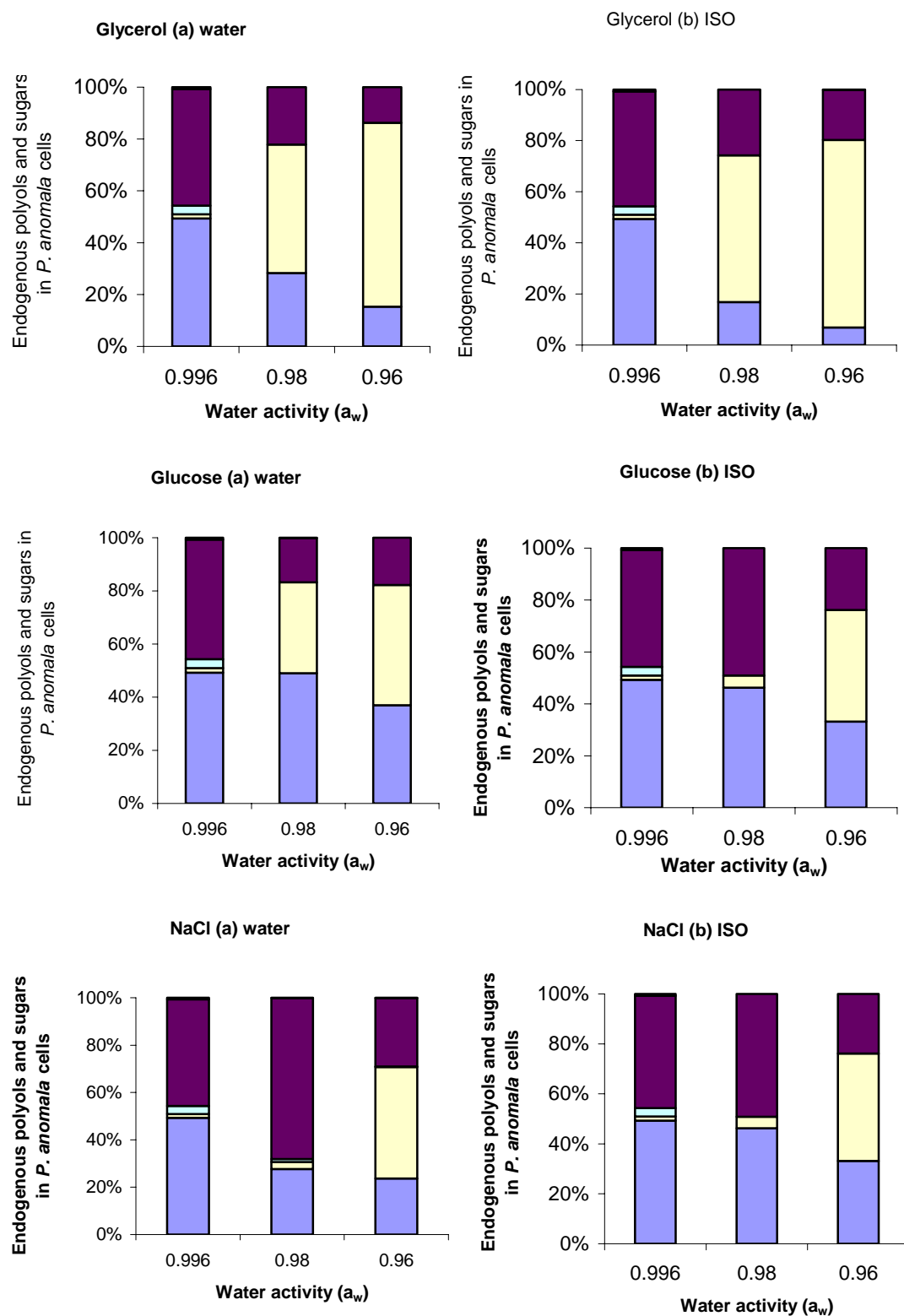
Figure 3.9. Ratio of sugars [trehalose (⬜) and glucose (🟩)] and sugar alcohols [(glycerol (⬜), erythritol (⬜), arabitol (⬛) and mannitol (🟧)] in *P. anomala* cells grown in molasses-based media unmodified (Control, 0.996 a_w) and modified with glycerol, glucose, NaCl, to achieve 0.98 and 0.96 a_w and left to incubate for 72 h. Cells were washed post-harvest with (a) water and (b) PEG 200 isotonic solution.
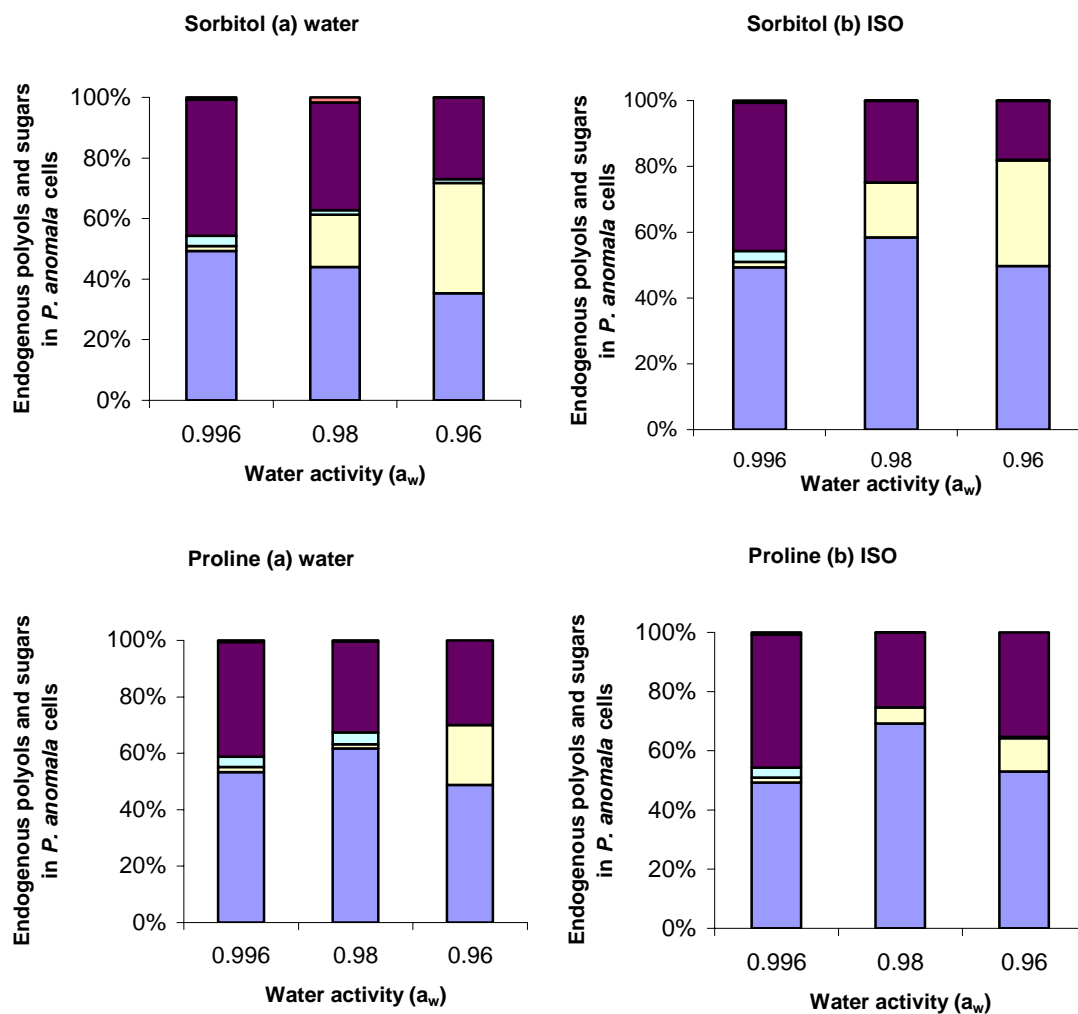
Figure 3.10 Ratio of sugars [trehalose (⬛) and glucose (⬛)] and sugar alcohols [(glycerol (⬜), erythritol (⬜) , arabitol (⬛) and mannitol (⬛)] in *P. anomala* cells grown in molasses-based media unmodified (Control, 0.996 a_w) and modified with sorbitol and proline to achieve 0.98 and 0.96 a_w and left to incubate for 72 h. Cells were washed post-harvest with (a) water and (b) PEG 200 isotonic solution.

Table 3.4. Amounts of sugars and sugar alcohols (mg compatible solute g^{-1} *P. anomala* fresh weight) detected in *P. anomala* cells grown in molasses-based media unmodified (Control, 0.996 a_w) and modified with glycerol, glucose, NaCl, sorbitol and proline to achieve 0.98 and 0.96 a_w and left to incubate for 72 h. Cells were washed post-harvest with (a) water and (b) PEG 200isotonic solution. All data are means of three replicates per treatment. Least significant differences (LSD) (*P*<0.05) between values from different treatments are shown for each solute.

aw	Solute	trehalose	glucose	glycerol	erythritol	arabitol	mannitol
0.996	Control	34.88	0	1.19	2.36	31.85	0.498
0.98	Glycerol	41.87	0	73.3	0	32.76	0
	ISO	**24.4**	**0**	**83.3**	**0**	**37.3**	**0.01**
	Glucose	55.93	0	38.97	0	18.99	0.15
	ISO	**13.9**	**0**	**49**	**0**	**30.7**	**0**
	NaCl	29.42	0	3.19	1.3	72.23	0.14
	ISO	**130.1**	**0**	**13.2**	**0**	**138.2**	**0**
	Sorbitol	36.34	0	14.29	1.24	29.39	1.4
	ISO	**85.6**	**0**	**24.3**	**0.1**	**36.2**	**0.16**
	Proline	61.24	0	1.47	4.14	31.85	0.49
	ISO	**134.7**	**0**	**10.5**	**0.01**	**49.1**	**0.28**
0.96	Glycerol	27.28	0	126.65	0	24.55	0
	ISO	**12.7**	**0**	**136.7**	**0**	**36.6**	**0**
	Glucose	48.41	0	59.11	0	23.24	0.06
	ISO	**113.4**	**0**	**69.1**	**0**	**42.5**	**0.05**
	NaCl	32.57	0	64.54	0.37	39.62	0.23
	ISO	**57.3**	**0**	**74.5**	**0**	**41.2**	**0**
	Sorbitol	46.83	0	48.12	0	35.6	0.169
	ISO	**89.8**	**0**	**58.1**	**0.21**	**32.3**	**0.22**
	Proline	48.36	0	21.05	0	29.68	0.05
	ISO	**123.7**	**0**	**26.3**	**0.87**	**82.4**	**0**
P		**	n/a	**	*	**	NS
LSD (P<0.05)		15.12	n/a	18.55	0.732	17.64	n/a

3.4.4   Loss of endogenous reserves of *P.anomala* cells during hypo-osmotic shock

Table 3.5 shows the amounts of sugars and polyols that were detected in the water used for washings from *P. anomala* cells. Trehalose was detected in cell washings in the sorbitol 0.96 a_w and NaCl 0.98 a_w treatments in significantly ($P<0.05$) higher amounts than the control treatment. Glucose was also significantly detected in washings of the glucose treatment in very high amounts. There was a similar trend for glycerol cell treatments with very high amounts of glycerol detected in cell washings (>13369 ppm). High glycerol amounts were also detected in washings of the glucose and NaCl cell treatments, with sorbitol and proline cell ones following. Arabitol was significantly washed out in glucose treatment cells and mannitol was found in washings of the sorbitol cell treatments.

Table 3.5. Amounts of solutes (ppm) detected in the water used to wash *P. anomala* cells after harvest. All data are means of three replicates per treatment. Least significant differences (LSD) ($P<0.05$) between values from different treatments are shown for each solute.

aw	Solute	trehalose	glucose	glycerol	erythritol	arabitol	mannitol
0.996	Control	91.3	12	52	8.7	156	9.04
0.98	Glycerol	6.3	7	13369	0	158	3.62
	Glucose	0	8187	4787	0	547	1.29
	NaCl	300	331	573	6.21	32	0
	Sorbitol	97.9	4	92	1.78	150	44.47
	Proline	30.3	40	41	2.32	123	7.15
0.96	Glycerol	0	7	23160	0	158	1.63
	Glucose	0	15933	6374	0	560	3.19
	NaCl	29.2	9	1532	2.79	0	0
	Sorbitol	301	0	461	0.36	88	36.62
	Proline	42.2	4	200	1.71	147	11.23
P		**	**	**	NS	**	**
LSD ($P<0.05$)		87.22	2528.30	1161.50	-	174.80	9.61

3.5   Storage stability of *P. anomala* cells grown in NYBD and molasses modified media when formulated as wet pastes

Storage stability of *P. anomala* cells from unmodified and modified NYDB and molasses media formulated as wet pastes and stored at 4°C and ambient (22°C) temperature are shown in figures 3.11-3.14. Cells were plated on NYDA media with (a) fully available water and (b) 0.96 a_w (water-stressed).

Storage stability of NYDB grown *P. anomala* cell treatments kept at 4°C lost viability by a factor of 0-0.7 log units within 30 days. At 90 days, viability decreased by a factor of 2-2.5 log units and by 180 days, by 3-4 log factor. After 365 days, cells lost viability by a factor of 3.5-4.5 log units, with the 0.98 a_w glycerol and glucose treatments being most viable. Cells responded in a similar way when plated on both water treatments. However, after 365 days of storage, cell viability further decreased by a 0.1-0.3 log factor when exposed to water stress (a_w).

Storage stability of molasses grown *P. anomala* cell treatments kept at 4°C lost viability by a factor of 0-0.7 log units within 30 days. At 90 days, viability dropped by a 1-1.9 log factor and at 180 days, by a 3-3.7 log factor. After 365 days, the cells had lost viability by a factor of 3.5-4.5 log units. There were no differences in final storage stability when cells were plated on water stress media; the best cell treatments were glucose 0.98 a_w and proline 0.96 a_w. In general, molasses grown cell treatments were more viable than those grown on NYDB during the first 90 days and then any advantage in storage stability was lost.

When NYDB grown *P. anomala* cell treatments were stored at 22°C, viability was rapidly lost within the first 30 days by a 1.7-2.1 log factor, 2-3 log factor at 90 days, 3.5-5 log factor at 180 days to end up at a 4-6 log factor lower viability. Viability when cells were plated on water stress media followed a similar pattern.

Storage stability of *P. anomala* cells grown on molasses modified media was much higher than that exhibited by NYDB grown cells within the first 30, being reduced by 1-1.3 log factor with NaCl 0.98 a_w treatment being an exception (viability decreased by 2 log factor). Viability then decreased by 2-3 log factors after 90 days and 4-5 log factors after 180 days. Viability after 365 days, decreased by a 5.7-6.3 log factors. It

should be noted that, even though molasses grown cells were more stable initially during storage, storage stability of NYDB grown cells tended to stabilise after 180 days, while molasses grown cells continued to lose viability.
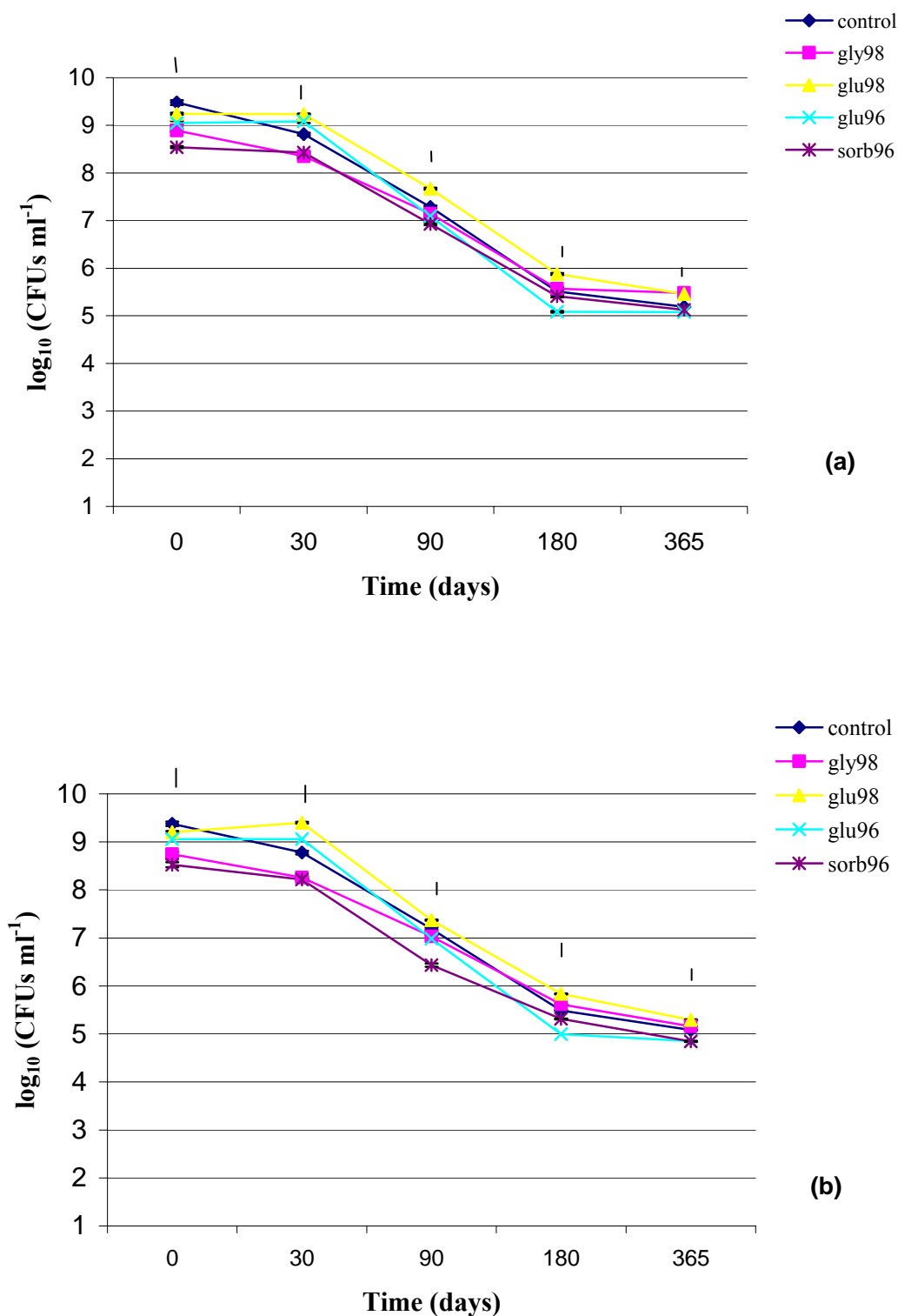
Figure 3.11. Storage stability of *P. anomala* cells grown in NYBD media unmodified (control) and modified with glycerol 0.98 a_w (gly98), glucose 0.98 a_w (glu98), glucose 0.96 a_w (glu96) and sorbitol 0.96a_w (sorb96) for 72 h at 25^oC and formulated as wet pastes. Cells were kept at fridge temperature (4^oC) and spread-plated on NYDA 0.996 a_w (a) and 0.96 a_w (b). All data are means of nine replicates per treatment.Bars=LSD (*P*<0.05).

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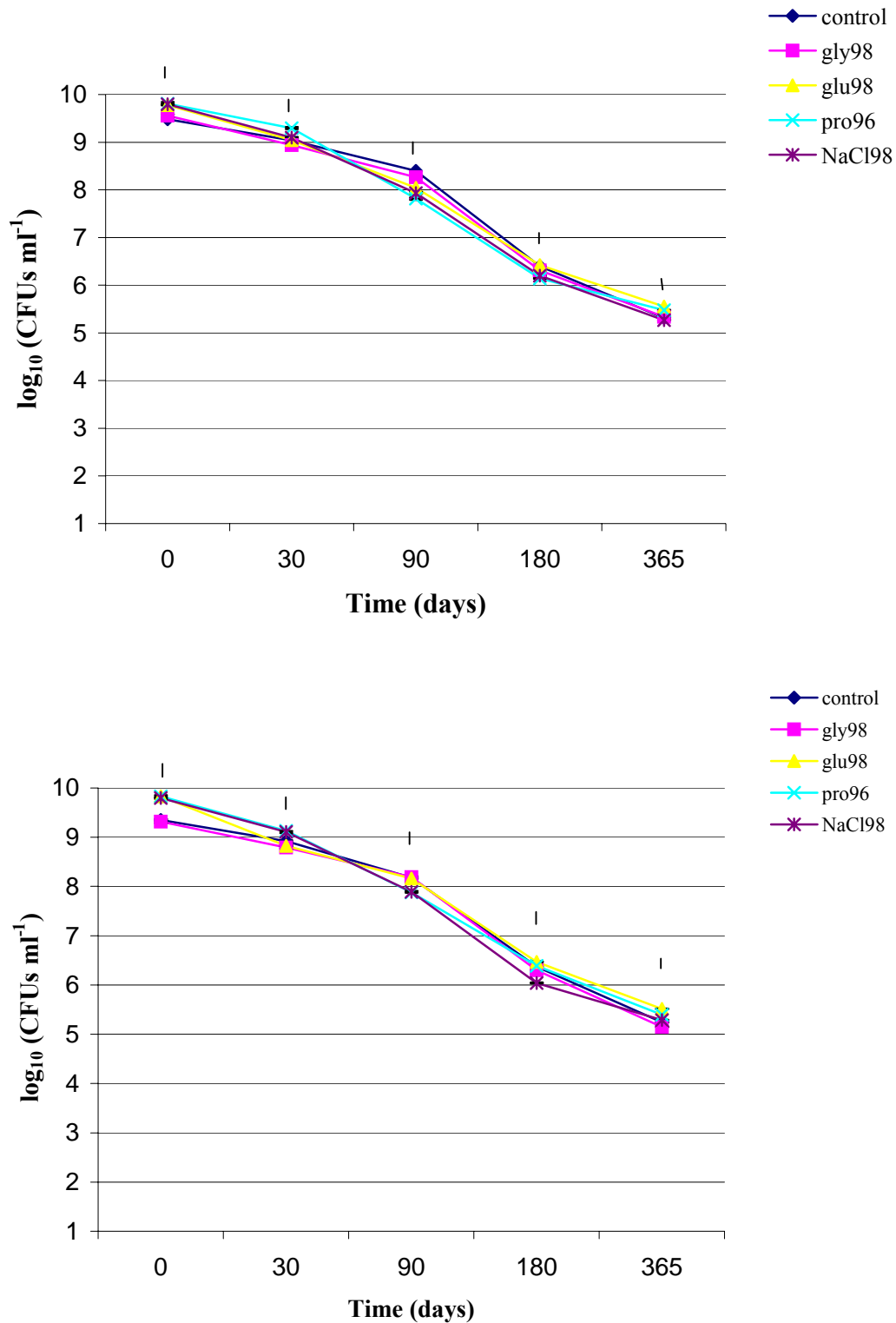
Figure 3.12. Storage stability of *P. anomala* cells grown in molasses media unmodified (control) and modified with glycerol 0.98 a_w (gly98), glucose 0.98 a_w (glu98), proline 0.96 a_w (pro96) and NaCl 0.98 a_w (NaCl98) for 72 h at 25°C and formulated as wet pastes. Cells were kept at fridge temperature (4°C) and spread-plated on NYDA 0.996 a_w (a) and 0.96 a_w (b). All data are means of nine replicates per treatment. Bars=LSD (*P*<0.05).
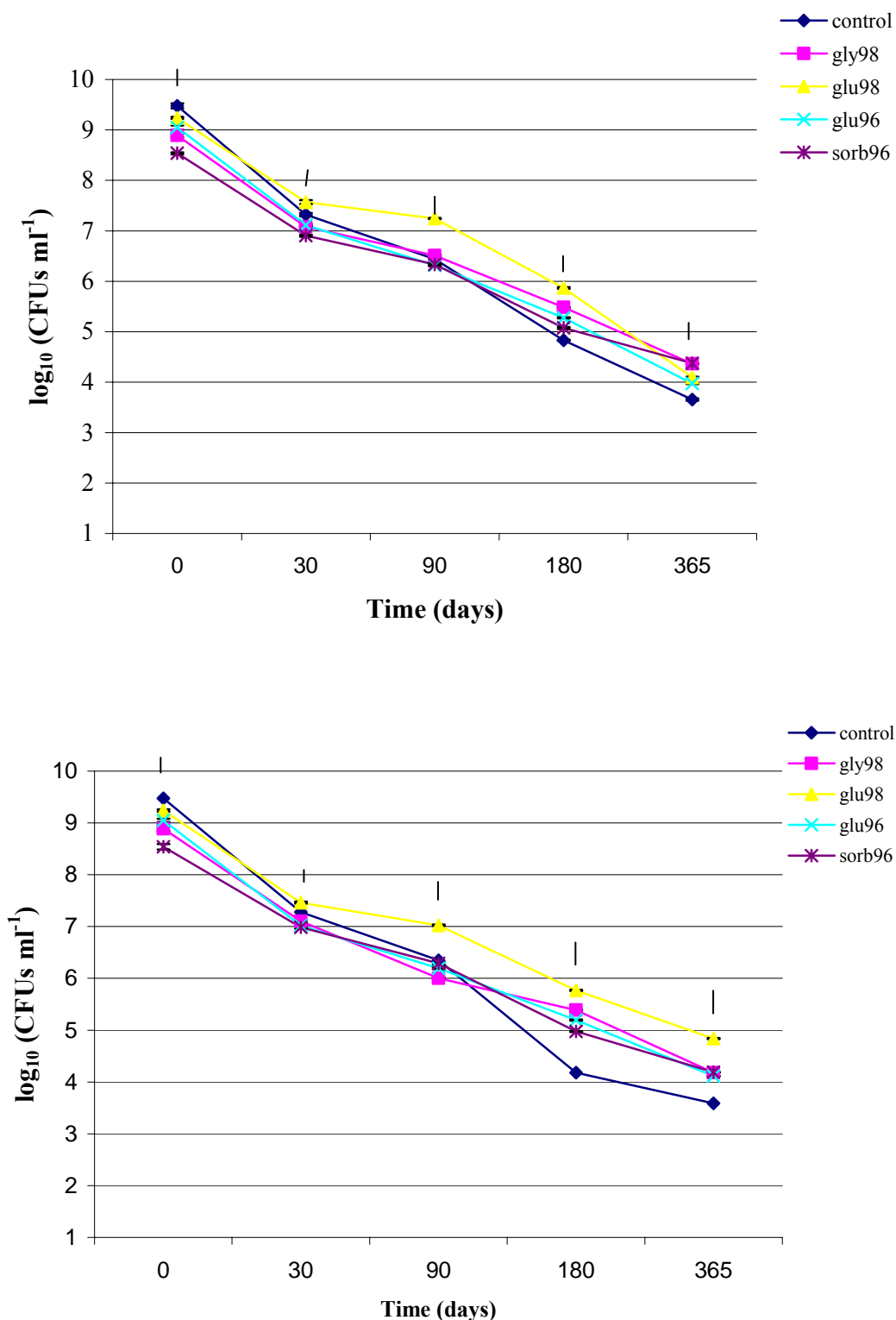
Figure 3.13. Storage stability of *P. anomala* cells grown in NYBD media unmodified (control) and modified with glycerol 0.98 a_w (gly98), glucose 0.98 a_w (glu98), glucose 0.96 a_w (glu96) and sorbitol 0.96 a_w (sorb96) for 72 h at 25°C and formulated as wet pastes. Cells were kept at ambient temperature (22°C) and spread-plated on NYDA 0.996 a_w (a) and 0.96 a_w (b). All data are means of nine replicates per treatment. Bars=LSD (*P*<0.05).
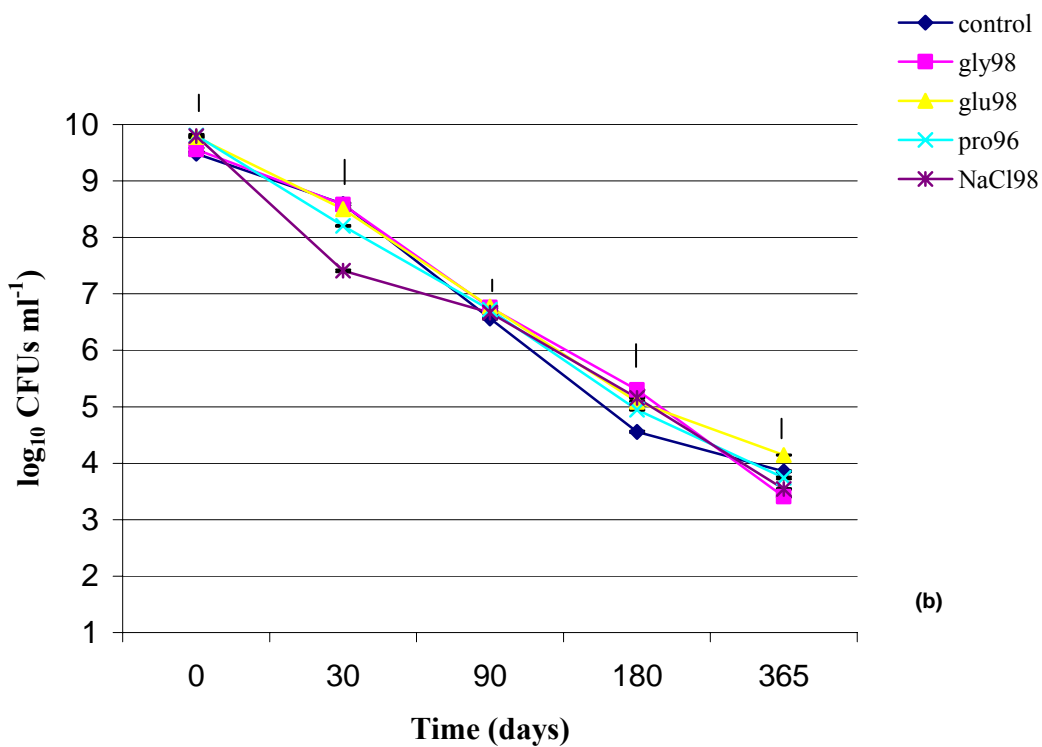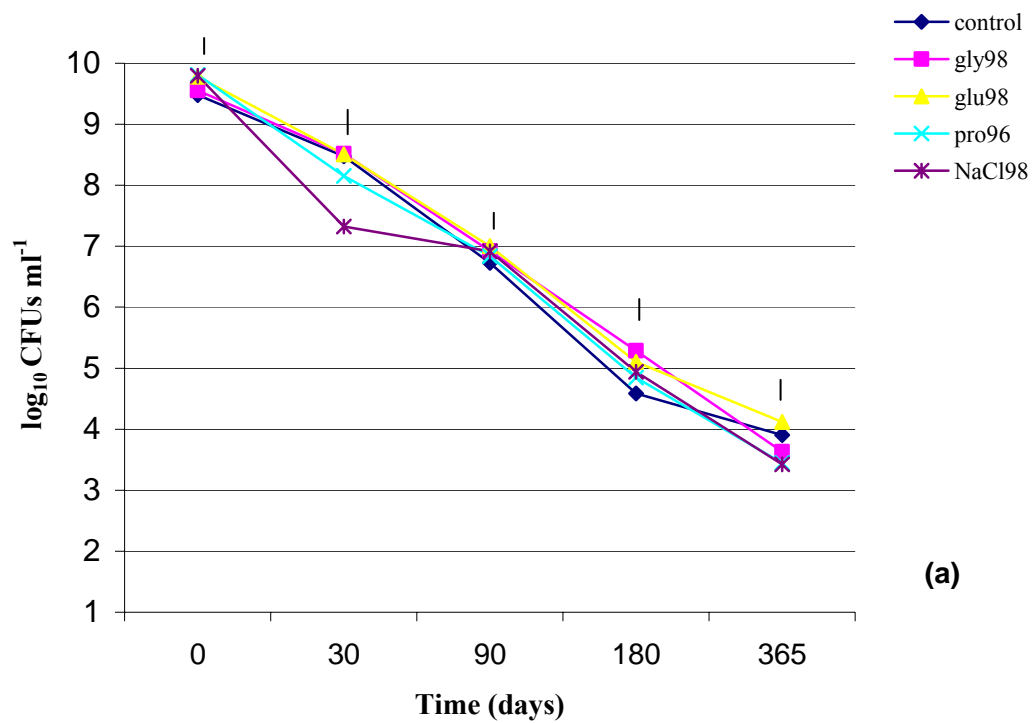
Figure 3.14. Storage stability of *P. anomala* cells grown in molasses media unmodified (control) and modified with glycerol 0.98a_w (gly98), glucose 0.98a_w (glu98), proline 0.96a_w (pro96) and NaCl 0.98a_w (NaCl98) for 72 h at 25°C and formulated as wet pastes. Cells were kept at ambient temperature (22°C) and spread-plated on NYDA 0.996a_w (a) and 0.96a_w (b). All data are means of nine replicates per treatment. Bars=LSD (*P*<0.05).

3.6 VALIDATION OF VIABLUE STAIN FOR DRYING FORMULATIONS

A rapid and reliable method to differentially determine viable yeast cells in fluidised bed drying, freeze drying and storage stability studies was required. Viablue staining method provided a great means of viability determination for *S. cerevisiae* cells (Hutchenson *et al.,* 1988). Stain was, therefore, checked with *P. anomala* cells. Figure 3.8 shows the good correlation (r=0.992) between the true viability of *P. anomala* yeast cells and "stain viability" when mixtures of dead (heat-killed) and viable yeast cells were made and tested. In order to confirm viability, total cell counts were taken and then serial dilutions of these preparations were made and scored by plate counts. All cells present in the viable preparations gave rise to viable colonies, whereas the heat-killed preparations showed zero tolerance. The authenticity of these preparations was therefore confirmed. The Viablue staining procedure, with *P. anomala* cells, behaved in an identical manner to the viability testing described above. All cells present in the viable preparation stained viable whereas all cells present in the heat–killed preparation stained as dead. Stained cells under fluorescent microscopy were scored as viable when fluorescence was restricted to a bright outline (halo), a sign that the cell membrane remained intact, whereas dead cells fluoresced brightly throughout. This technique was used for all studies with fluidised bed drying, freeze drying and formulations storage stability.

3.7 FLUIDISED BED DRYING OF *P. anomala* CELLS

3.7.1 Optimisation of fluidised bed drying procedure

Optimising conditions (temperature and time) in fluidised bed drying procedure was the first step in this study. Figure 3.16 shows the effect of temperature and duration of drying on viability and final moisture content of *P. anomala* cells using a fluidised bed-dryer. Viability decreased with increasing temperature and duration of drying. Statistical analysis (<0.05) showed a significant two-way interaction between temperature and duration of drying on cell viability (Appendix I); Viability was about 65% for cells dried at 50°C for 10 min and at 60°C for both 10 and 20 min. At 70°C, viability was dramatically decreased to about 7%.

Moisture content of dried *P. anomala* cells was significantly affected by a two-way interaction between temperature and duration of drying (Appendix I). Moisture content of cells decreased as time and drying temperature increased. Moisture content of cells was 5.85% (drying at 70°C for 20 min) and resulted in very high cell mortality. However, moisture cell content of 7.15% resulted in 66.7% cell viability (drying at 50°C for 20 min). Thus, 50°C and 20 min were used for subsequent fluidised bed drying studies.
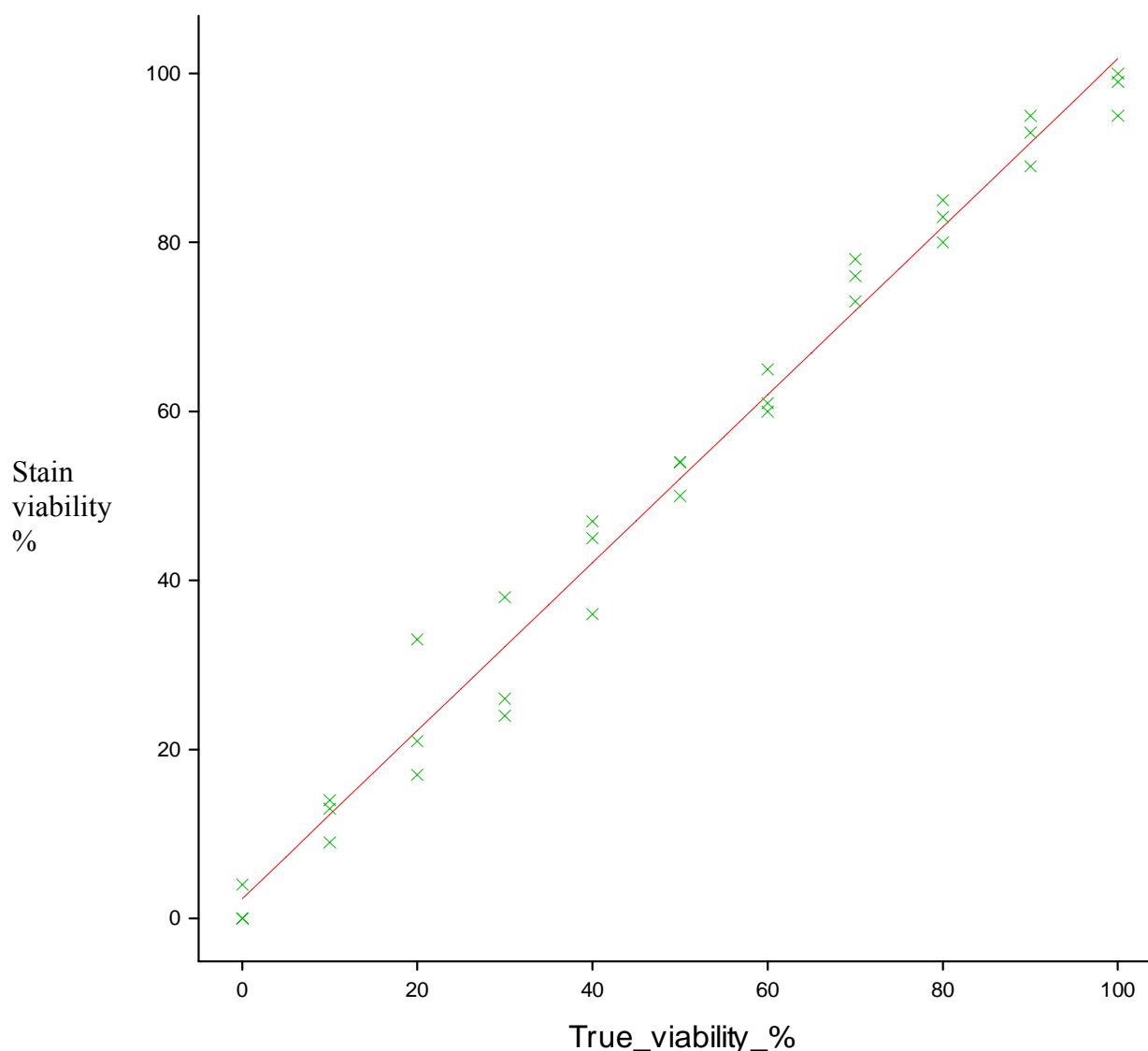
Figure 3.15. Relationship between the number of *P. anomala* cells staining viable with Viablue and the number of viable cells actually present in mixed preparations of viable and dead (heat-killed) yeast cells. The total number of yeast cells present was about 10^{9} ml^{-1}, and the number of viable yeast cells present varied from 0 to 100% as shown in the graph. Line represents fitted regression line (y = 0.9942x+2.32; r =0.992).
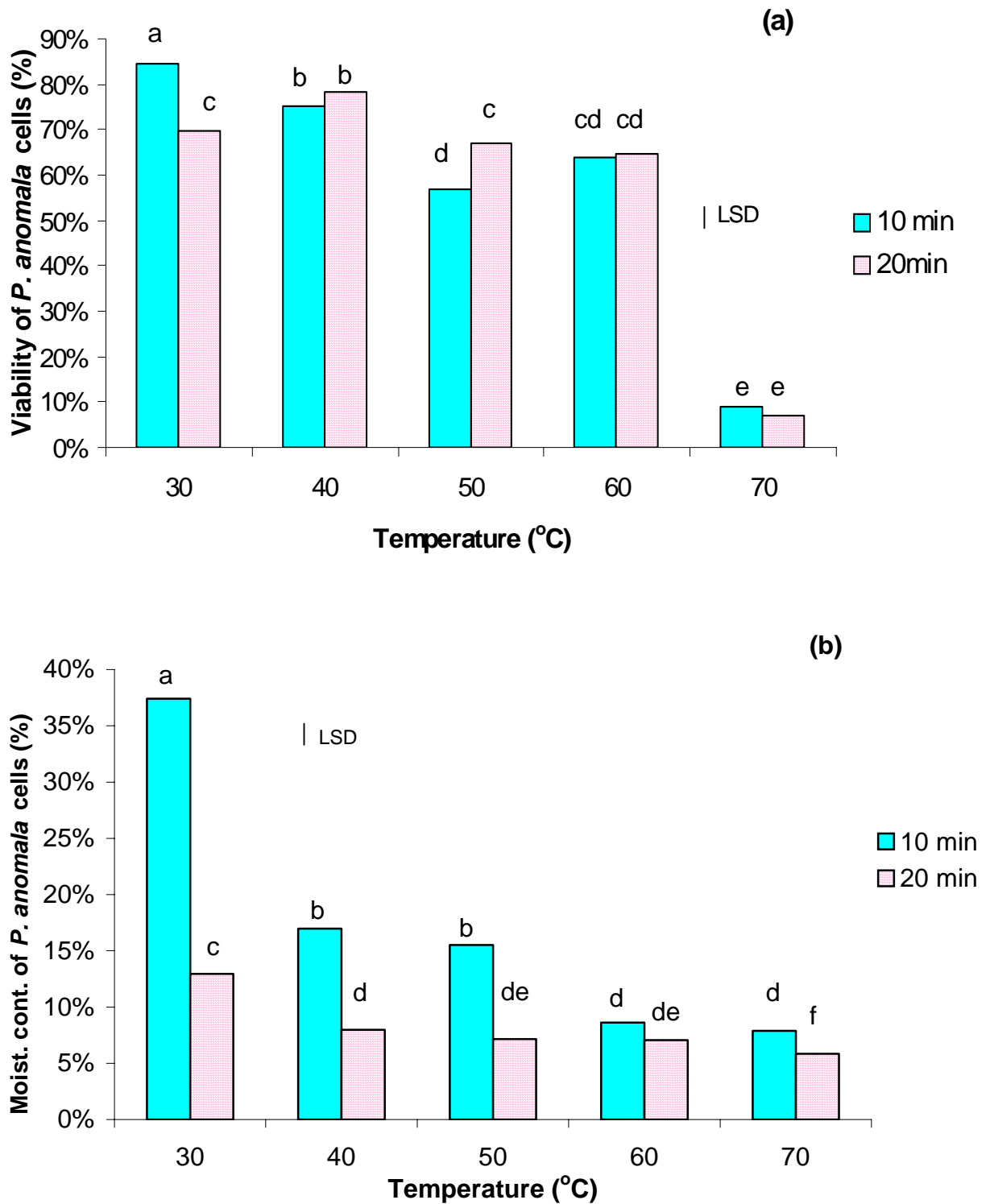
Figure 3.16. Effect of drying temperature and duration on *P. anomala* cells (a) viability and (b) final moisture content using a fluidised bed dryer. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).

3.7.2 Effect of isotonic solutions used prior to fluidised bed drying on *P. anomala* cells

P. anomala cell osmoprotection, by using different isotonic solutions, and its effect on cell tolerance against fluidised bed drying was also checked. Post-harvest washing of yeast cells grown in different molasses-modified media (NaCl $0.98a_w$, proline $0.98\ a_w$, glycerol $0.98\ a_w$, glucose $0.98\ a_w$ and sorbitol $0.96\ a_w$) with PEG 200, glucose and NaCl resulted in combined effect on cell viability and moisture content as shown in Figure 3.17. Statistical analysis ($P<0.05$) showed that there was a two-way interaction between different yeast cells and isotonic washings that significantly affected both cell viability and final moisture content.

P. anomala cell viability ranged from 2 to 70%. Use of PEG 200 post-harvest isotonic solution resulted in high cell mortality indicating a toxic effect on *P.anomala* cells. The highest cell viability occurred when *P. anomala* cells were grown in proline 0.98 a_w molasses media and washed with NaCl isotonic solution, followed by NaCl 0.98 a_w grown cells washed with water and NaCl isotonic solution. Cells grown in glycerol 0.98 a_w media had about 65% viability when washed with NaCl and glucose. Overall, use of NaCl isotonic solution resulted in either similar or higher *P.anomala* cell viability in all cell treatments when compared with post-harvest water washing treatment.

In general, washing with isotonic solutions resulted in significantly increased *P. anomala* cell moisture content. Use of isotonic glucose solution resulted in significantly increased cell moisture content and in a sticky appearance of dried cells. Interestingly, *P. anomala* grown in glucose 0.98 a_w molasses media had significantly increased moisture content and also had a sticky appearance after drying. Washing with NaCl solution resulted in similar *P. anomala* cell final moisture content regardless of cell treatment with the exception of glucose cell treatment.
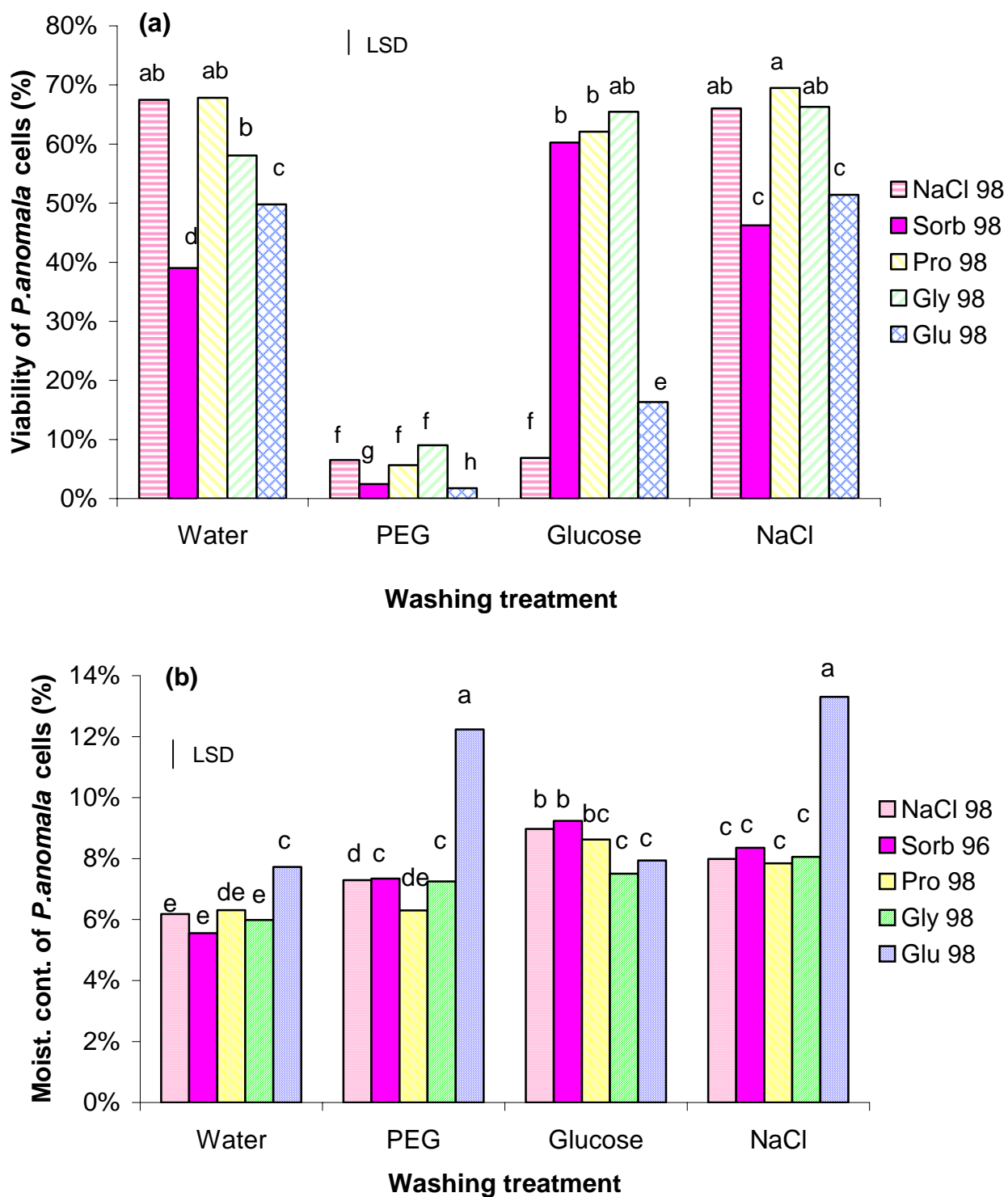
Figure 3.17. Effect of post-harvest washing treatment on *P. anomala* different treatment cells (a) viability and (b) final moisture content after drying at 50°C for 20 min using a fluidised bed dryer. Post-harvest washing treatment was done with either water or solutions isotonic to the growth media those being PEG 200, glucose or NaCl. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).

Plate 3.1. Fluidised bed dried of unformulated *P. anomala* cells (x10)

3.7.3 Effect of addition of protectants (adjuvants and carriers) prior to fluidised bed drying on *P. anomala* cells

Addition of several protectants at different amounts prior to fluidised bed drying and their impact on *P. anomala* cell viability and moisture content was the next study step (Figure 3.18-3.19). Statistical analysis (*P*<0.05) showed that there was a 3x3 significant interaction between three factors (±additive, different type and different percentage of additive) affecting both viability and final moisture content of *P. anomala* cells after fluidised bed drying at 50°C for 20 min (Appendix I). The control cell treatment (no additives) significantly differed from other treatments. When protectants were added at 5% and 10% (w/w) they had an adverse effect on *P. anomala* dried cells, resulting in decreased viability. Increased use of protectants (20% to 100%) increased cell viability, which was significantly higher than the control (no additives) in SM 100% and WS 20% treatments. Addition of protectants at higher amounts, 100% (w/w), resulted in an overall higher final viability after drying. Usage of talc as a protectant adversely affected cell viability no matter which amount was used. CM addition at different amounts slightly affected cell final viability after drying.

The overall lowest final moisture content occurred when protectans were used at the highest percentage (100%). However, there was no pattern in the way final moisture content changed with increased amount of protectants (5%-100%). In general, CM and CSF usage resulted in lowest final cell moisture content, reaching 5.18% and 6.86% respectively, when both were added at 100% (w/w). Even though an increase in cell viability was not always concomitant with a decrease in final cell moisture content, there was such a trend in CSF and WS treatments when used at 20% and 100% (w/w) amounts.
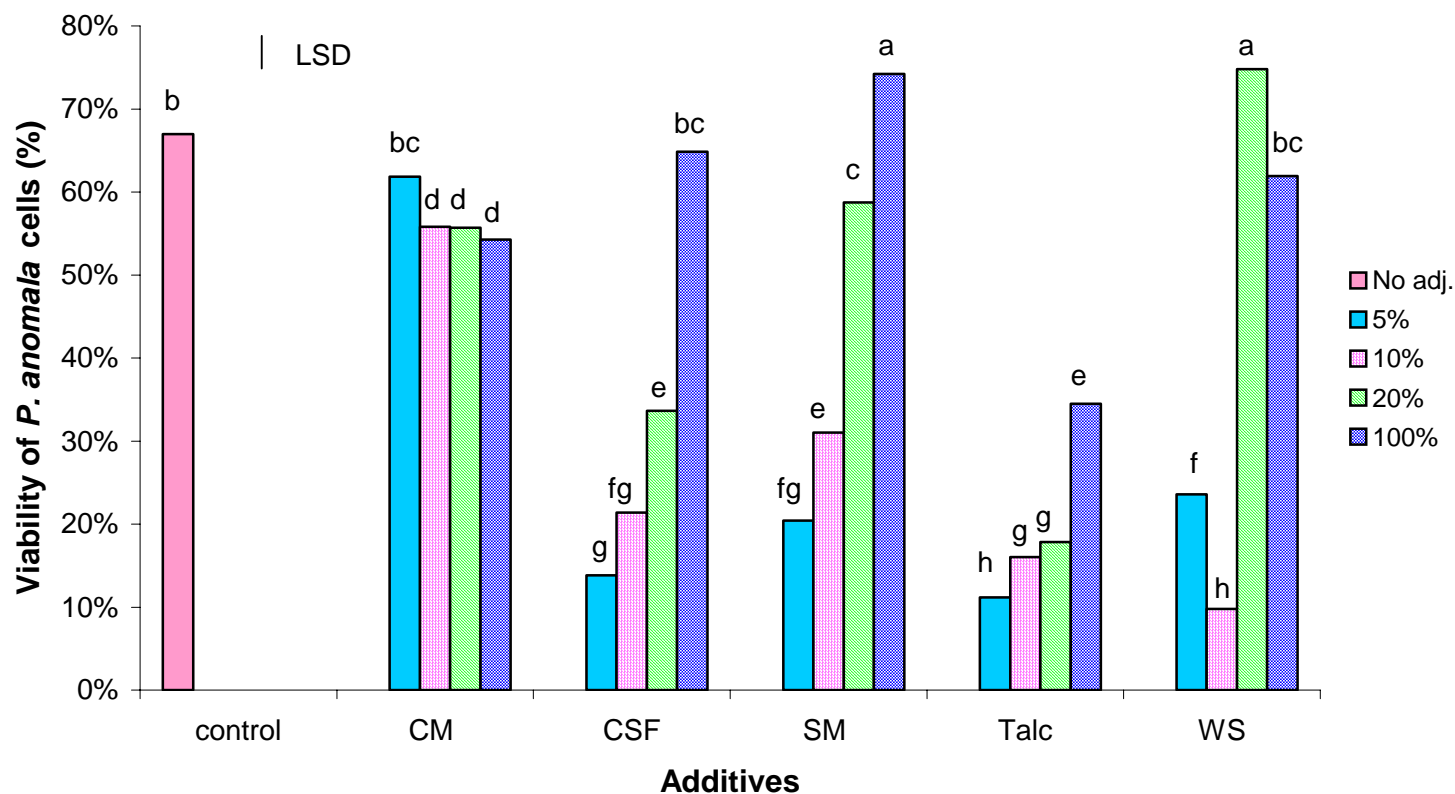
Figure 3.18. Effect of type and percentage (% additive:fresh cells) of additives on *P. anomala* cells on viability after drying at 50°C for 20 min using a fluidised bed dryer. Additives were corn meal (CM), cottonseed flour (CSF), skimmed milk (SM), Talc and wheat starch (WS) at (a) 5% and 10% (a) and (b) 20% and 100%. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).
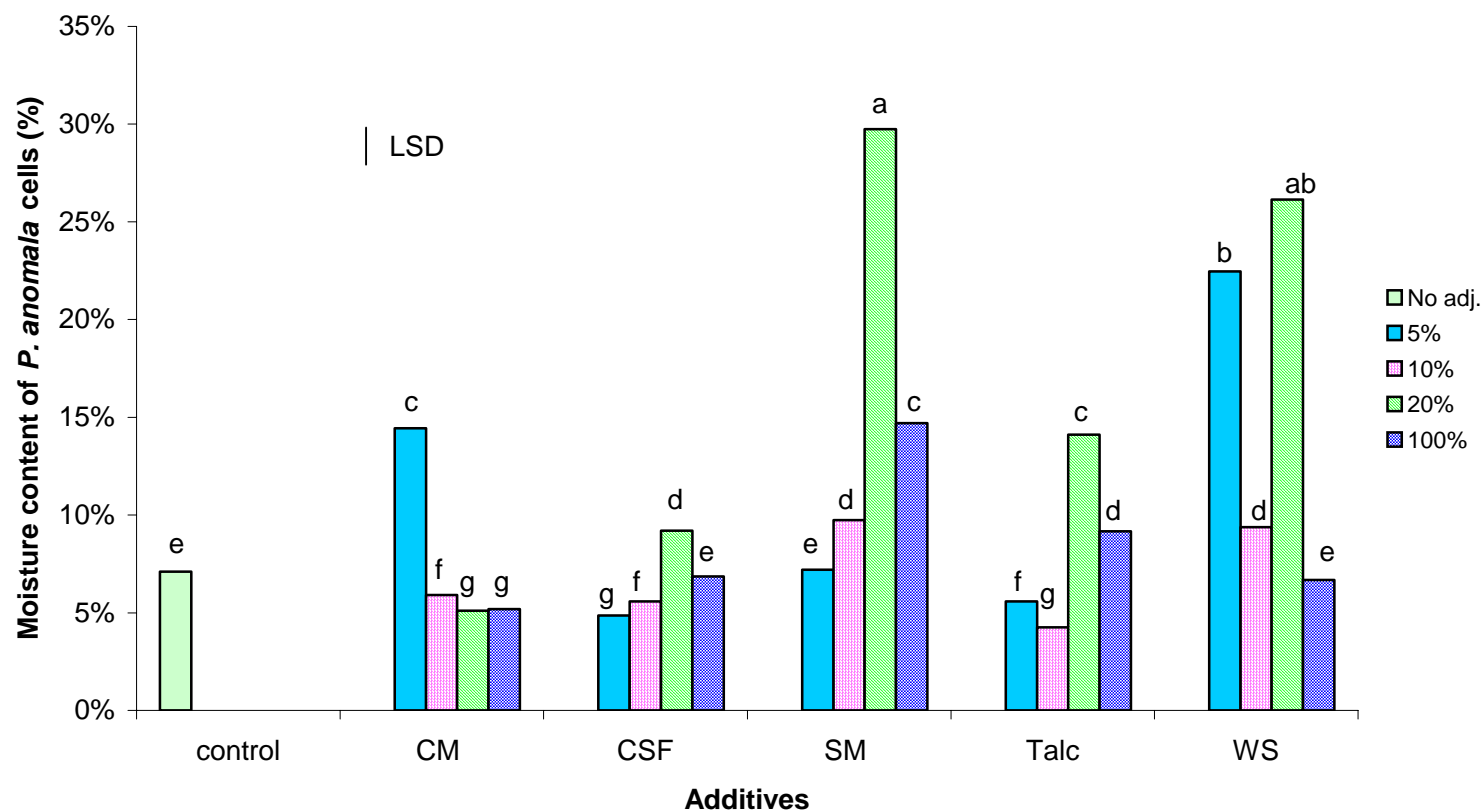
Figure 3.19. Effect of type and percentage (% additive: fresh cells) of additives on *P. anomala* cells on final moisture content after drying at 50°C for 20 min using a fluidised bed dryer. Additives were corn meal (CM), cottonseed flour (CSF), skimmed milk (SM), Talc and wheat starch (WS) at (a) 5% and 10% (a) and (b) 20% and 100%. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).

3.7.4 Screening for final *P. anomala* formulations

Viability of *P. anomala* formulation treatments, when fluidised bed dried for 20 min at 50°C, significantly differed from the control treatment (unformulated cells). Significant differences ($P<0.05$) between treatments were also noted (Appendix I). Of all the treatments compared, CSF:1+10% SM (w/w, additives/fresh cells) resulted in significantly increased final cell viability (Fig. 3.20a). Addition of several adjuvants to carriers resulted in increased or similar cell viability. Final cell moisture content did not significantly differ ($P<0.05$) between formulated and unformulated treatments (Fig. 3.20b). However, the CSF:1+10% SM treatment, interestingly, had a significantly lower final cell moisture content.

3.7.5 Effect of best *P. anomala* formulation treatments combined with isotonic solutions and best fresh cell treatments on final cell viability, moisture content and CFUs mg^{-1} of final product

(a) *P. anomala* cells grown in control molasses media

Final viable CFUs mg^{-1} formulation is an important parameter in formulating yeast cells and was thus, measured. Figure 3.21 shows the effect of different formulation treatments on viable CFUs mg^{-1} formulation. ANOVA showed significant ($P<0.05$) differences between treatments at both media used, stress free and water stressed with a similar pattern. Formulation B resulted in the highest CFUs mg^{-1} formulation (4.45 x 10^6 and 4.35 x 10^6 CFUs mg^{-1} formulation, respectively) followed by control (unformulated cells) and formulation C treatments. Formulation A resulted in the lowest viable CFUs mg^{-1} formulation.
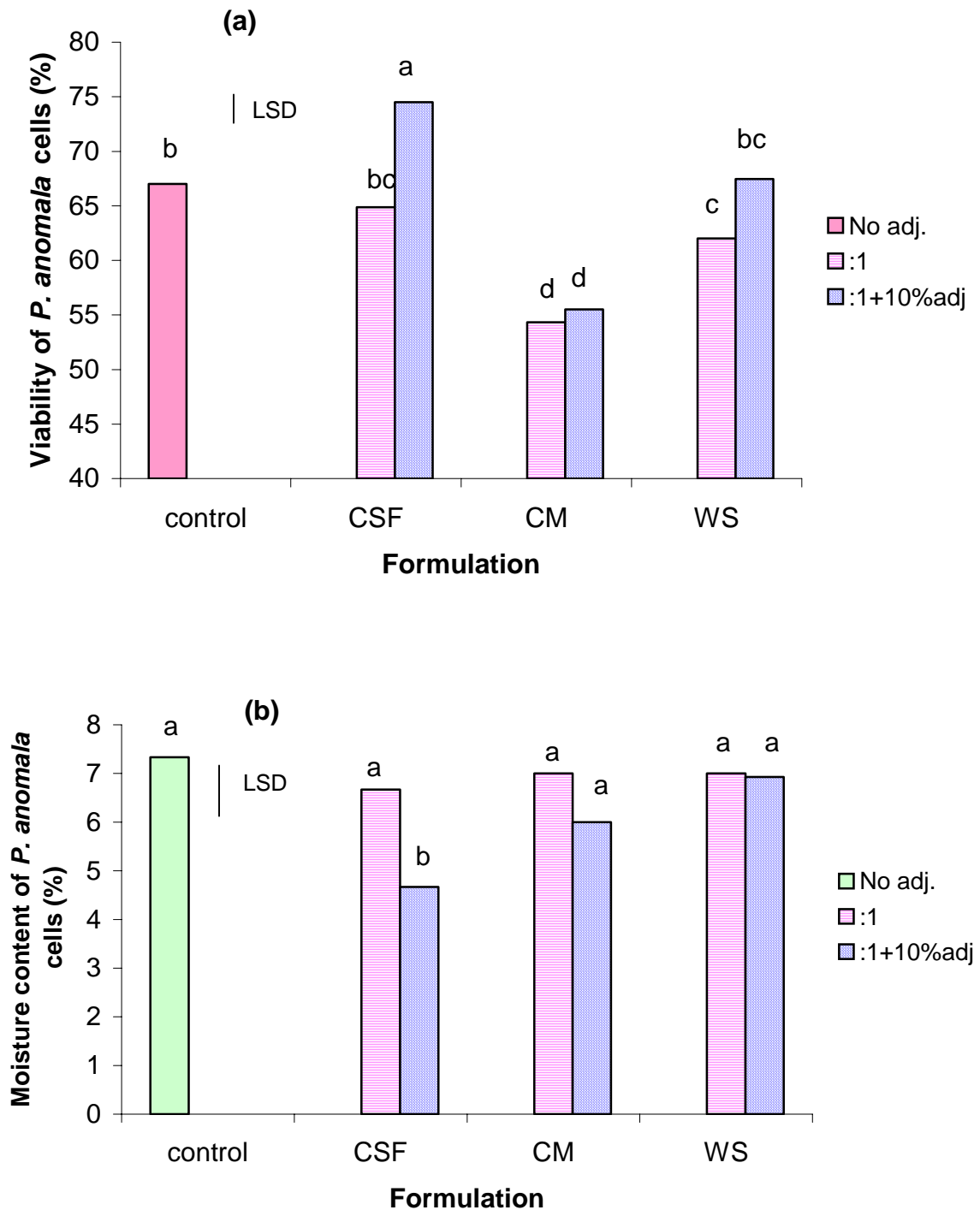
Figure 3.20. Effect of additives used as carriers (100% or 1:1 w/w) like corn meal (CM), cottonseed flour (CSF), and wheat starch (WS) alone or with adjuvants (10% skimmed milk for CSF and WS and glycerol for CM) on *P. anomala* cell viability and final moisture content after drying at 50°C for 20 min using a fluidised bed dryer. Control is untreated *P. anomala* cells. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).
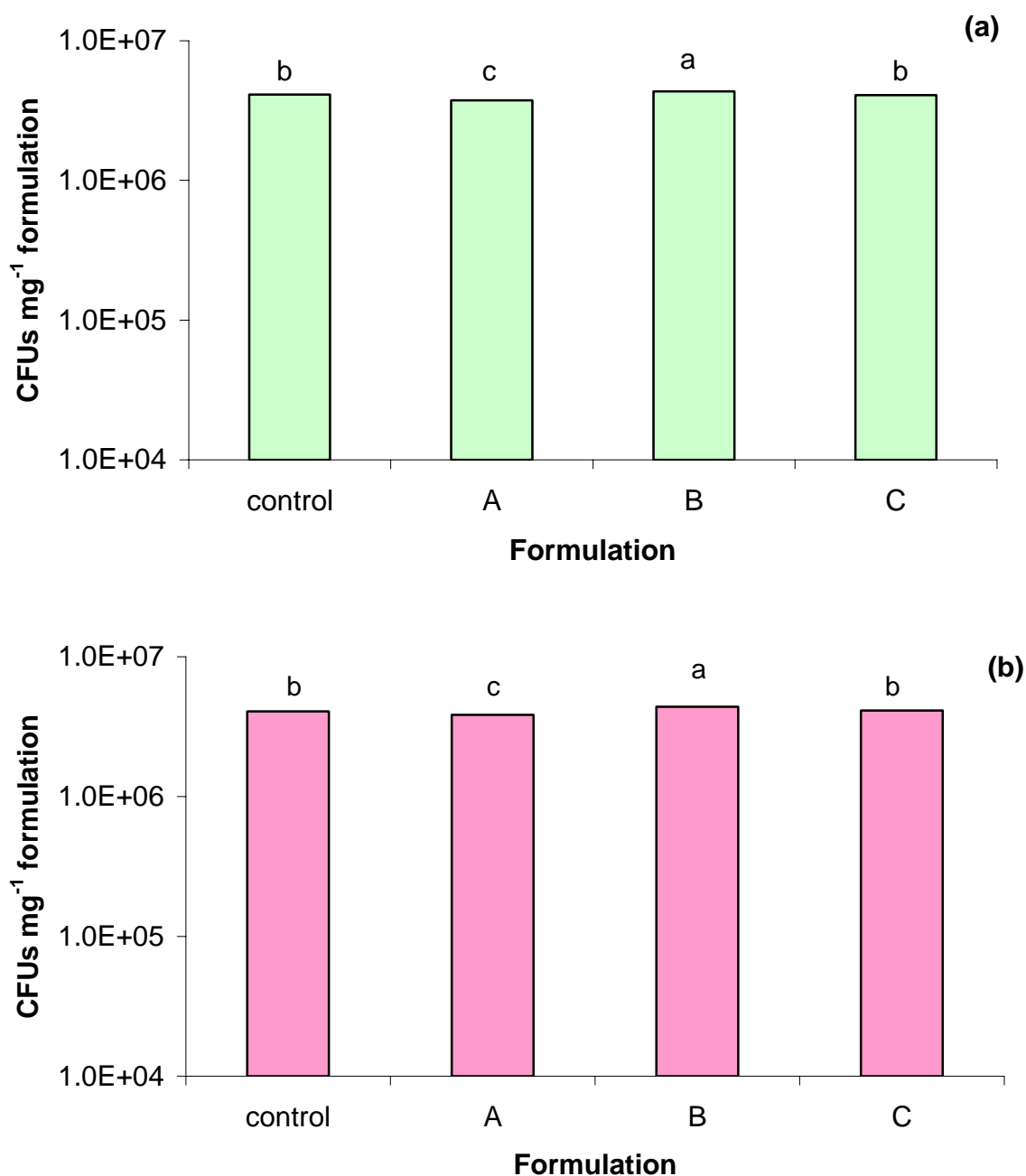
Figure 3.21. Effect of control *P. anomala* cell formulations on final CFUs mg^{-1} formulation on water stressed-free (0.996 a_w) NYDA medium (a) and water stressed (0.96 a_w) NYDA medium (b). Formulations are A: corn meal:1+10% glycerol (w/w), B:cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreated (unformulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means.

(b) *P. anomala* cells grown in proline 0.98 a$_w$ media

As previously shown, *P. anomala* cells grown in proline 0.98 a$_w$ media accumulated high trehalose amounts which doubled when isotonic solution was used as post-harvest washing treatment. Viability and moisture content of proline 0.98 a$_w$ *P. anomala* formulated treatments washed with either water or NaCl isotonic solution, when fluidised bed dried for 20 min at 50°C, showed no significant interaction among the different formulation treatments and different washing treatments (Figures 3.22-3.23). The ANOVA (*P*<0.05) also showed no significant differences between the two washing treatments (see Appendix I). However, there was a significant difference on cell viability among the different formulations. Formulation C [wheat starch:1+10% skimmed milk (w/w)] resulted in similar cell viability when compared to control cells with formulation B [cottonseed flour:1+10% skimmed milk (w/w)] the next best. Formulation A [corn meal:1+10% glycerol (w/w)] cells resulted in the lowest viability of all the treatments. Formulation A and C treatments resulted in signifantly lower and higher cell final moisture content, respectively.

Regarding final CFUs of *P.anomala* cells mg^{-1} formulation, when plated on freely available water NYDA medium (0.996a$_w$), ANOVA (*P*<0.05) showed no significant 2 x 2 interaction (Appendix I). Only different formulation treatments had a significant effect on the CFUs mg^{-1} formulation with all treatments (control included) yielding the similar viable CFUs mg^{-1} formulation. Formulation A had significantly lower viable CFUs mg^{-1} of formulation. Data are presented in Figure 3.24 so that, comparison with CFUs mg^{-1} of formulation of cells plated on water-stress NYDA medium (0.96 a$_w$), could be made.

Figure 3.25 shows that there was a significant 2 x 2 interaction on final CFUs of *P. anomala* cells mg^{-1} of formulation, when plated on water stressed NYDA medium (0.96a$_w$) (Appendix I). Formulation B, when cells were washed with water, provided the highest CFUs mg^{-1} formulation; isotonic washing of cells yielded a significantly lower CFUs mg^{-1} formulation. Unformulated cells (control) gave similar CFUs mg^{-1} viable cells. In formulations A and C, there were no differences between the different washing treatments.
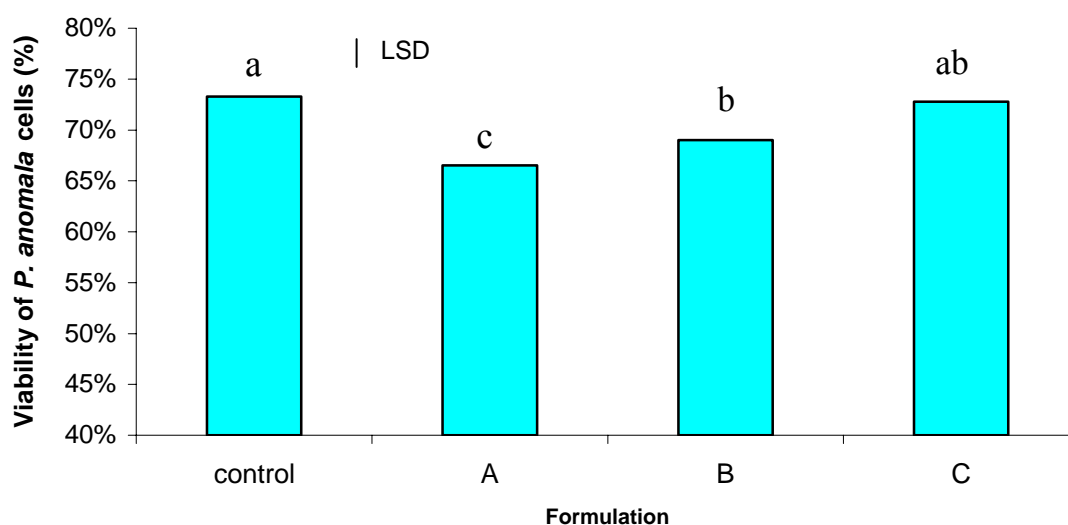
Figure 3.22. Effect of different proline 0.98 a_w *P. anomala* cell formulations on cell viability. Formulations are A: corn meal:1+10% glycerol (w/w), B:cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreated (no formulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).
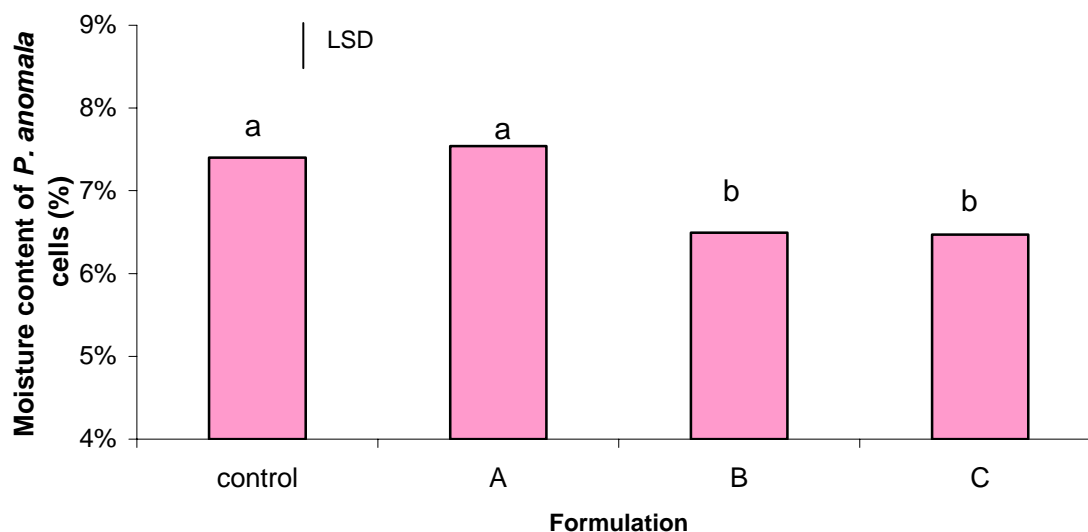
Figure 3.23. Effect of different proline 0.98 a_w *P. anomala* cell formulations on final cell moisture content. Formulations are A: corn meal:1+10% glycerol (w/w), B:cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreatedv(unformulated) *P.anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).
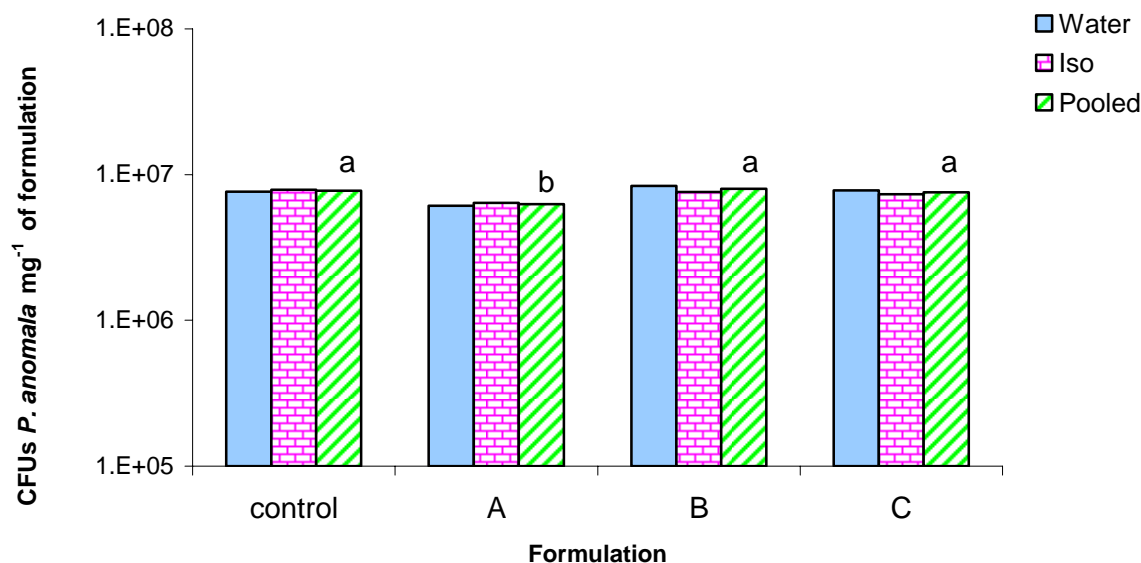
Figure 3.24. Effect of different proline 0.98 a_w *P. anomala* cell formulations on final CFUs mg^{-1} formulation on water stress-free NYDA medium (0.996a_w). Formulations are A: corn meal:1+10% glycerol (w/w), B: cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreated (no formulated) *P.anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. Cells were washed with water ☐ and NaCl ☐ isotonic solution. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means.
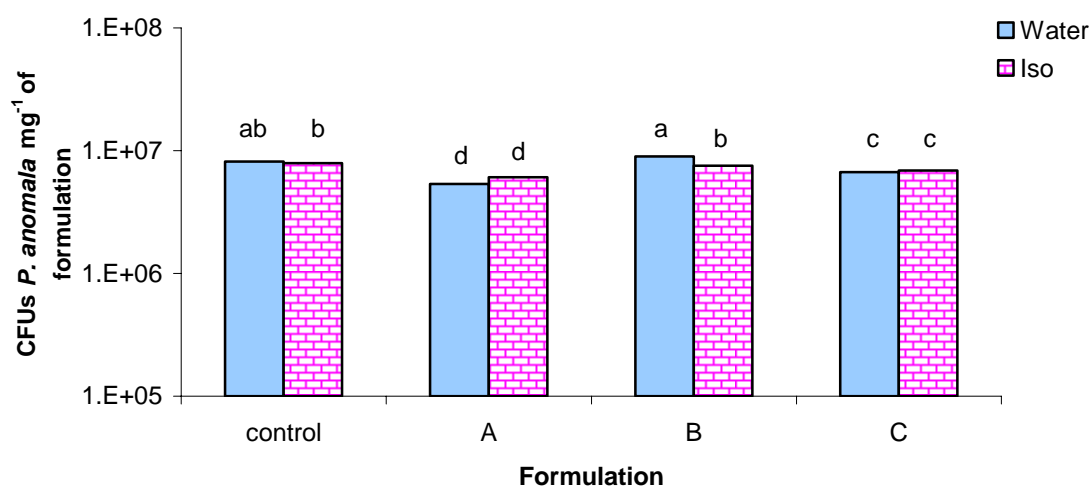
Figure 3.25. Effect of different proline 0.98 a_w *P. anomala* cell formulations on final CFUs mg^{-1} formulation on water stressed NYDA medium (0.96 a_w). Formulations are A: corn meal:1+10% glycerol (w/w), B: cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreated (no formulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. Cells were washed with water ☐ and NaCl ☐ isotonic solution. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means.

Plate 3.2. *P. anomala* cells formulated as A formulation**:** corn meal:1+10% glycerol (w/w) (x1).

Plate 3.3. *P. anomala* cells formulated as B formulation**:** cottonseed flour:1+10% skimmed milk (w/w) (x1).

Plate 3.4. *P. anomala* cells formulated as C formulation**:** wheat starch: 1+10% skimmed milk (w/w) (x1).

(c) *P. anomala* cells grown in NaCl 0.98 a_w

As previously shown, *P. anomala* cells grown in NaCl 0.98 a_w media accumulated high trehalose amounts which quadraplied when isotonic solution was used as post-harvest washing treatment. Viability and moisture content of cells formulated treatments washed with either water or NaCl isotonic solution, when fluidised bed dried for 20 min at 50^oC showed a significant interaction among the different formulation treatments and different washing treatments (Figures 3.26-3.27). Control cells washed with isotonic solution resulted in the highest viability (75%). In general, use of isotonic solution increased final viability of the cells in a remarkable way. Formulation A [corn meal:1 + 10% glycerol (w/w)] resulted in the lowest viability of all formulation treatments while formulation B [cottonseed flour:1+10% skimmed milk (w/w)] were themost viable. The lowest final viability of cells occurred with formulation A when cells were washed with water. In control and formulation B treatments, use of isotonic solutions resulted in increased final cell moisture content (about 1.5-2% compared with the water washing treatments), while in the formulation C treatment there was a significant decrease when isotonic solution was used.

Regarding final CFUs of *P. anomala* cells mg^{-1} formulation, when plated on water stress-free NYDA medium ($0.996 a_w$), ANOVA ($P<0.05$) showed no significant 2x2 interaction. However, both different formulation and washing treatments had a significant effect on viable CFUs mg^{-1} formulation acting independently. All different formulation treatments (control included) yielded similar CFUs mg^{-1} formulation and only formulation A had significantly lower CFUs mg^{-1} formulation. Use of isotonic solution resulted in an increase in final CFUs mg^{-1} formulation. Data are presented on Figure 3.28 so that a comparison can be made of viability of formulations when cells were plated on water-stress NYDA medium ($0.96 a_w$).

Figure 3.29 shows that there was a significant 2x2 interaction on final CFUs of *P. anomala* cells mg^{-1} of formulation, when plated on water stressed NYDA medium ($0.96 a_w$). Formulation B, when cells were washed with isotonic solution, provided the highest CFUs mg^{-1} of formulation. In general, use of isotonic solution resulted in similar or significantly higher CFUs mg^{-1} formulation. Formulation A cells washed with water resulted in the lowest CFUs mg^{-1} formulation.
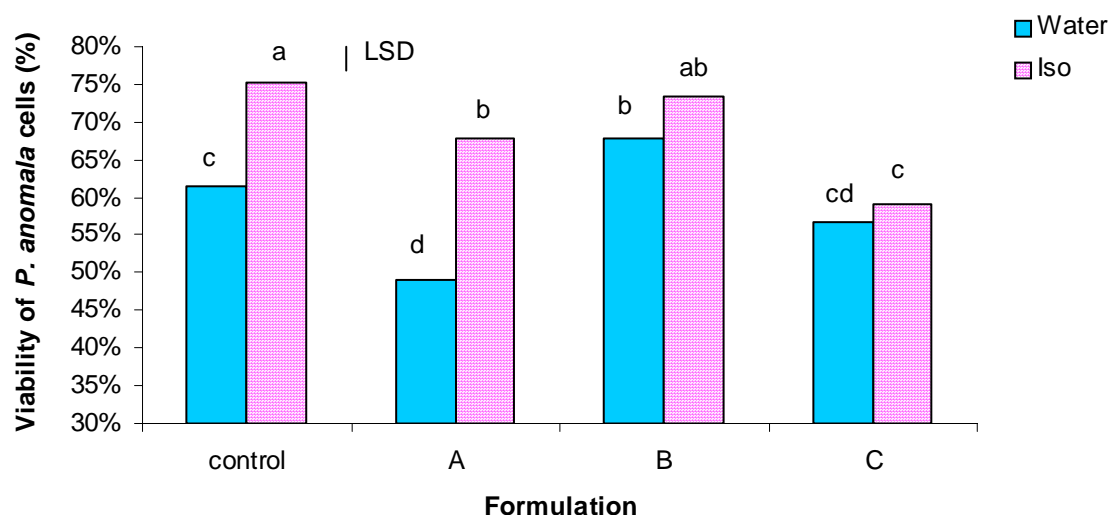
Figure 3.26. Effect of different NaCl 0.98 a_w *P. anomala* cell formulations on cell viability. Formulations are A: corn meal: 1 + 10% glycerol (w/w), B:cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1 + 10% skimmed milk (w/w). Control is untreated (no formulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. Cells were washed with water ☐and NaCl ☒ isotonic solution. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).
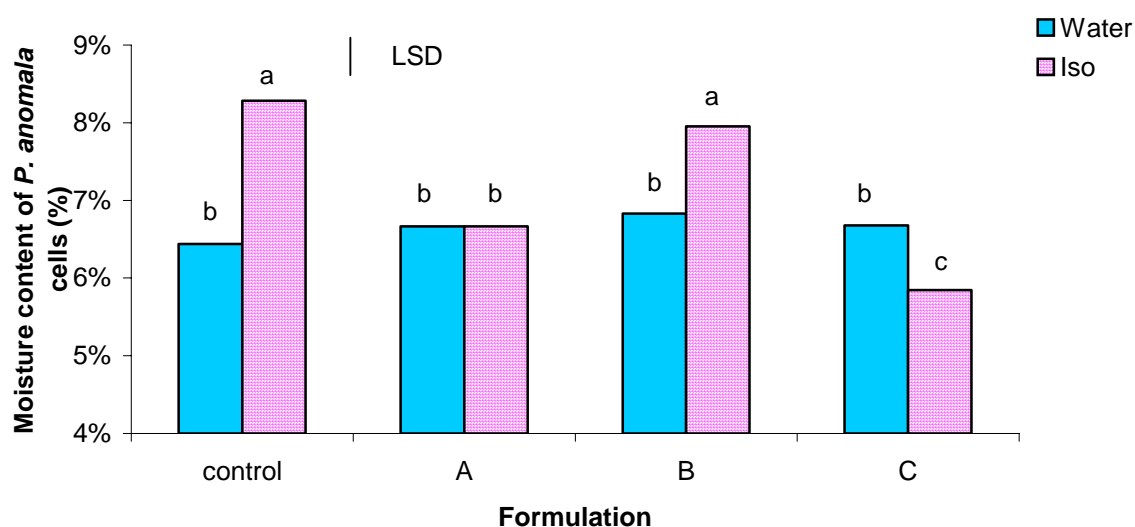
Figure 3.27. Effect of different NaCl 0.98 a_w *P. anomala* cell formulations on final cell moisture content. Formulations are A: corn meal:1+10% glycerol (w/w), B:cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreatedv(no formulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. Cells were washed with water ☐ and NaCl ☒ isotonic solution.All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bars=LSD (*P*<0.05).
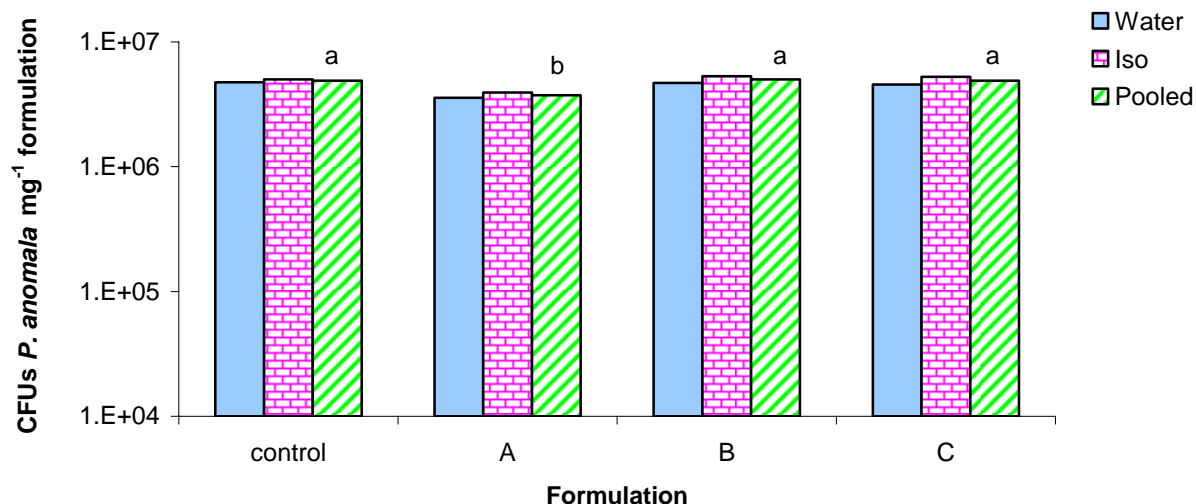
Figure 3.28. Effect of different NaCl 0.98 a_w *P. anomala* cell formulations on final CFUs mg^{-1} formulation on water stress-free NYDA medium (0.996a_w). Formulations are A: corn meal:1+10% glycerol (w/w), B:cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreated (no formulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. Cells were washed with water ☐ and NaCl ▦ isotonic solution. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means
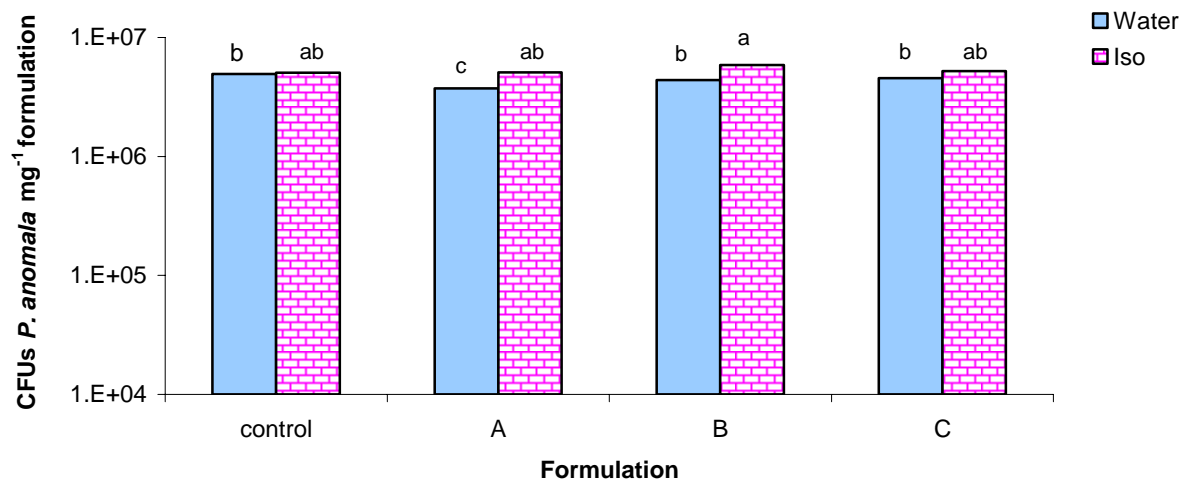
Figure 3.29. Effect of different NaCl 0.98 a_w *P. anomala* cell formulations on final CFUs mg-1 formulation on water stressed NYDA medium (0.96 a_w). Formulations are A: corn meal:1+10% glycerol (w/w), B:cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreated (no formulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. Cells were washed with water ☐ and NaCl ▦ isotonic solution. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means

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3.7.6 Storage stability of *P. anomala* fluidised bed-drying formulations

In all of the treatments, storage at 25°C resulted in a higher decrease in viability than that obtained at 4°C after 150 days of storage (Figures 3.30-3.36). In particular, proline 0.98 a_w *P. anomala* cell formulation C [wheat starch:1 (w/w) +10% (w/w) skimmed milk] when stored at 4°C, resulted in only a very small viability loss over time (from 73.5% to 69.2%).

Storage stability was also affected by the different formulations used. Generally, formulation A [corn meal:1 (w/w) +10% (w/w) skimmed milk] treatment resulted in drastically decreased viability after 150 days storage (20% in control *P. anomala* cells treatment). Formulations B [cottonseed flour: 1 (w/w) +10% (w/w) skimmed milk] and C resulted in higher viability overtime when cells were stored at both ambient and 4°C (about 45% and 55%, respectively).

Different post-harvest washing treatments (water or isotonic) affected storage stability, in particular in NaCl; when isotonic solutions were used, storage stability of the formulated product was higher than that of the water post-harvest washing treatment at all different formulations used and at both ambient and 4°C. In the proline 0.98 a_w treatment, use of an isotonic post-harvest treatment resulted in increased storage stability only when formulation C was used. It is interesting to note that in the NaCl 0.98 a_w fresh cell treatment, trehalose was strongly retained in the cell when isotonic solution was used as a post-harvest washing treatment.

Different *P. anomala* cell fresh treatments also affected storage stability but it seems that there is a complex interaction among the fresh cell treatments, post-harvest treatments, different formulations used and storage temperature.
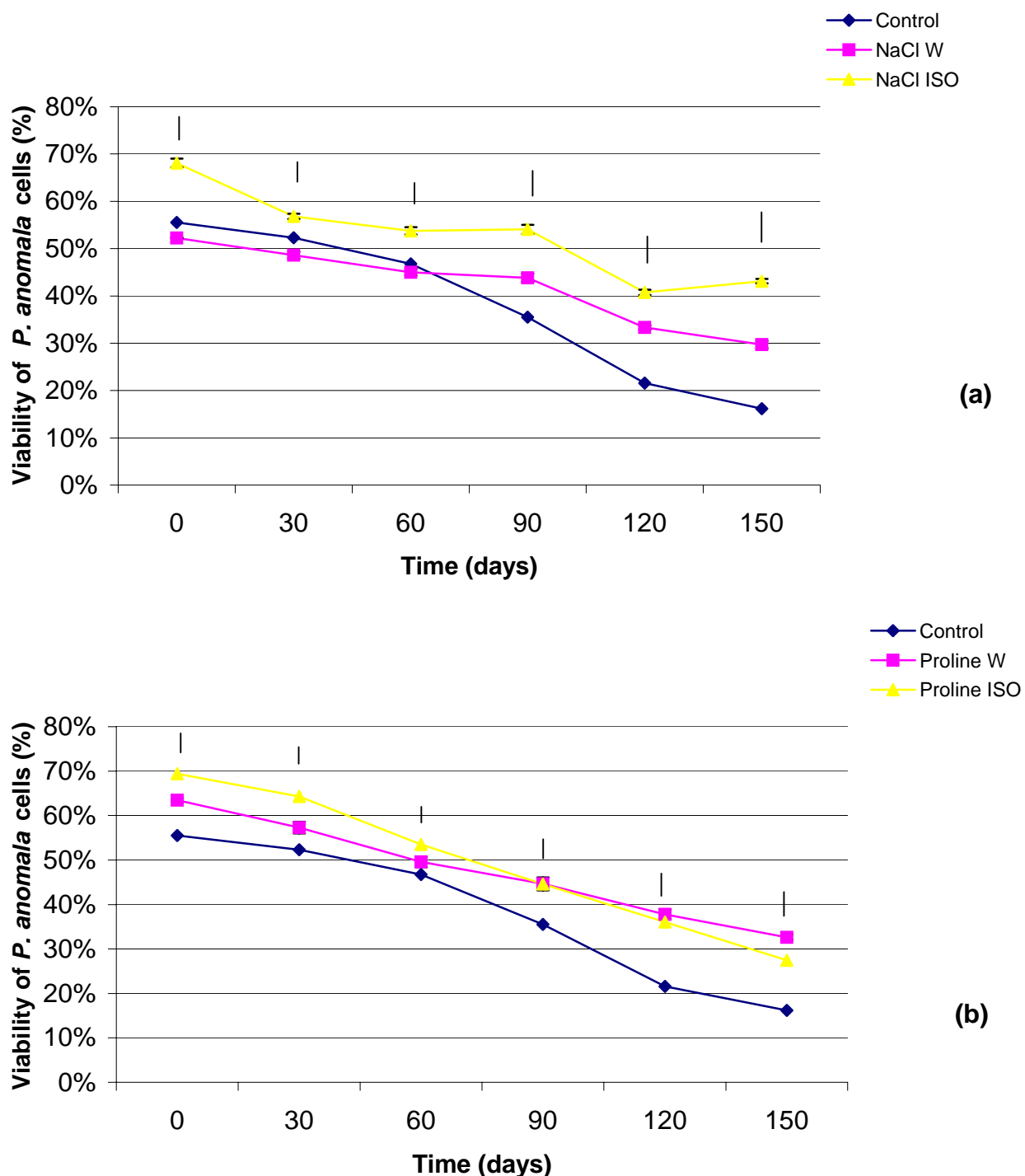
Figure 3.30. Storage stability of *P. anomala* cells formulated with corn meal (CM) 1:1 (w/w) and 10% (w/w) glycerol (formulation A) after drying at 50°C for 20 min using a fluidised bed dryer when stored at ambient temperature (20-22°C) over a period of 5 months (150 days). *P. anomala* cells were grown in NaCl 0.98 a$_w$ modified molasses media (a) and proline 0.98 a$_w$ modified molasses media (b). Control cells were grown in unmodified molasses media. Post-harvest washing treatments were water (W) and NaCl isotonic solution (ISO). All data are means of three replicates per treatment. Bars = LSD (*P*<0.05).
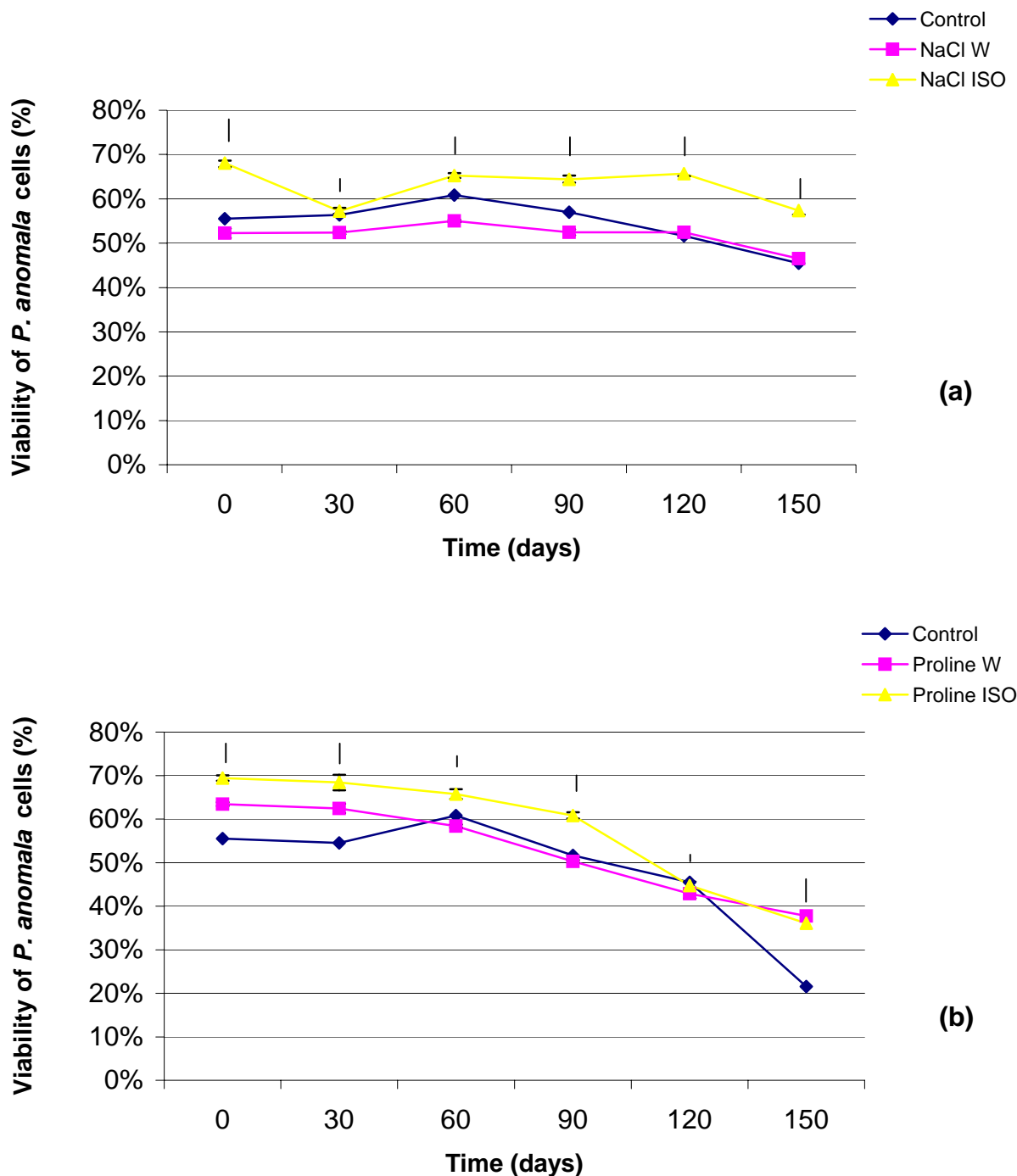
Figure 3.31. Storage stability of *P. anomala* cells formulated with corn meal (CM) 1:1 (w/w) and 10% (w/w) glycerol (formulation A) after drying at 50°C for 20 min using a fluidised bed dryer when stored at fridge temperature (4°C) over a period of 5 months (150 days). *P. anomala* cells were grown in NaCl 0.98 a$_w$ modified molasses media (a) and proline 0.98 a$_w$ modified molasses media (b). Control cells were grown in unmodified molasses media. Post-harvest washing treatments were water (W) and NaCl isotonic solution (ISO). All data are means of three replicates per treatment. Bars = LSD (*P*<0.05).
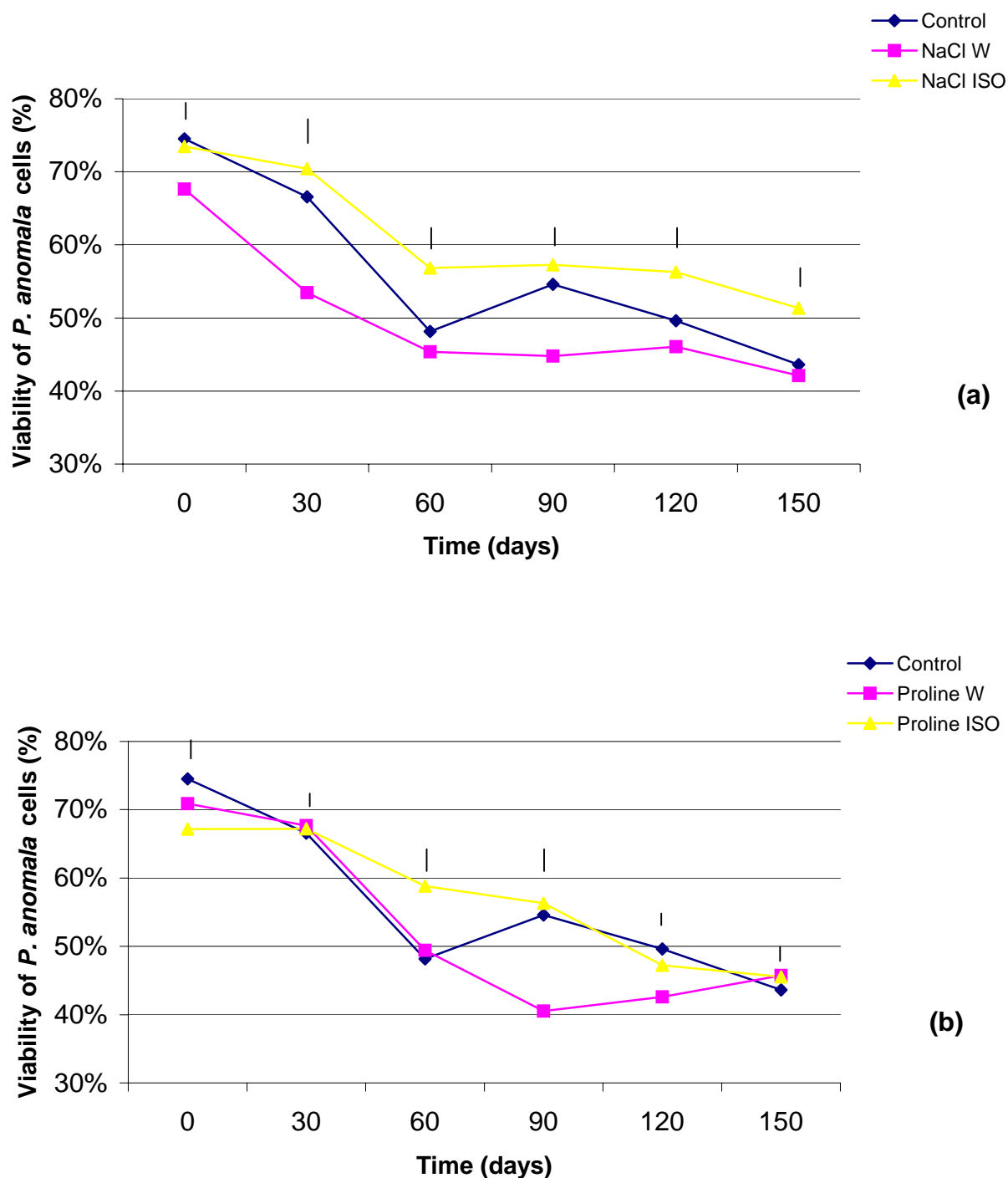
Figure 3.32. Storage stability of *P. anomala* cells formulated with cottonseed flour (CSF) 1:1 (w/w) and 10% (w/w) skimmed milk (formulation B) after drying at 50°C for 20 min using a fluidised bed dryer when stored at ambient temperature (20-22°C) over a period of 5 months (150 days). *P. anomala* cells were grown in NaCl 0.98 a$_w$ modified molasses media (a) and proline 0.98 aw modified molasses media (b). Control cells were grown in unmodified molasses media. Post-harvest washing treatments were water (W) and NaCl isotonic solution (ISO). All data are means of three replicates per treatment. Bars = LSD (*P*<0.05).
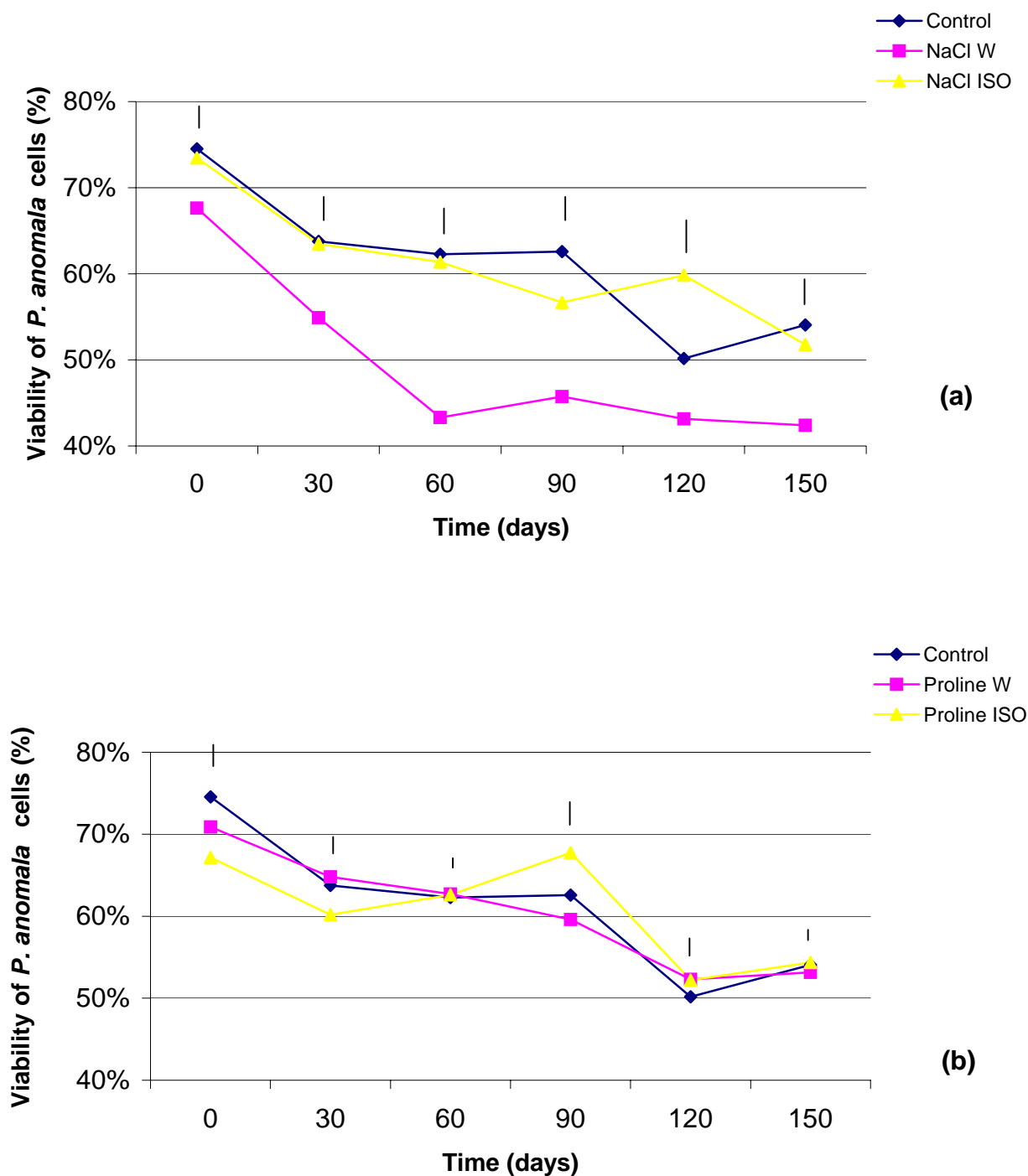
Figure 3.33. Storage stability of *P. anomala* cells formulated with cottonseed flour (CSF) 1:1 (w/w) and 10% (w/w) skimmed milk (formulation B) after drying at 50°C for 20 min using a fluidised bed dryer when stored at fridge temperature (4°C) over a period of 5 months (150 days). *P. anomala* cells were grown in NaCl 0.98 a_w modified molasses media (a) and proline 0.98 a_w modified molasses media (b). Control cells were grown in unmodified molasses media. Post-harvest washing treatments were water (W) and NaCl isotonic solution (ISO). All data are means of three replicates per treatment. Bars = LSD (*P*<0.05).

Figure 3.34. Storage stability of *P. anomala* cells formulated with wheat starch (WS) 1:1 (w/w) and 10% (w/w) skimmed milk (formulation C) after drying at 50°C for 20 min using a fluidised bed dryer when stored at ambient temperature (20-22°C) over a period of 5 months (150 days). *P. anomala* cells were grown in NaCl 0.98 a$_w$ modified molasses media (a) and proline 0.98 a$_w$ modified molasses media (b). Control cells were grown in unmodified molasses media. Post-harvest washing treatments were water (W) and NaCl isotonic solution (ISO). All data are means of three replicates per treatment. Bars = LSD (*P*<0.05).
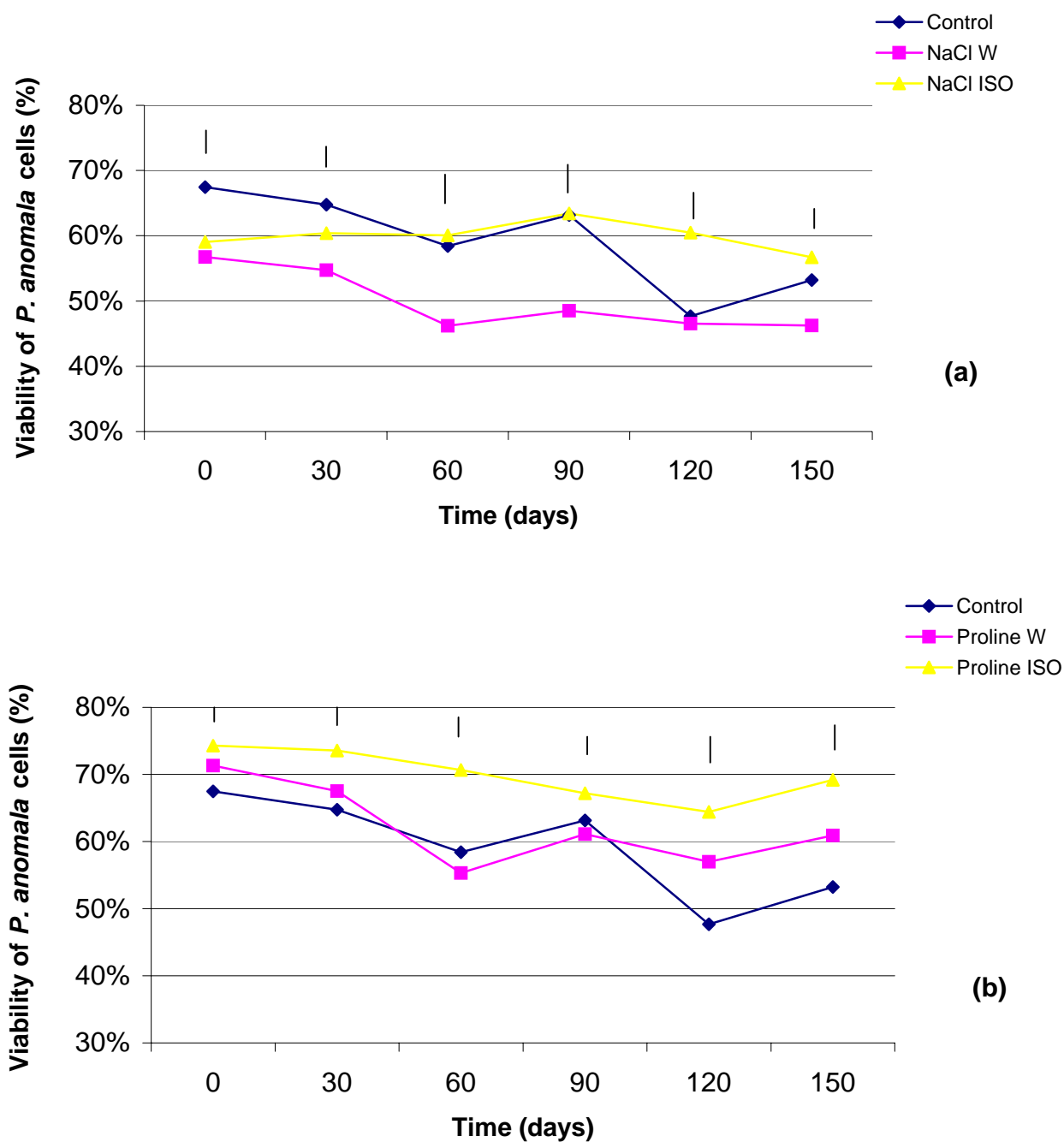
Figure 3.35. Storage stability of *P. anomala* cells formulated with wheat starch (WS) 1:1 (w/w) and 10% (w/w) skimmed milk (formulation C) after drying at 50°C for 20 min using a fluidised bed dryer when stored at fridge temperature (4°C) over a period of 5 months (150 days). *P. anomala* cells were grown in NaCl 0.98 a_w modified molasses media (a) and proline 0.98 a_w modified molasses media (b). Control cells were grown in unmodified molasses media. Post-harvest washing treatments were water (W) and NaCl isotonic solution (ISO). All data are means of three replicates per treatment. Bars = LSD ($P<0.05$)

3.8   FREEZE DRYING OF *P. anomala* CELLS

3.8.1   Effect of protective solutions and rehydration media on *P. anomala* cell freeze, thawing and freeze drying tolerance

The first step in the freeze drying procedure was to study the effect of several protective media and their combinations (water, 10% and 20% skimmed milk, 10% glucose, 10% sucrose, 10% skimmed milk+10%glucose and 10% skimmed milk+10% sucrose) and 2 rehydration media (water and 10% skimmed milk) on *P. anomala* cells after (a) freezing and thawing and (b) freeze drying and subsequent rehydration after freeze drying. The main aim was to investigate whether they conferred any cell protection and tolerance against freeze drying shock. Statistical analysis ($P<0.05$) (Appendix I) showed that the best thawing protection was achieved by use of 10% SM+10% sucrose protective solution followed by 10% SM (Figure 3.36).

Cell freeze drying protection has shown to be affected by a significant ($P<0.05$) 2x2 interaction among the different protective solutions and rehydration media treatments (Figure 3.37) (Appendix I). The best freezing protection was conferred by use of 20% skimmed milk rehydrated in both media with use of 10% skimmed milk being slightly better. However, use of 20% skimmed milk solution was not easy to handle while autoclaving rendering its potential use on a commercial base difficult. Use of 10% skimmed milk+10% sucrose and rehydration on water conferred the sond best cell protection against freeze drying (94.2%). Cells with no protective solution, when rehydrated in water were only 18% viable, reaching 46% viability when rehydrated in 10% skimmed milk. In several protective solution treatments (control, 10% glucose, 10% skimmed milk) use of 10% skimmed milk as a rehydration medium increased cell protection. However, at 10% skimmed milk+10% sucrose treatment, use of skimmed milk before freeze drying resulted in a high final cell protection which was not further increased during rehydration. Therefore, 10% skimmed milk+10% sucrose for protective medium and water as a rehydration medium was used in subsequent studies.
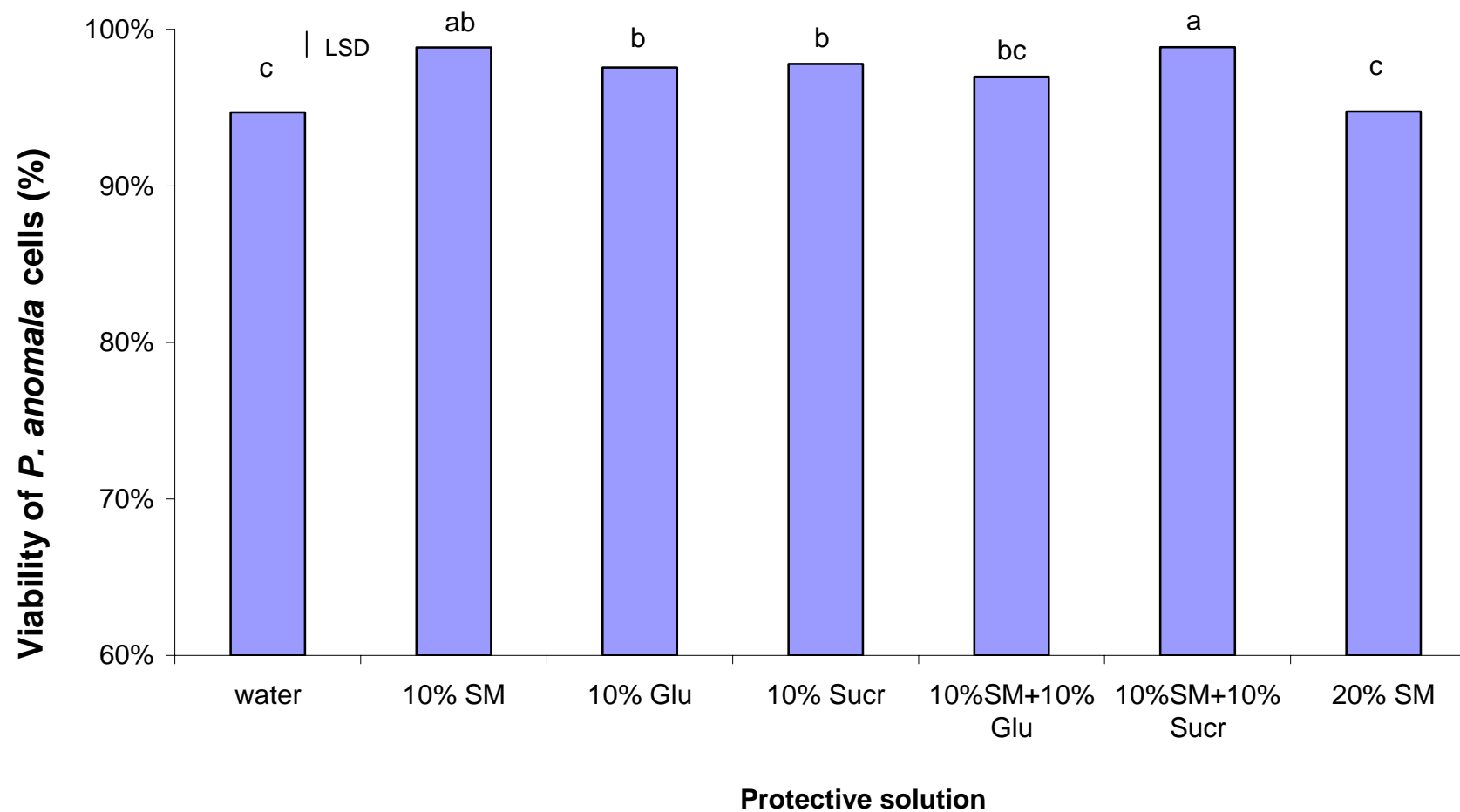
Figure 3.36. Effect of protective solutions on *P. anomala* cell freeze tolerance. SM: skimmed milk, Glu: glucose, Sucr: sucrose. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bar = LSD (*P*<0.05).
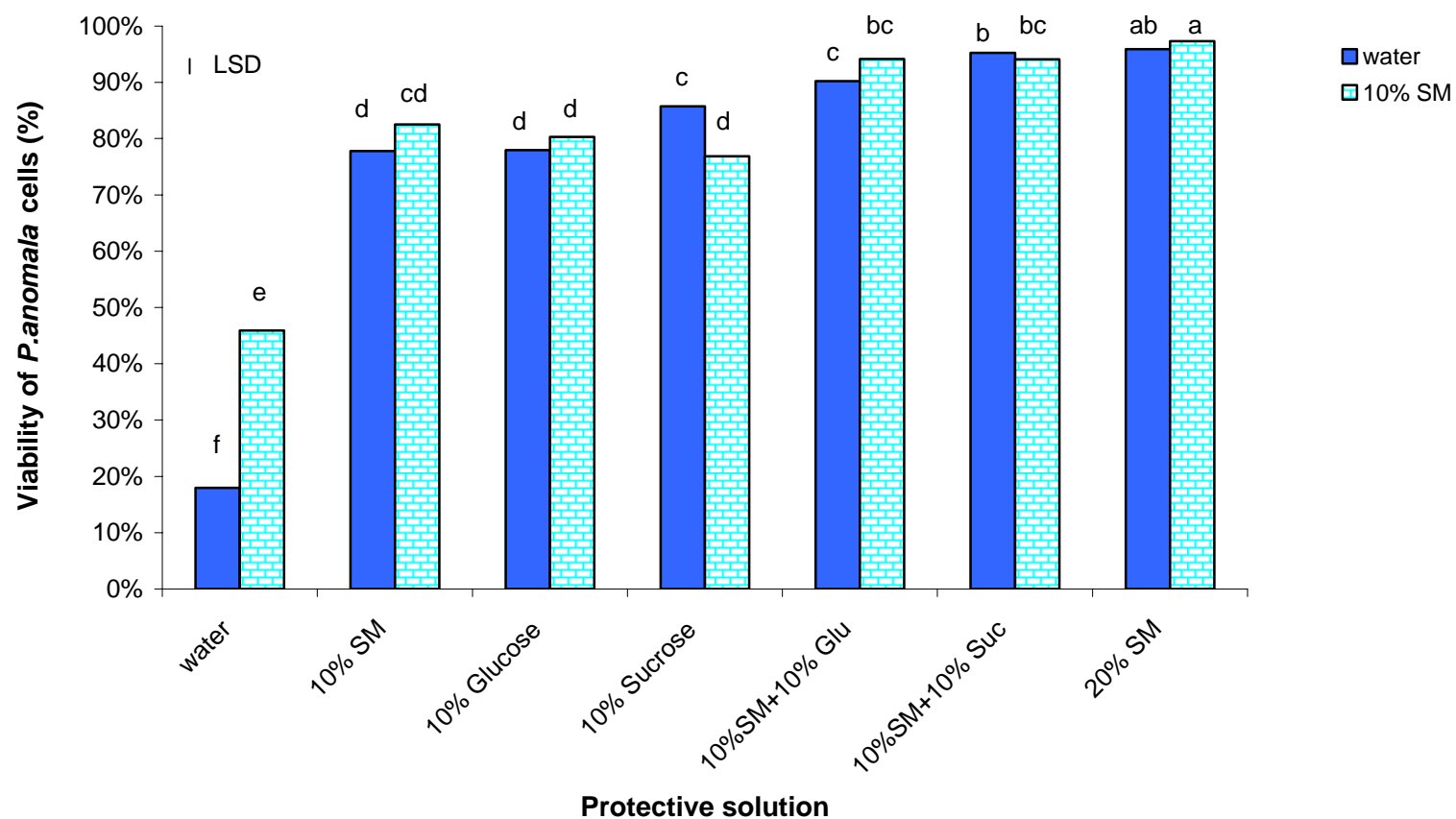
Figure 3.37. Effect of protective solutions (water, 10% and 20% skimmed milk, 10% glucose, 10% sucrose, 10% skimmed milk+10%glucose and 10% skimmed milk+10% sucrose) and 2 rehydration media (10% skimmed milk and water) on *P. anomala* freeze drying tolerance. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P<0.05*) between means. Bar = LSD (*P<0.05*)

3.8.2   Study of effect of different isotonic solutions used for post-harvest cell washing on *P. anomala* cell tolerance against thawing and freeze drying

P. anomala cell osmoprotection by use of post-harvest washing of yeast cells grown in glucose 0.98 a_w molasses-modified media with PEG 200, glucose and NaCl resulted in combined effects on viability after (a) freezing and thawing and (b) freeze drying (Fig 3.38). Statistical analysis ($P<0.05$) showed that there was a significant 2 x 2 interaction between protective solutions and isotonic washings affecting cell freezing and thawing and freeze drying tolerance. Use of NaCl post-harvest isotonic solution resulted in high cell viability after thawing (99%, when 10% SM + 10% sucrose was the protective medium) which was not significantly higher than water post-harvest treatment. Use of PEG 200 resulted in the lowest tolerance after freezing (67% when water was used) followed by the glucose isotonic treatment (82%). Use of 10% SM + 10% sucrose as the protective medium conferred increased freezing tolerance and only PEG 200 isotonic treatment was significantly worse than the others. In general, use of a protective medium masked the protective effect of different post-harvest treatments.

Regarding *P. anomala* tolerance against freeze drying, when cells were freeze dried in water alone, use of isotonic solutions post-harvest (NaCl and PEG 200) significantly increased cell tolerance. However, when cells were freeze dried in 10% SM + 10% sucrose protective solution were efficiently protected in a similar way, PEG 200 treatment being significantly worse. To investigate any possible effect of post-harvest isotonic solution treatments in freeze dried cells storage stability, NaCl isotonic solution was chosen.
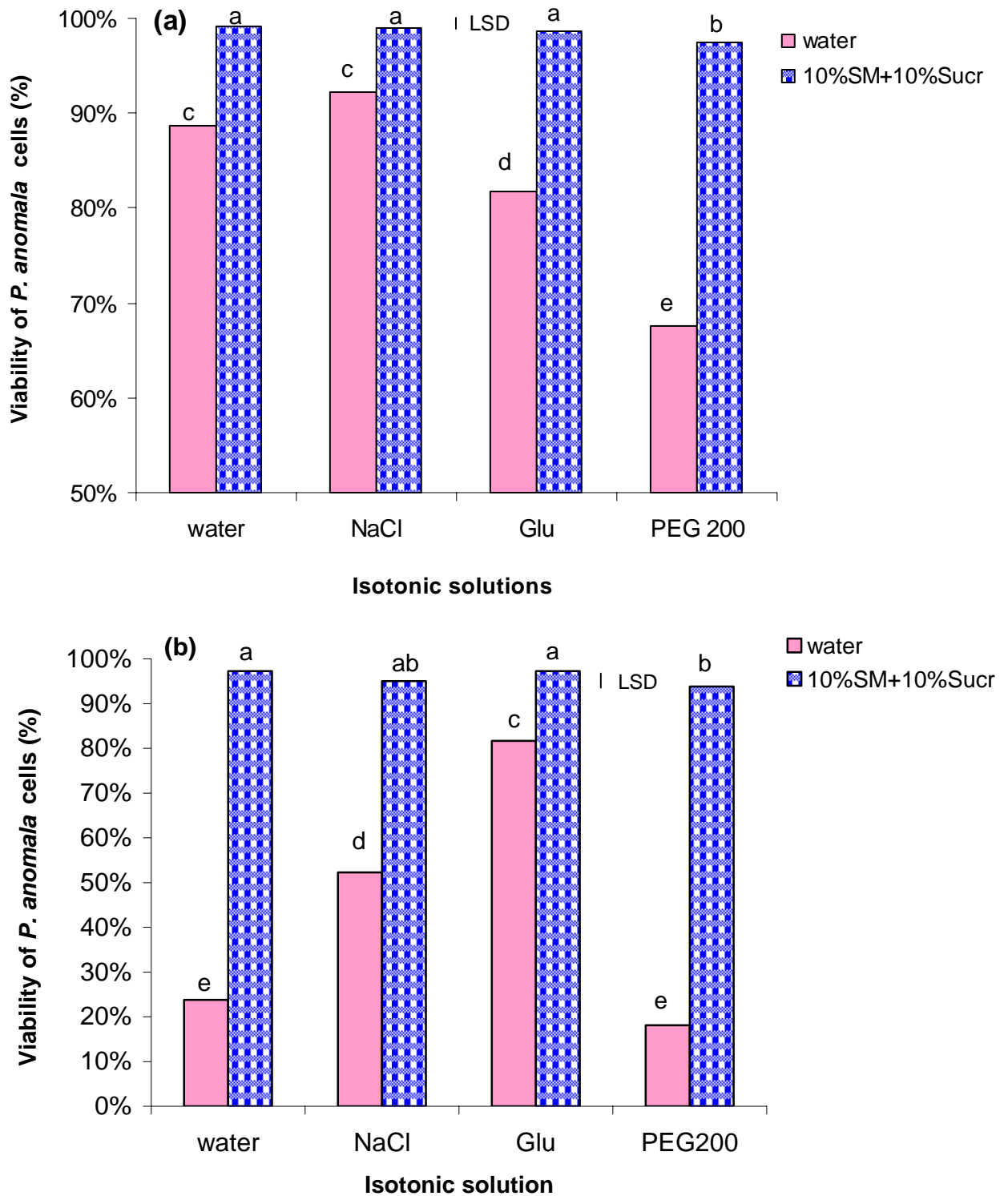
Figure 3.38. Effect of post-harvest washing of *P. anomala* cells grown in glucose 0.98 a_w molasses-modified media with PEG 200, glucose and NaCl and different protective media (water and 10%SM+10% sucrose) on cell tolerance after (a) freezing and thawing and (b) freeze drying. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bars = LSD (*P*<0.05).

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3.8.3 Studies with best fresh *P. anomala* treatments and best isotonic post-harvest washing treatment prior to freeze drying

Freeze drying tolerance of modified *P. anomala* cells with use of isotonic post-harvest washing treatment and ± protective media is shown on Figure 3.39. Yeast cell viability was significantly ($P<0.05$) affected by a 3x3 interaction between three factors (±media modification, type of solute used for media modification, post-harvest washing treatment) when both water and 10% SM + 10% sucrose were used as suspension media.

When water was used as suspension media, unmodified cells had the lowest viability (24%) of all treatments with proline 0.98 a_w treatment having the highest one (57%) when no isotonic solution was used. Use of isotonic solution significantly increased, in all treatments, cell tolerance against freeze drying with proline 0.98a_w exhibiting the highest tolerance of all (64% viability).

When a protective medium (10%SM+10% sucrose) was used, different modified *P. anomala* cells significantly differed in freeze drying tolerance both among them and from the control treatment (Fig. 3.39b). In all treatments, viability was higher than 93.9% reaching 99% in NaCl 0.98a_w and proline 0.98a_w case. However, use of isotonic NaCl solution did not significantly affect final cell viability after freeze drying. Use of a protective medium, once more, masked the protective effect of isotonic post-harvest washing treatment.
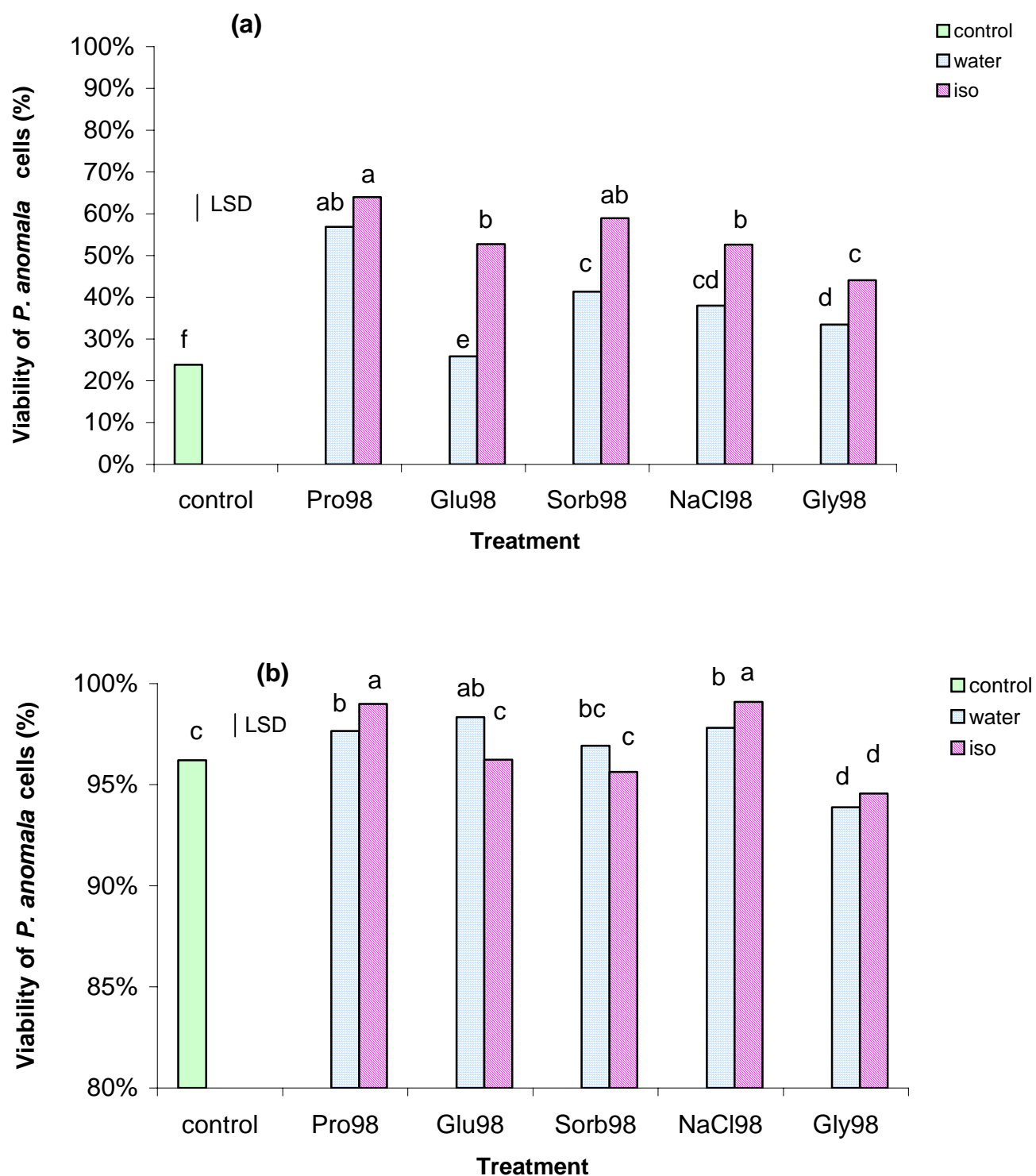
Figure 3.39. Effect of use of isotonic NaCl solution on freeze drying tolerance of *P. anomala* cells grown in molasses-modified media with proline (Pro98), glucose (Glu98), sorbitol (Sorb98), NaCl and glycerol (Gly98) at 0.98a_w and when (a) water and (b) protective solution (10%SM+10% sucrose) were used. All data are means of three replicates per treatment. Different letters indicate statistical differences (*P*<0.05) between means. Bars = LSD (*P*<0.05)

3.8.4 Storage stability of modified *P. anomala* freeze dried cells

Storage stability of *P. anomala* freeze dried cell treatments when (a) water or isotonic NaCl solution (b) were used as post-harvest washing is shown on Figs 3.40 (storage at 4°C) and on Fig. 3.41 (storage at 22°C). Statistical analysis ($P<0.05$) showed that there was a significant two-way interaction between *P. anomala* cell treatments and storage time (Appendix I) for both temperature and post-harvest washing treatments.

When stored at 4°C, viability of *P. anomala* freeze dried control cells decreased between 96% to 85.5% after 150 days storage. Glucose 0.98 a$_{w}$ treatment cells exhibited high storage stability (viability fell from 98.3% to 95.8% after 150 days of storage). In all other treatments, viability decreased to 88.3-90.6%. When isotonic solution was used, viability decreased from 96.3 to 95.5% in glucose 0.98 a$_{w}$ treatment, and from 99 to 96% for NaCl 0.98 and proline 0.98 a$_{w}$ treatments. In other treatments viability decreased from 94.6-96% to 87-88%. Different post-harvest washing treatments (water or isotonic) affected storage stability, in NaCl 0.98 and proline 0.98 a$_{w}$ treatments. In all cases, viability was higher than 85% after 150 days of storage at 4°C.

Regardless of post-harvest washing treatment used, when stored at 22°C, viability of *P. anomala* freeze dried cell treatments decreased rapidly. After 30 days storage, control cells decreased viability from 96.2 to 24.2%. All of the other treatments followed the same decrease pattern. For the water post-harvest washing treatment cell viability decreased from 94-99% to 27-35%. The pattern was similar for the isotonic NaCl post-harvest washing treatment.
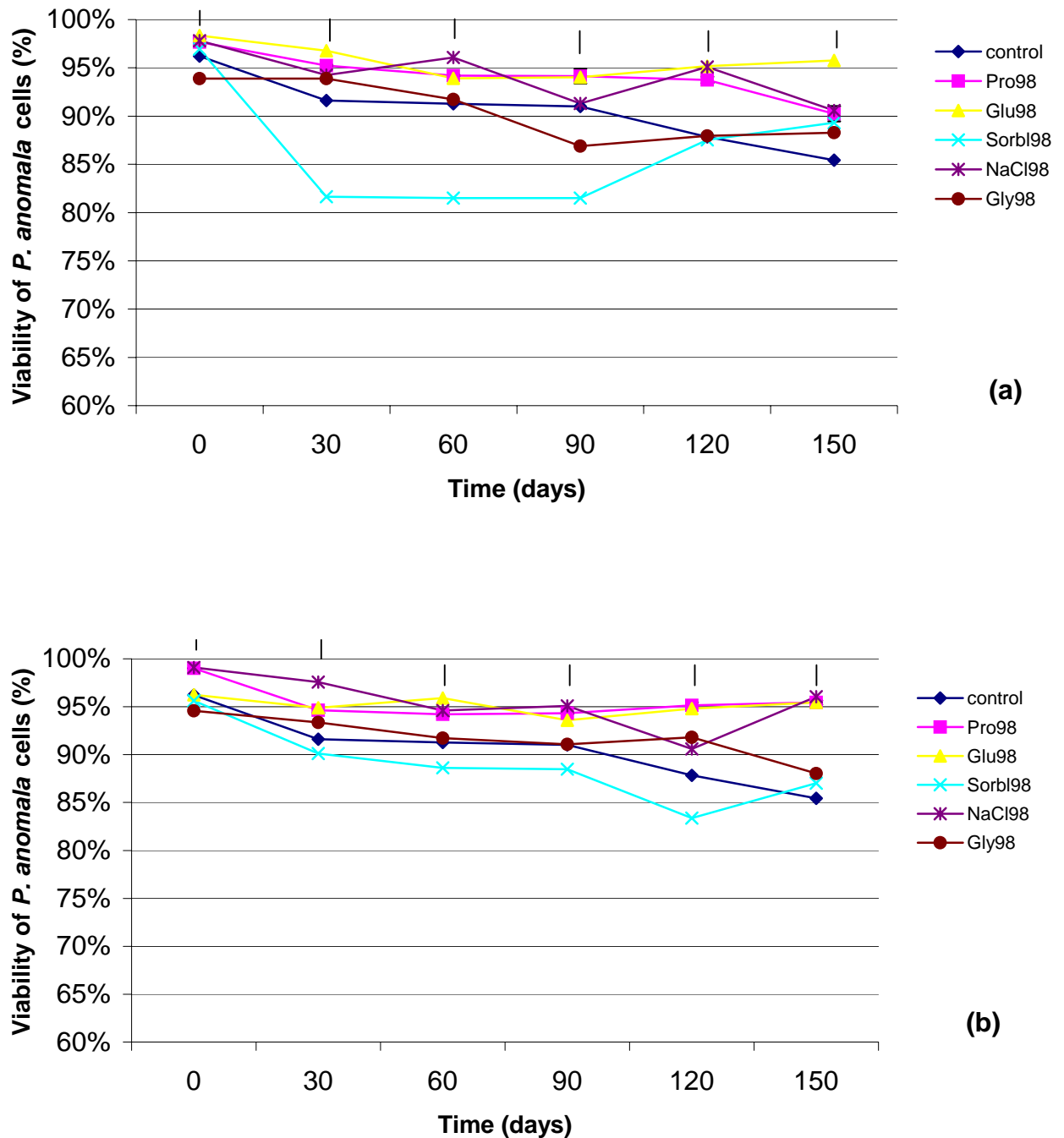
Figure 3.40. Storage stability at 4°C of *P. anomala* freeze dried cell treatments when (a) water or (b) isotonic NaCl solution were used as post-harvest washing treatments. Modified *P. anomala* cells were grown in modified molasses media with proline (pro98), glucose (glu98), sorbitol (sorb98), NaCl (NaCl98) and glycerol (gly98); control cells were grown in unmodified molasses media. All data are means of three replicates per treatment. Bars = LSD (*P*<0.05).
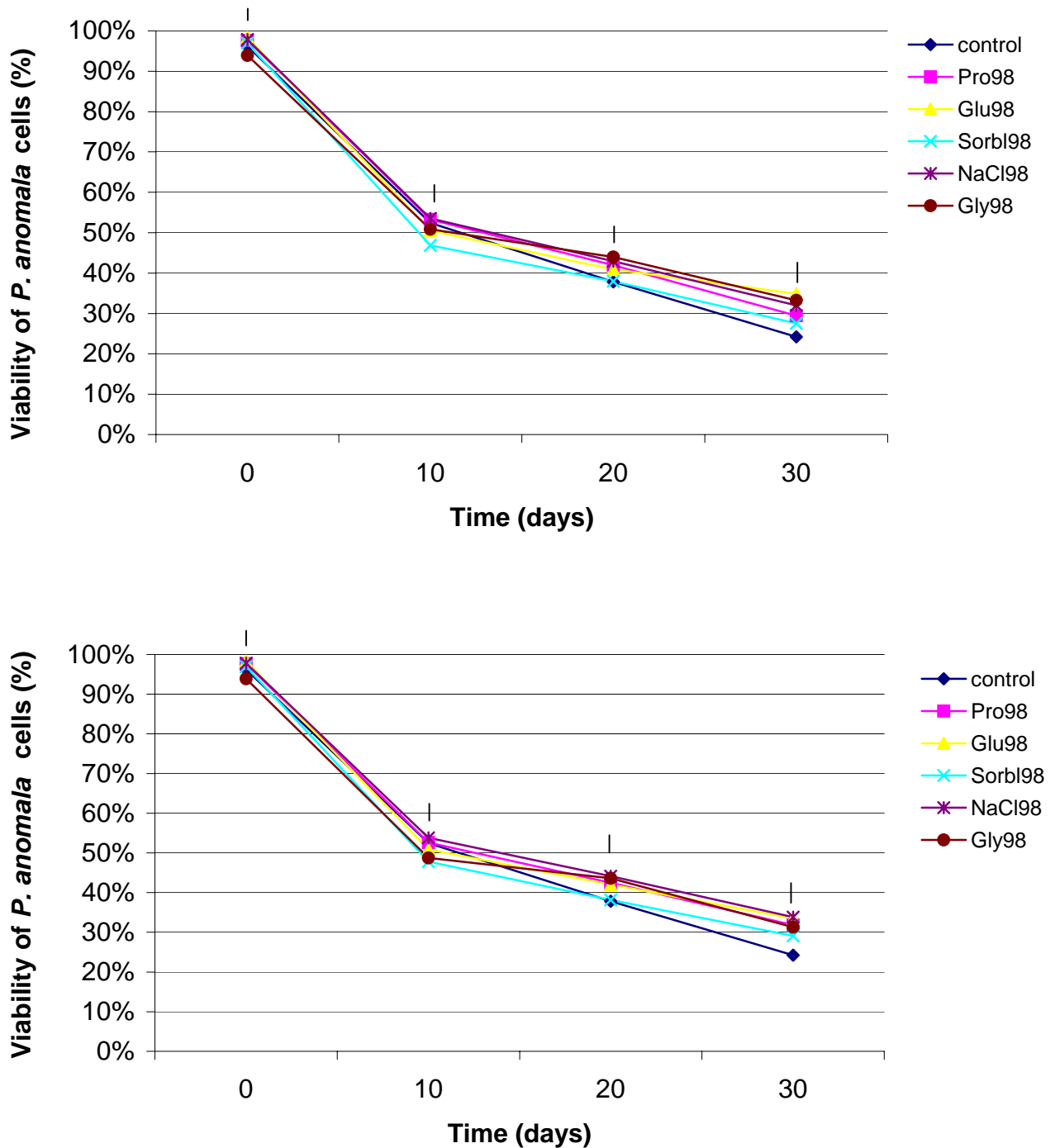
Figure 3.41. Storage stability at 22°C of *P. anomala* freeze dried cell treatments when (a) water or (b) isotonic NaCl solution were used as post-harvest washing treatments. Modified *P. anomala* cells were grown in modified molasses media with proline (pro98), glucose (glu98), sorbitol (sorb98), NaCl (NaCl98) and glycerol (gly98); control cells were grown in unmodified molasses media. All data are means of three replicates per treatment. Bars represent standard errors of the means.

3.9 ENZYME PRODUCTION BY *P. anomala*

3.9.1 Screening for enzyme production by use of API ZYM system

API ZYM system was used to quickly screen the possibility of several enzyme production by *P. anomala*. Analysis of enzymatic activity of *P. anomala* cells grown in Czapek salt minimal medium supplemented with CWP of *P. verrucosum* (P.v.) 2 mg ml-1, CWP of *P. verrucosum*+glucose (each 2 mg ml^{-1}) and CWP of *P. roqueforti* (P.r.) 2 mg ml-1 (Fig. 3.42) demonstrated that butyrate esterase, caprylate esterase lipase, acid phosphatase, napthol-AS-BI-phosphohydrolase and β-glucosidase were detected in all treatments. Enzymatic activity of butyrate esterase and caprylate esterase lipase increased overtime (3rd and 4rth day respectively) and was high for *P. verrucosum* CWP+glucose treatment (20 and 15 nmoles respectively) and scarcely detected for the other two treatments. Acid phosphatase activity slightly increased with time and then remained stable for both CWP treatments alone while it slightly decreased to be non detectable for *P. verrucosum* CWP+glucose treatment; in all cases it was no higher than 10 nmoles. For all treatments, napthol-AS-BI-phosphohydrolase activity increased up to day 2 and then started decreasing. β-glucosidase activity was low for all treatments (up to 5 nmoles).

Analysis of temporal enzymatic activity of *P. anomala* cells grown in unmodified (control) and modified with proline (pro), glucose (glu) and NaCl (NaCl) molasses media, post-harvest washed with water or NaCl isotonic solution (Iso) for 72 h at 25°C that were subsequently grown in Czapek salt minimal medium supplemented with *P. verrucosum* CWP+glucose (each 2 mg ml^{-1}) is shown on Fig. 3.43 for (a) butyrate esterase, (b) caprylate esterase lipase, (c) acid phosphatase, (d) napthol-AS-BI-phosphohydrolase and (e) β-glucosidase. Butyrate esterase activity, for all but control treatments, was low and progressively decreased over time. Caprylate esterase lipase activity increased after day 3 to remain stable or decrease by day 5 for all but the proline water post-harvest treatments. Acid phosphatase increased at day 3 to remain stable at day 5 with only exception being proline water post-harvest treatment that increased at day 5. The pattern was similar for napthol-AS-BI-phosphohydrolase activity. However, proline cells washed post- harvest with both water and NaCl isotonic solution increased enzyme activity over time. β-glucosidase activity was low

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(<10 nmoles) and slightly increased overtime to all but the glucose treatments. In general, when the same treatment cells were washed post-harvest with isotonic solution there was slightly higher enzyme activity. However, it has to be stressed that API-ZYm system kit is at best semi-quantitative.
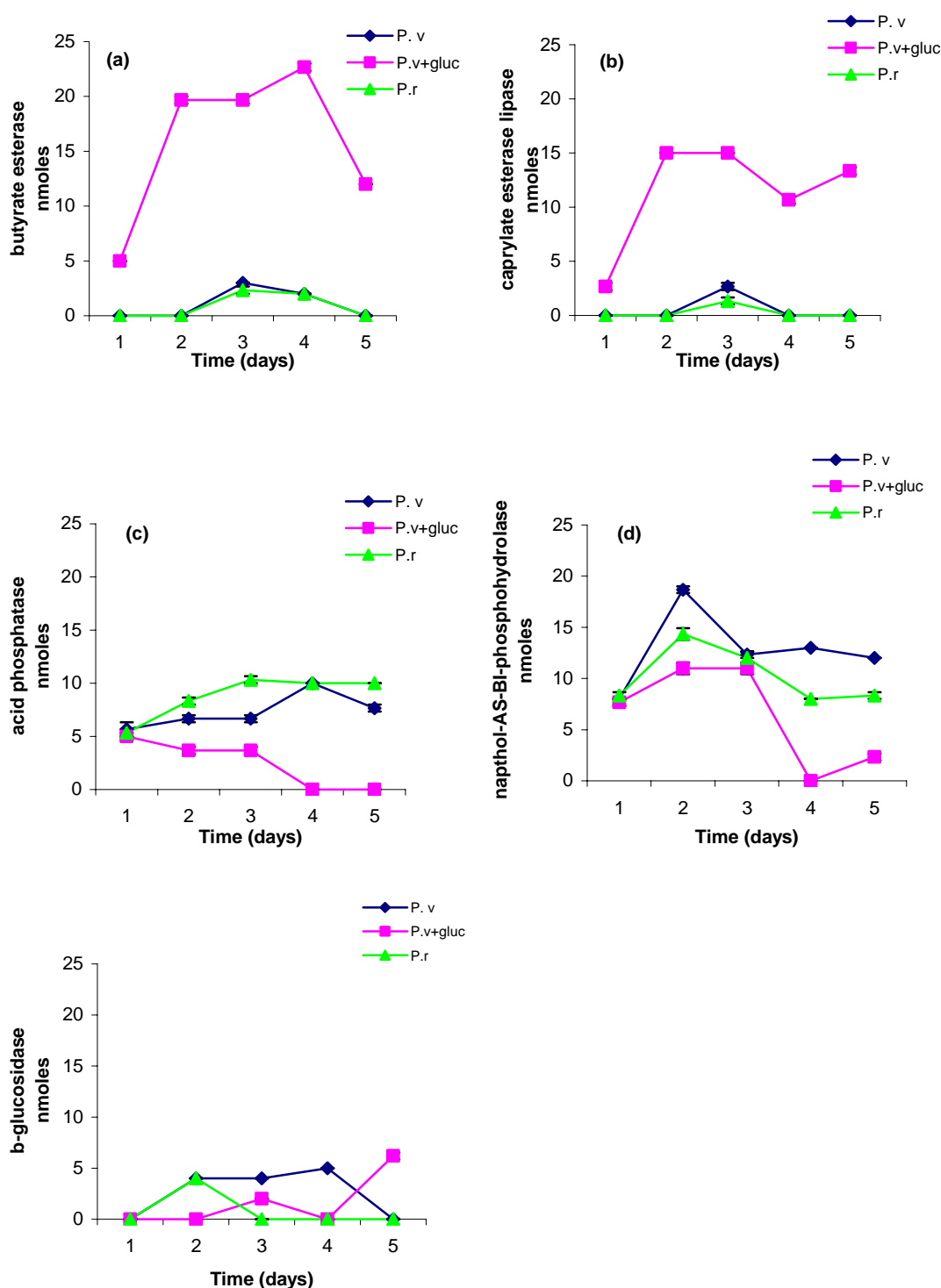
Figure 3.42. Temporal enzymatic activity of *P. anomala* cells when grown in Czapek salt minimal medium in the presence of *P. verrucosum* CWP (P.v.) 2 mg ml^{-1}, *P. verrucosum* CWP +glucose (each 2 mg ml^{-1}) and of *P .roqueforti* CWP (P.r.) 2 mg ml^{-1} Cells were incubated at 25°C. Enzymatic activity in nmoles was scored for (a) butyrate esterase, (b) caprylate esterase lipase, (c) acid phosphatase, (d) napthol-AS-BI-phosphohydrolase and (e) β-glucosidase according to API ZYM system manufacturers colour chart. There were three replicates per treatment. Bars represent standard error (SE) of the means.
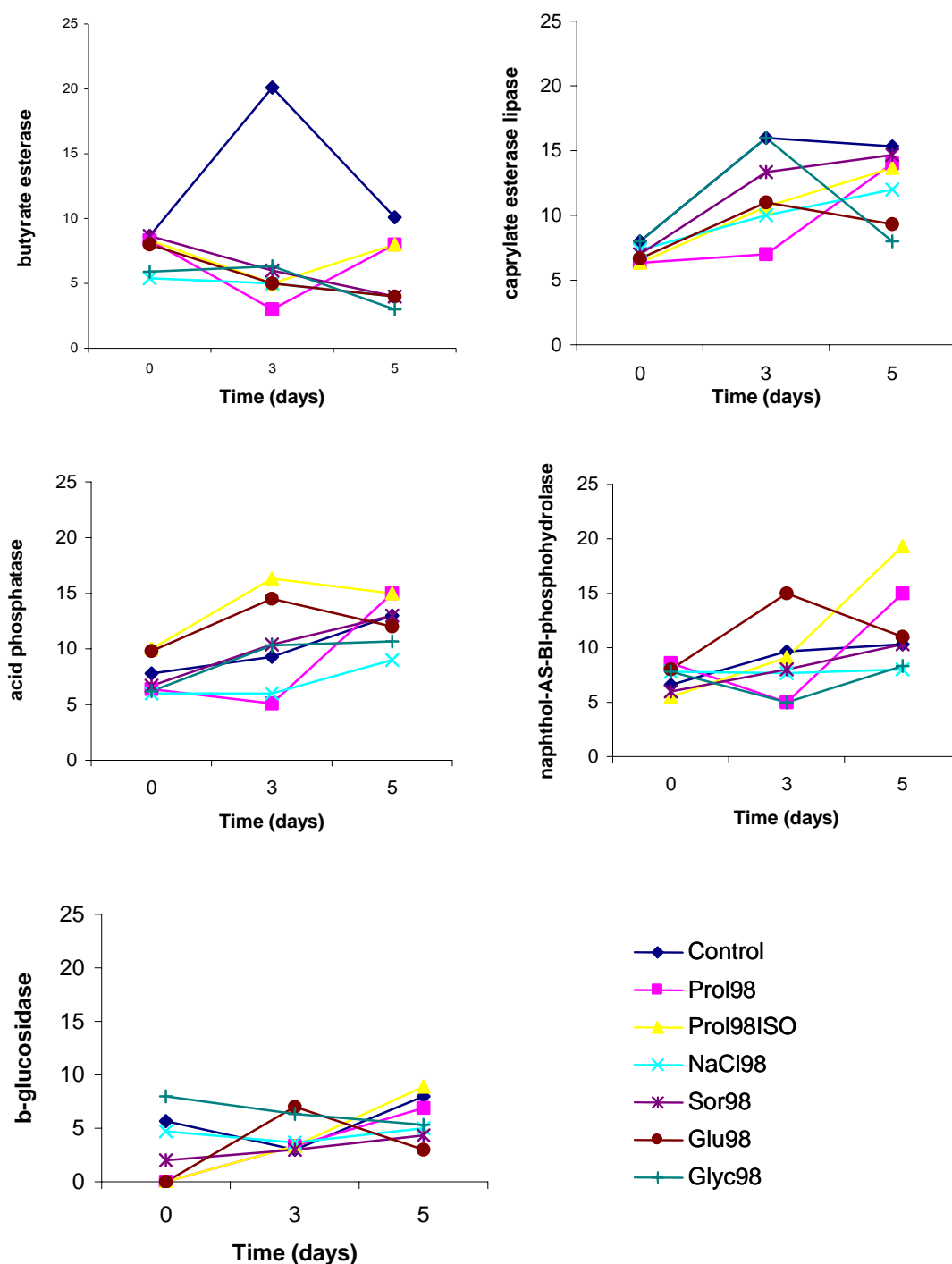
Figure 3.43. Temporal enzymatic activity of *P. anomala* cells from modified molasses media with proline (Pro98), glucose (Glu98) and NaCl (NaCl98), all at 0.98 a_w level. Control cells grew in unmodified molasses media and washed post-harvest with water. Cells were post-harvest washed with water and NaCl isotonic solution and subsequently grown in Czapek salt minimal medium in the presence of CWP of *P. verrucosum*+glucose (each 2mg ml^{-1}).Cells were incubated at 25°C. Enzymatic activity in nmoles was scored for (a) butyrate esterase, (b) caprylate esterase lipase, (c) acid phosphatase, (d) napthol-AS-BI-phosphohydrolase and (e) β-glucosidase according to API ZYM system manufacturers colour chart. There were three replicates per treatment. Bars represent standard error (SE) of the means.

3.9.2   Production of exo β-1,3-glucanase in *vitro*

Effect of different carbon sources when added to Czapek minimal salt medium and different incubation times on exo β-1,3-glucanase produced by *P. anomala* cells is shown on Fig. 3.44. Statistical analysis (*P*<0.05) showed that there was a two-way significant interaction between substrate used and time, affecting exo β-1,3-glucanase activity (Appendix I). Enzyme activity in each sole carbon source increased with incubation time. The only case where a reduction in enzyme activity was noticed was the combination of chitin+laminarin at 72 h incubation. Laminarin addition resulted in the highest enzyme activity after 72 h incubation (1.2 units), followed by chitin (1.02 units) and glucose (0.96 units). The lowest enzyme activity at 72 h was detected in chitin+glucose treatment (0.36 units).
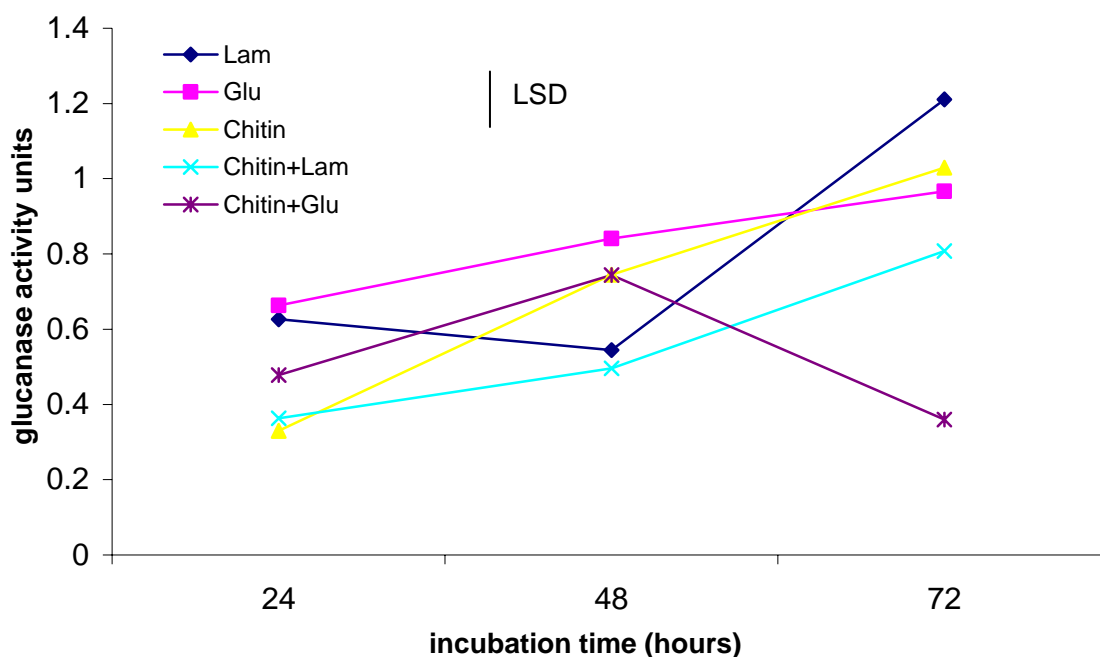
Figure 3.44. Exo β-1,3-glucanase activity of *P. anomala* grown in Czapek minimal salt medium in the presence of laminarin (Lam) 2 mg ml^{-1}, glucose (Glu) 2mg ml^{-1}, chitin 10 mg ml^{-1} and their combinations. Cells were incubated at 25°C. Bar indicates least significant differences (LSD) (*P*<0.05) of the means.

Figure 3.44 shows the effect of Cell Wall Preparations (CWP) of *P. verrucosum*, *P. roqueforti* and combination of *P. verrucosum*+glucose used as carbon sources to supplement Czapek salt minimal medium and incubation time on exo β-1,3-glucanase activity produced by *P.anomala*. Statistical analysis (P<0.05) showed that there was no significant interaction between those two factors; however, each of them significantly affected exo β-1,3-glucanase activity (Appendix I). Enzyme activity increased with incubation time and the combination of *P. verrucosum* CWP+glucose resulted in the significantly highest exo β-1,3-glucanase activity for all incubation times reaching 2.23 units after 120 h. CWP derived from *P. verrucosum* resulted in significantly higher enzyme activity compared to CWP from *P. roqueforti*.
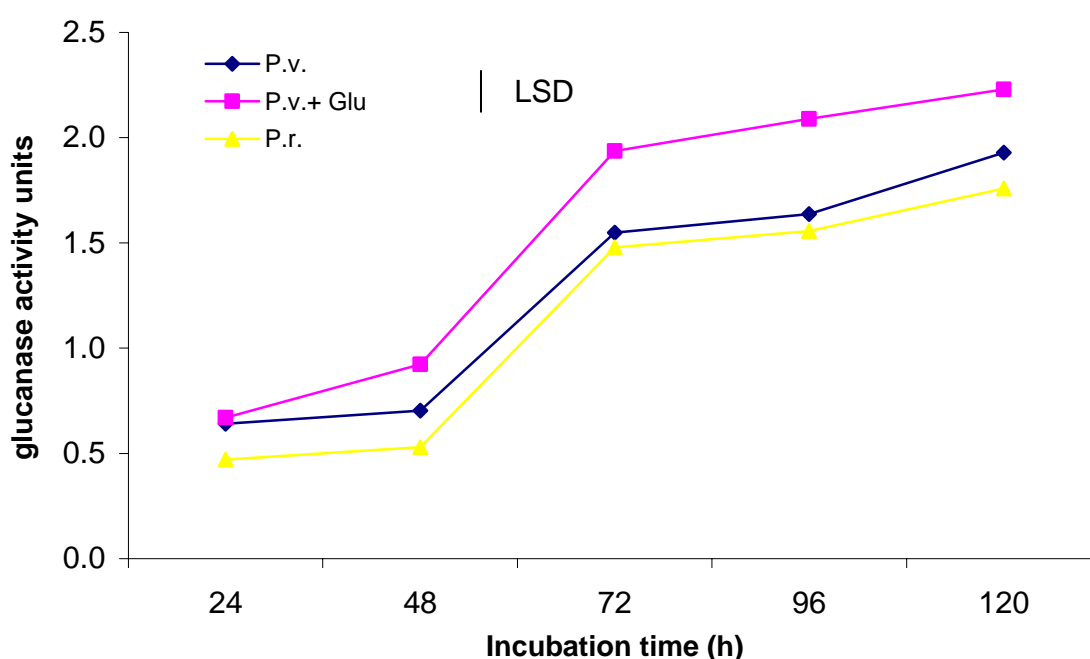
Figure 3.45. Exo β-1,3-glucanase activity of *P. anomala* grown in Czapek salt minimal medium in the presence of CWP of *P. verrucosum* (P.v.) 2 mg ml^{-1}, CWP of *P. verrucosum*+glucose (each 2mg ml^{-1}) and CWP of *P. roqueforti* (P.r.) 2 mg ml^{-1}. Cells were incubated at 25°C. Bar indicates least significant differences (LSD) (*P*<0.05) of the means.

Figure 3.46 shows the effect of different *P. anomala* cell treatment and different post-harvest washing treatments (water and NaCl isotonic solution) on exo β-1,3-glucanase activity after 120h incubation. Statistical analysis ($P<0.05$) showed that there was no significant interaction between different cell and post-harvest treatments (Appendix I). However, main factor effects were significant. Data of different post-harvest treatments were pooled together and means of different cell treatments are also shown. Proline 0.98 a_w cells resulted in similar enzyme activity compared to control treatment while glucose 0.98 and NaCl 0.98 cells resulted in a significantly lower enzyme activity and did not differ between them.
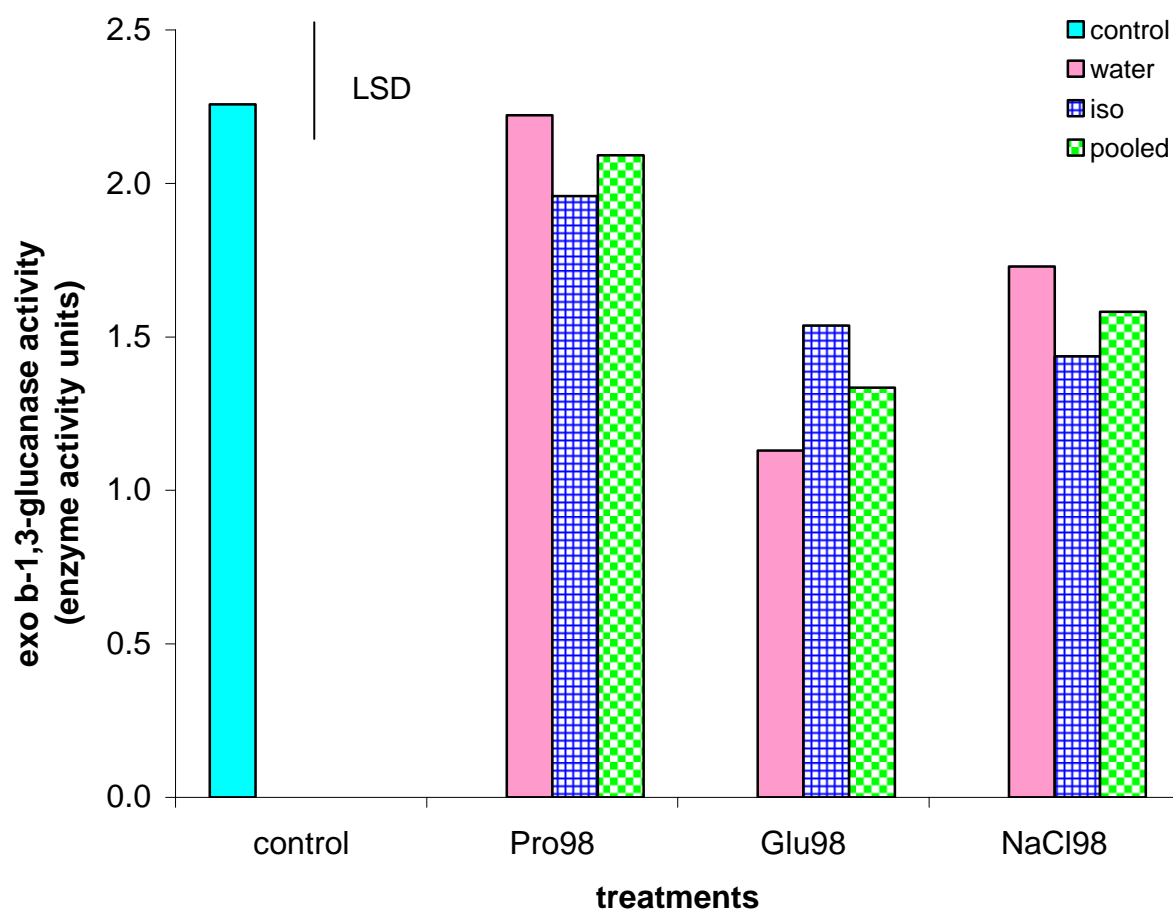
Figure 3.46. Exo β-1,3-glucanase activity of *P. anomala* cells from modified molasses media with proline (Pro98),glucose (Glu98) and NaCl (NaCl98), all at 0.98 a_w level. Control cells grew in unmodified molasses media and washed post-harvest with water.Cells were post-harvest washed with water ▨ and NaCl isotonic solution ▨ and subsequently grown in Czapek salt minimal medium in the presence of CWP of *P. verrucosum*+glucose (each 2mg ml^{-1}). Cells were incubated at 25°C. Bar indicates least significant differences (LSD) (P<0.05) of the means.

3.9.3 Production of exochitinase *in vitro*

Effect of different carbon sources when added to Czapek minimal salt medium and different incubation times on exochitinase produced by *P. anomala* cells is shown on Figure 3.47. Statistical analysis (*P*<0.05) showed that those two factors had a significant combined effect on exochitinase activity (Appendix I). Enzyme activity in each sole carbon source increased with incubation time. The chitin + glucose combination treatment resulted in a significantly higher enzyme activity after 72 h incubation (1.2 units), followed by chitin+laminarin (0.7 units), glucose (0.7 units). Chitin (0.63) did not significantly differ between treatments. The lowest enzyme activity at 72 h was detected in laminarin treatment (0.39 units).
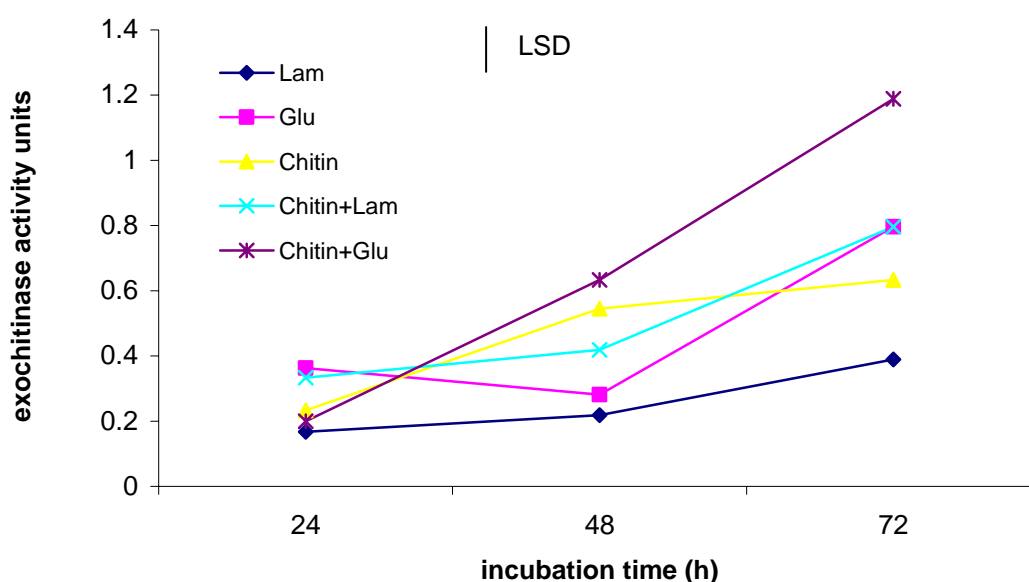
Figure 3.47. Exochitinase activity of *P. anomala* grown in Czapek salt minimal medium in the presence of laminarin (Lam) 2 mg ml^{-1}, glucose (Glu) 2mg ml^{-1}, chitin 10 mg ml^{-1} and their combinations. Cells were incubated at 25°C. Bar indicates least significant differences (LSD) (*P*<0.05) of the means.

Figure 3.48 shows the effect of Cell Wall Preparations (CWP) of *P. verrucosum*, *P. roqueforti* and combination of *P. verrucosum*+glucose used as carbon sources to supplement Czapek salt minimal medium and incubation time on exochitinase activity produced by *P. anomala*. Statistical analysis ($P<0.05$) showed that there was a significant two-way interaction between those two factors with the main effect of each of them being also significant (Appendix I). Enzyme activity increased with incubation time and the combination of *P. verrucosum* CWP+glucose resulted in significantly higher exochitinase activity for all incubation times reaching 0.9 units after 120 h incubation. *P. verrucosum* and *P. roqueforti* CWP did not significantly differ in conferred enzyme activity. Exochitinase activity was no higher than 0.93 units (*P. verrucosum* CWP+glucose after 96 h incubation).
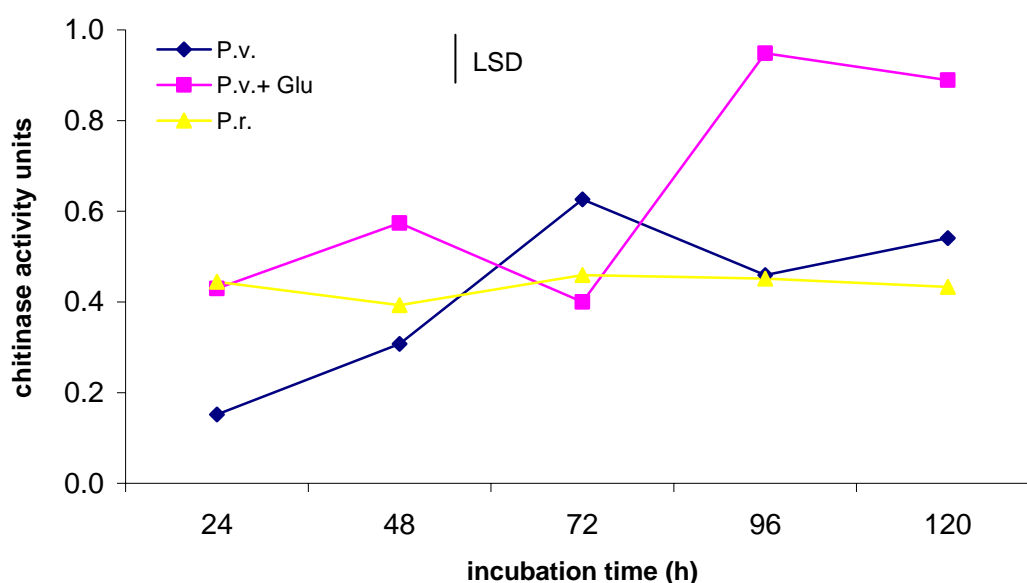
Figure 3.48. Exochitinase activity of *P. anomala* grown in Czapek salt minimal medium in the presence of CWP of *P. verrucosum* (P.v.) 2 mg ml^{-1}, CWP of *P.verrucosum*+glucose (each 2 mg ml^{-1}) and CWP of *P.roqueforti* (P.r.) 2 mg ml^{-1}. Cells were incubated at 25$^{\mathrm{o}}$C. Bar indicates least significant differences (LSD) ($P<0.05$) of the means.

Figure 3.49 shows the effect of different *P. anomala* cell treatment and different post-harvest washing treatment (water and NaCl isotonic solution) on exochitinase activity after 120 h incubation. Statistical analysis ($P<0.05$) showed that there was a significant 3x3 interaction between ±media modification, different media modification and post-harvest treatments (Appendix I). Glucose 0.98 a_w cells washed post-harvest with NaCl isotonic solution resulted in the significantly highest enzyme activity (1.3 units), which differed to that conferred when cells were washed post-harvest with water (0.99 unit). NaCl 0.98 98 a_w cells washed post-harvest with NaCl isotonic solution resulted in 0.90 unit enzyme activity, again significantly higher than the one when cells were washed post-harvest with water. However, NaCl 0.98 a_w cells washed with isotonic solution resulted in significantly lower enzyme activity compared to NaCl 0.98 a_w cells washed with water.
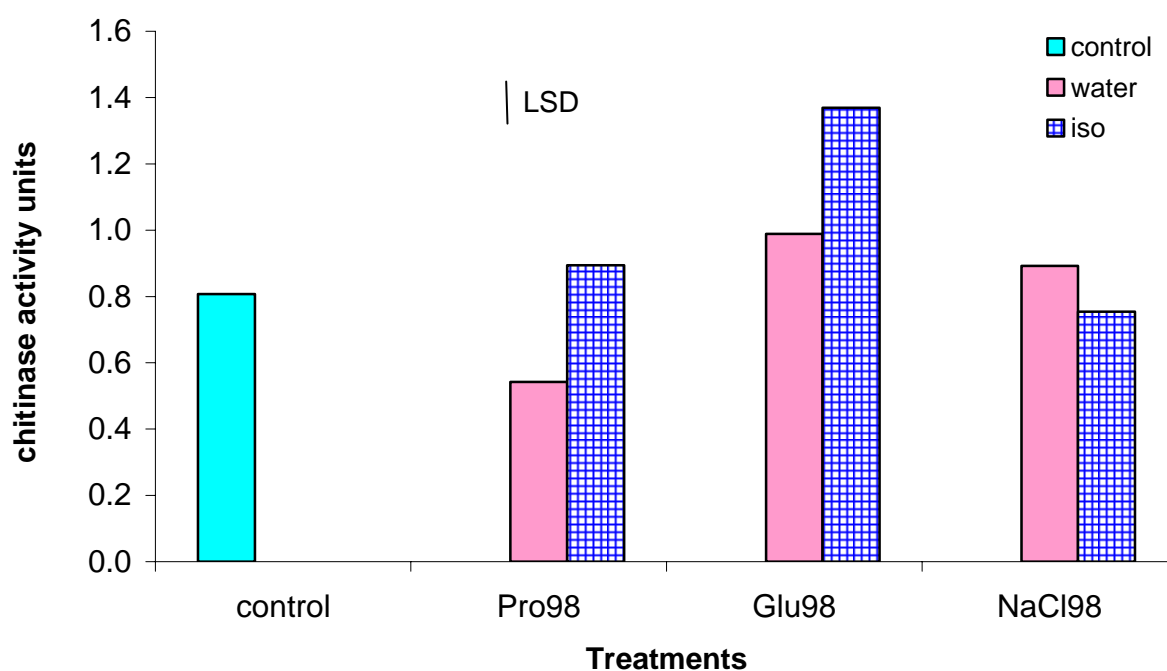
Figure 3.49. Exochitinase activity of *P. anomala* cells from modified molasses media proline (Pro98), glucose (Glu98) and NaCl (NaCl98), all at 0.98 a_w level. Control cells grew in unmodified molasses media and washed post-harvest with water. Cells were post-harvest washed with water☐ and NaCl isotonic solution☐and subsequently grown in Czapek salt minimal medium in the presence of CWP of *P. verrucosum*+glucose (each 2 mg ml^{-1}) and CWP of *P. roqueforti* (P.r.) 2 mg ml^{-1}. Cells were incubated at 25°C. Bar indicates least significant differences (LSD) ($P<0.05$) of the means.

3.9.4 Production of β-glucosidase *in vitro*

Figure 3.50 shows (a) total (μmol 4-nitrophenol min^{-1}) and (b) specific (nmol 4-nitrophenol min^{-1} μg^{-1} protein) enzyme activity of β-glucosidase produced by *P. anomala*. The factors tested were the different *P.anomala* cell treatment and different post-harvest washing treatment (water and NaCl isotonic solution). For total enzyme activity, statistical analysis ($P<0.05$) showed that interaction of two factors was not significant and that only different *P. anomala* treatment had a significant effect on it. Modification of molasses media with several solutes (NaCl, glucose and proline) did not significantly affect total β-glucosidase activity. Proline treatment resulted in significantly higher total enzyme activity (0.85 μmol 4-nitrophenol min^{-1}) with the rest of the treatments having no differences between them. For specific enzyme activity, statistical analysis ($P<0.05$) showed that interaction of two factors was not significant and that only different *P.anomala* treatments had a significant effect. Control treatment had the highest specific enzyme activity (3 nmol 4-nitrophenol min^{-1} μg^{-1} protein); glucose and NaCl treatments followed with no significant differences between them (2.48 and 2.41 nmol 4-nitrophenol min^{-1} μg^{-1} protein). Proline treatment resulted in the lowest specific enzyme activity (0.91 nmol 4-nitrophenol min^{-1} μg^{-1} protein).
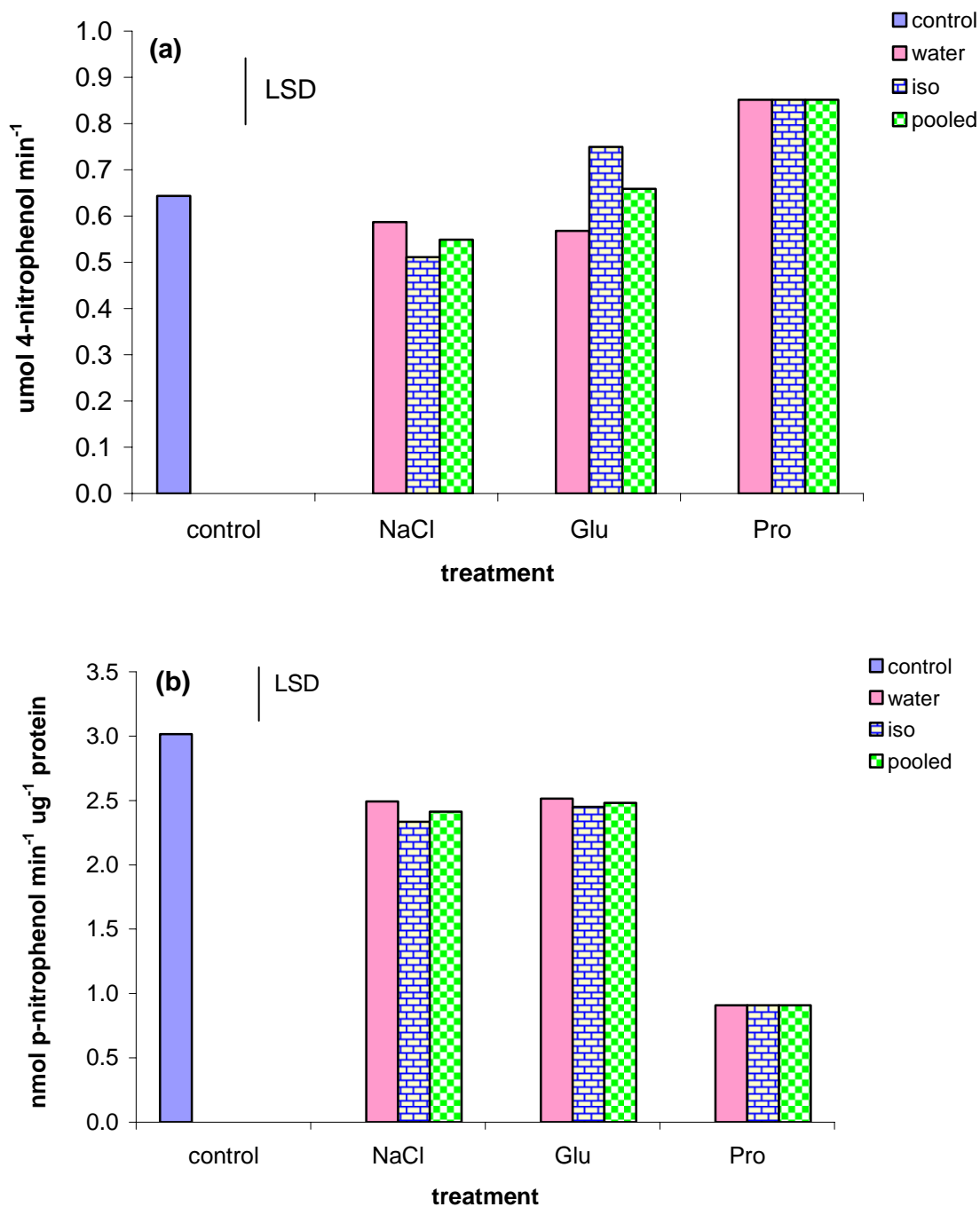
Figure 3.50. Total (μmol 4-nitrophenol min^{-1}) (a) and specific (nmol 4-nitrophenol min^{-1} μg^{-1} protein) (b) enzyme activity of β-glucosidase produced by *P. anomala*. Treatment cells grew in m olasses medium modified with proline (Pro), glucose (Glu) and NaCl (NaCl), all at 0.98 a$_w$ level. Control cells grew in unmodified molasses media and washed post-harvest with water.Cells were post-harvest washed with water ▢ and NaCl isotonic solution ▢ and subsequently grown in Czapek salt minimal medium in the presence of *P. verrucosum* CWP + glucose (each 2 mg ml^{-1}). Cells were incubated at 25°C for 120 h. Bars indicate least significant differences of the means (LSD) (*P*<0.05).

3.9.5 Endo protein production of *P. anomala* cell treatments when post-harvest washed with water and NaCl isotonic solution

Figure 3.51 shows the effect of different solutes added to modify molasses media, a_w of media and use of different post-harvest washing treatments on endo protein production by *P. anomala*. Modification of media itself was used as an added factor. Statistical analysis ($P<0.05$) showed that there was a significant interaction between modification of media itself, different cell modification, a_w of media and post-harvest treatments. All factors significantly affected endo protein production. Glucose 0.96 a_w cells when washed NaCl isotonic solution were found to contain the highest amount of total protein (19.39 mg g^{-1} f.w.). Other than this treatment, the control was found to contain significantly higher total endoprotein compared to all other treatments (10.29 mg g^{-1} f.w.). Glucose treatment contained, in general, higher total endo protein amounts than other treatments. When cells were post-harvest washed with water, media modification to the lower a_w level resulted in lower total protein content. However, when cells were washed post-harvest with NaCl isotonic solution there were no significant differences between different a_w levels for same modification treatment, with glucose only exception. Proline and NaCl modified media contained the lowest total protein at 0.96 a_w level when washed post-harvest with water (4.45 and 4.47 mg g^{-1} f.w respectively).
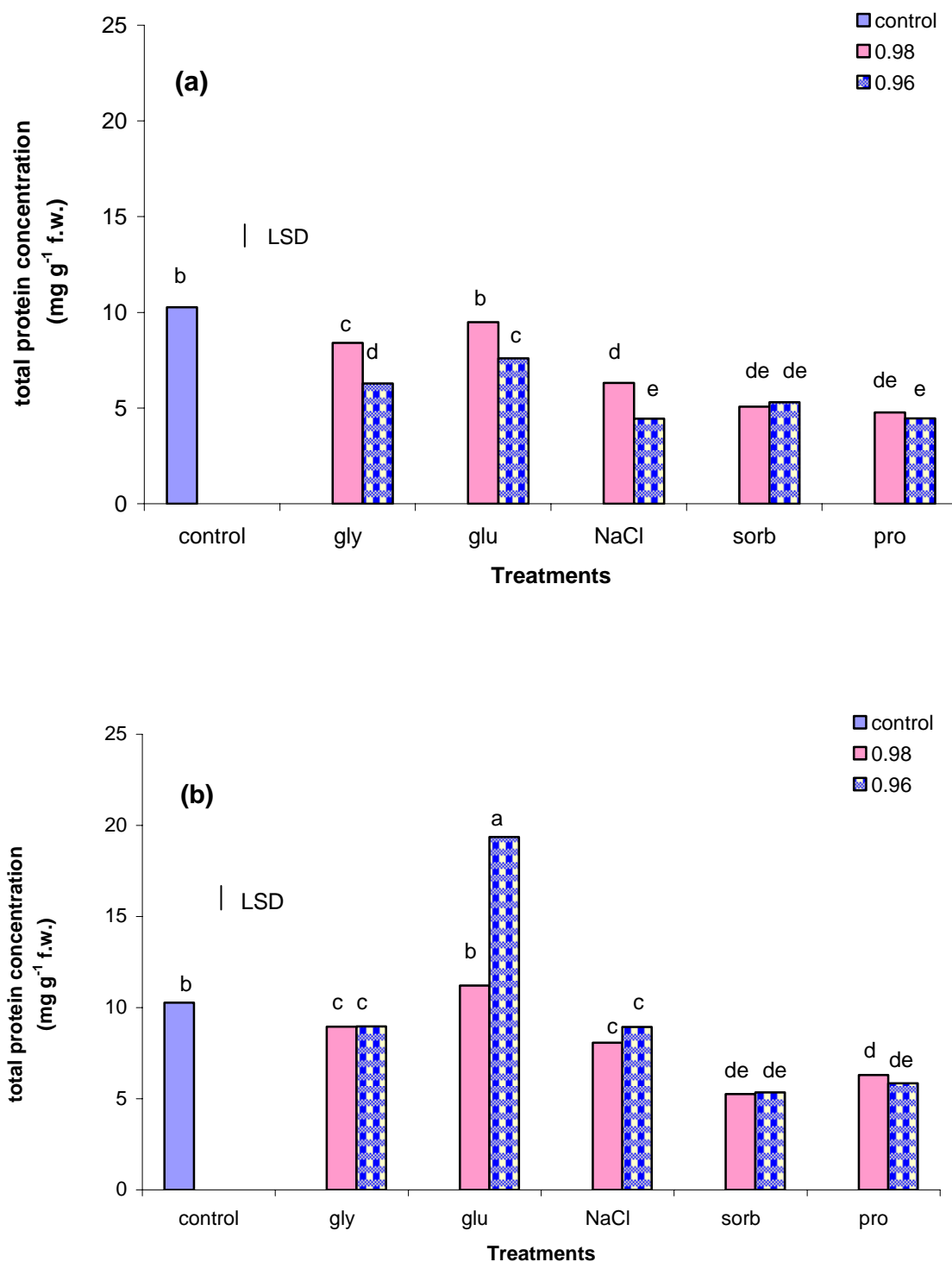
Figure 3.51. The effect of different solutes (glycerol-gly, glucose-glu, NaCl, sorbitol-sorb and proline-pro), a_w level (0.98 □ and 0.96 ▨ and post-harvest treatment [water (a) and NaCl isotonic solution (b)] on endogenous total protein content in *P. anomala* cells grown in unmodified (control) and modified with above the solutes molasses media. Cells were grown at 25°C for 72 h. All data are means of three replicates per treatment. Different letters indicate statistical (*P*<0.05) differences between means. Bars = LSD (*P*<0.05).

3.10 BIOASSAYS

3.10.1 Influence of *P. anomala* on growth of *P. verrusocum* in γ-irradiated wheat grain

Effect of *P. anomala* on *P. verrucosum* growth in γ-irradiated wheat grain at 25°C for 1, 10, 20 and 30 days is shown on Fig. 3.52 at (a) 0.95 and (b) 0.93 a_w levels. Statistical analysis (*P*<0.05) showed that the mould's growth was a result of 3x3 significant interaction between different treatments used, wheat grain a_w and time. 2x2 interactions and the main effects of each factor were also significant (Appendix I). *P. verrucosum* alone reached significantly higher population at 0.95 a_w, starting from 1 x 10^3 CFUs g^{-1} one day after inoculation, to reach 6.2 x 10^6 CFUs g^{-1} and 4.5 x 10^6 CFUs g^{-1} at 0.93 a_w after 30 days.When co-cultured with *P. anomala,* it reached a level of 3.2-3.75 x 10^5 CFUs g^{-1} with no statistical differences between the different treatments. However, there were statistical differences between treatments after 10 days.

3.10.2 *P. anomala* growth when co-cultured with *P. verrucosum* in γ-irradiated wheat grain

P. anomala growth alone or in co-culture with *P. verrucosum* in γ-irradiated wheat grain at 25°C for 1, 10, 20 and 30 days is shown on Fig. 3.53 for (a) 0.95 and (b) 0.93 a_w levels. Statistical analysis (*P*<0.05) showed that yeast's growth was a result of a 3x3 significant interaction between different treatments used, wheat grain a_w and time. 2x2 interactions and the main effects of each factor were also significant (Appendix I). *P. anomala* alone reached statistically higher growth at 0.95 a_w, starting from 5 x 10^5 CFUs g^{-1} one day after inoculation, to reach 8.8 x 10^7 CFUs g^{-1} after 30 days. At 0.93 a_w the yeast alone reached populations of 6.2 x 10^7 CFUs g^{-1} after 30 days. In general, lower wheat grain a_w resulted in significantly decreased yeast growth either alone or in co-culture with *P. verrucosum*. Co-culture with *P. verrucosum* also significantly decreased *P. anomala* growth; the co-culture treatment that gave higher CFUs g^{-1} was A, fresh *P. anomala* proline cells after 30 days of inoculation at 0.95 a_w (5.7 x 10^7 CFUs g^{-1}).

3.10.3 Influence of *P. anomala* on ochratoxin A (OTA) accumulation produced by *P. verrucosum* in γ-irradiated wheat grain

Ochratoxin A (OTA) accumulation produced by *P. verrucosum* alone or in co-culture with *P. anomala* in γ-irradiated wheat grain at 25°C for 1, 10, 20 and 30 days is shown on Fig. 3.54 for (a) 0.95 and (b) 0.93 a_w levels. Statistical analysis (*P*<0.05) showed that OTA accumulation was a result of a three-way significant interaction between the different treatments used, wheat grain a_w and time. Two and one-way interactions were also significant. OTA was significantly accumulated after 30 days of inoculation for all treatments. When *P. verrucosum* grew alone, OTA accumulated after 30 days of inoculation reached 56000 and 28700 µg K^{-1} for 0.95 and 0.93 wheat grain a_w levels respectively. Interestingly, at 0.95 wheat grain a_w level, co-culture with *P. anomala* resulted in significantly increased OTA accumulation for A, *P. anomala* proline modified fresh cells washed with NaCl isotonic solution, and C, *P. anomala* proline modified cells fluidised bed dried as cottonseed flour:1+10% skimmed milk formulation washed with water, treatments. At 0.93 wheat grain a_w level, co-culture with *P.anomala* resulted in similar OTA levels with C treatment, or significantly decreased in A, B and D treatments.
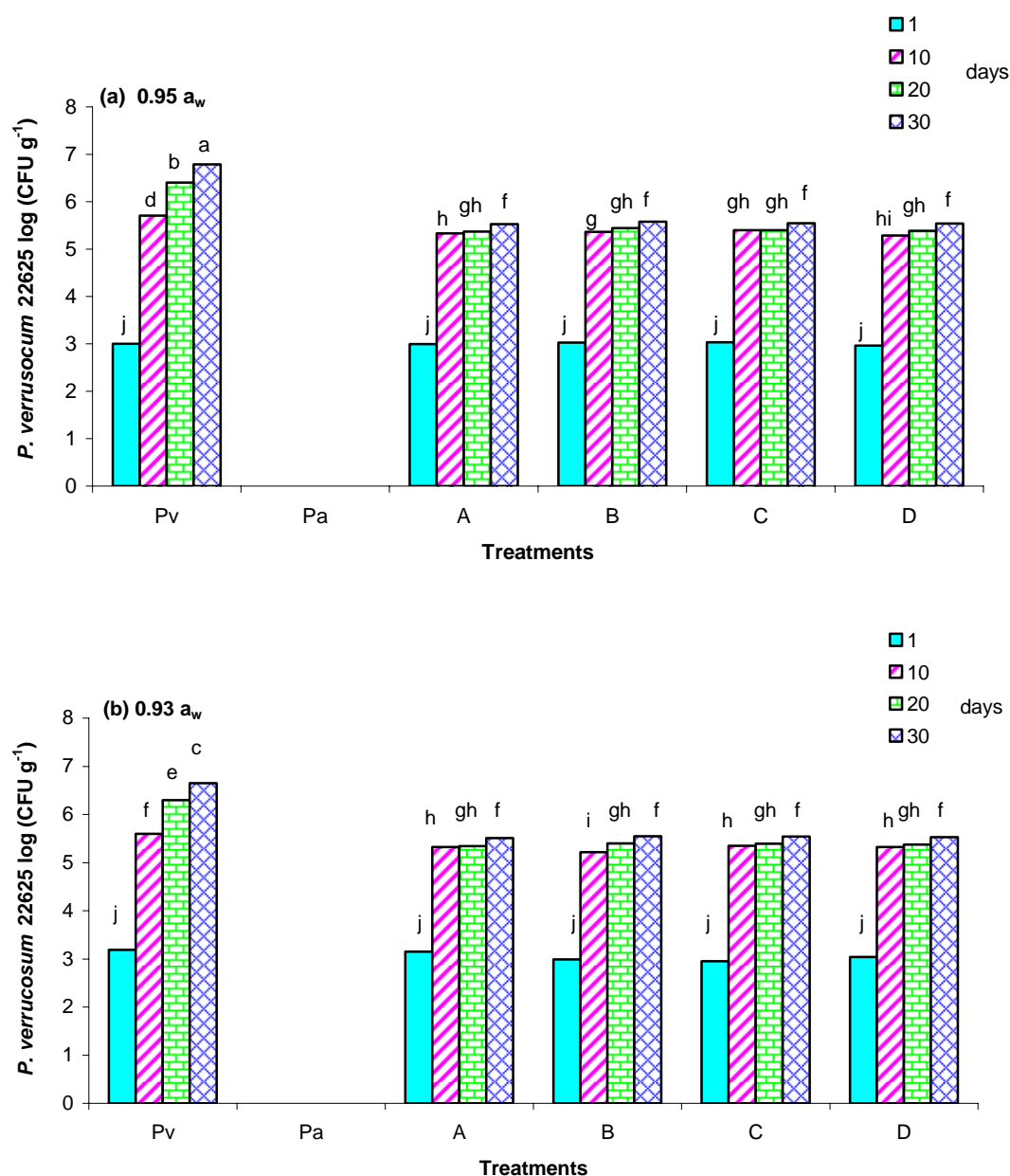
Figure 3.52. Effect of *P. anomala* on the growth and sporulation of *P. verrucosum* strain 22625 measured as CFUs in co-cultures in petri dishes with γ-irradiated wheat grain [a_w 0.95 (a) and a_w 0.93 (b)] at 25°C for 1 ▢ , 10 ▨ , 20 ▨ and 30 ▨ days. Different treatments were *P. verrucosum* alone (control), *P.anomala* unmodified fresh cells alone, *P. anomala* proline modified fresh cells washed with NaCl isotonic solution (A), *P. anomala* unmodified cells fluidised bed dried as cottonseed flour: 1 + 10% skimmed milk formulation (B), *P. anomala* proline modified cells fluidised bed dried as cottonseed flour:1+10% skimmed milk formulation washed with water (C), *P. anomala* proline modified cells fluidised bed dried as cottonseed flour: 1 + 10% skimmed milk formulation washed with NaCl isotonic solution (D). A, B, C, D treatments were co-cultures with *P. verrucosum*. All data are means of three replicates per treatment. Different letters indicate statistical (*P*<0.05) differences between means.

Figure 3.53. *P. anomala* growth measured as CFUs in co-cultures with *P. verrucosum* strain 22625 in petri dishes with γ-irradiated wheat grain [a_w 0.95 (a) and a_w 0.93 (b)] at 25°C for 1 ▢ , 10 ▨, 20 ▦ and 30 ▧ days. Different treatments were *P.verrucosum* alone (control), *P. anomala* unmodified fresh cells alone, *P. anomala* proline modified fresh cells washed with NaCl isotonic solution (A), *P. anomala* unmodified cells fluidised bed dried as cottonseed flour:1 + 10% skimmed milk formulation (B), *P. anomala* proline modified cells fluidised bed dried as cottonseed flour: 1 + 10% skimmed milk formulation washed with water (C), *P. anomala* proline modified cells fluidised bed dried as cottonseed flour: 1 + 10% skimmed milk formulation washed with NaCl isotonic solution (D). A, B, C, D treatments were co-cultures with *P. verrucosum*. All data are means of three replicates per treatment. Different letters indicate statistical (*P*<0.05) differences between means.

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Figure 3.54. Effect of *P. anomala* on the accumulation of ochratoxin A (OTA), µg kg^{-1}, produced by *P. verrucosum* strain 22625 alone or in co-cultures, in Petri dishes with γ-irradiated wheat grain [a$_w$ 0.95 (a) and a$_w$ 0.93 (b)] at 25°C for 1▢ , 10▨, 20▦ and 30▨ days. Different treatments were *P. verrucosum* alone (control), *P. anomala* unmodified fresh cells alone, *P. anomala* proline modified fresh cells washed with NaCl isotonic solution (A), *P. anomala* unmodified cells fluidised bed dried as cottonseed flour:1 + 10% skimmed milk formulation (B), *P. anomala* proline modified cells fluidised bed dried as cottonseed flour: 1 + 10% skimmed milk formulation washed with water (C), *P. anomala* proline modified cells fluidised bed dried as cottonseed flour:1 + 10% skimmed milk formulation washed with NaCl isotonic solution (D). A, B, C, D treatments were co-cultures with *P. verrucosum*. All data are means of three replicates per treatment. Different letters indicate statistical (*P*<0.05) differences between means

4   Chapter 4 DISCUSSION

4.1   YIELD OF *P. anomala* IN UNSTRESSED AND WATER-STRESSED LIQUID NYDB AND MOLASSES-BASED MEDIA

Impact of different solute stress on yield of *P. anomala* cells grown in liquid NYDB (a synthetic medium) and molasses based (a natural-complex) was measured. On both type of basal media, a decrease of a_w resulted in a decrease in *P. anomala* cell yield; a yield increase was, however, noted when glucose was added to media at 0.98 a_w. Reduction of cell yield or spore production when a decrease in a_w took place has been a common finding in several studies employing yeasts (Teixidó *et al.,* 1998b; Abadias *et al.*, 2000) and filamentous fungi (Inch & Trinci, 1987; Ypsilos & Magan 2004). Inch & Trinci (1987) attributed this response to the increased energy requirements for osmoregulation at lower water activities. The type of solute used to decrease media a_w had a differential effect on final yield due to its metabolic value and use by the yeast.

Studies on NYDB media demonstrated that the yeast grew better on the control treatment (5.9 mg ml^{-1} medium) and when glucose was used to modify the a_w. Yield was stimulated in glucose medium at 0.98 a_w (6.1 mg ml^{-1} medium). In all other treatments, yield decreased as medium a_w decreased. Regardless of the solute used to modify a_w, growth in all 0.96 a_w treatments was markedly lower than in 0.98 a_w treatments. Glucose 0.96 a_w treatment had a significant effect on the lag phase but the yeast population recovered quickly. The ionic solute NaCl was toxic at high concentrations (0.96 a_w) resulting in very low yield (0.35 mg ml^{-1} medium). Even at 0.98 a_w cells were less tolerant of NaCl than with non-ionic solutes. Previous studies on the growth of *C. sake* showed that yeast cells were less tolerant to NaCl than to non-ionic solutes (Teixidó *et al.,* 1998b; Abadias *et al.*, 2000). However, several *Candida* spp. were found to be more tolerant in the presence of NaCl than in the presence of the non-ionic solute glucose (Van Eck *et al.,* 1993). To a lesser extent, glycerol and proline resulted in good yield (4.4 and 3.6 mg ml^{-1} medium, respectively). *P. anomala* cells were more tolerant of glucose and proline at 0.96 a_w

when compared to the other solutes examined. Previously, proline was found to give particularly good yield results with *C. sake* cells (Abadias *et al.*, 2000).

Studies on molasses-based media demonstrated that yield followed the same pattern as on NYDB. In the control treatment better growth (2.9 mg ml^{-1} medium) was obtained than in others and only glucose modified medium at 0.98 a_w was superior (3.4 mg ml^{-1} medium) after 72 h incubation. Yield once more decreased as medium water stress was increased. Regardless of the solute used to modify a_w, yield in all 0.96 a_w treatments was significantly lower than in 0.98 a_w; however, the sorbitol 0.96 a_w treatment resulted in a higher yield after 72 h incubation. Glucose had an adverse effect on cell growth at the highest concentration used (0.96 a_w) with the yeast population not being able to recover over the period of the experiment. This may possibly be an indication that such a high level of glucose and sucrose cannot be tolerated by the yeast. NaCl was found to be toxic at 0.96 a_w. Interestingly, it gave good results when used to modify 0.98 a_w. Glycerol treatments resulted in good yields (2.7 and 2.5 mg ml^{-1} medium respectively) at 0.98 and 0.96 a_w especially after 72 h incubation. Previous studies with three entomopathogenic fungi showed that growth on glycerol-modified media was better than on glucose-modified media (Hallsworth & Magan, 1994b). They suggested that this could be due to the ability to take up glycerol directly from the medium; glycerol could also then act intracellularly in osmotic adjustment and as a compatible solute. This could explain the poor yield of *P. anomala* when medium a_w was modified with glucose to 0.96 a_w when compared with the glycerol-modified treatment. However, the general trend was that cells were more affected at 0.96 a_w when cells were grown in molasses-based media than when they were grown in NYDB. This favours the theory that molasses-based media exert stress due to its high sucrose content.

When comparing yield of the yeast cells (as expressed in mg dry weight ml^{-1} of the medium used) between the two different media, the rich defined media was found to result in higher yeast cell production than the heterogeneous undefined sugar cane molasses-based media. The chemical composition of cane molasses usually contains high sugar, mostly sucrose (about 30-40 %), total nitrogen of 0.5-1 %, ash 10%, moisture 9%, proteins, vitamin, amino acids, organic acids and several metals and salts such as iron, zinc, copper, manganese, magnesium and calcium; beet molasses

contain even higher sucrose levels (47-50%) (Roukas, 1998; Miranda *et. al.,* 1999; Costa *et al.,* 2001). In general, glucose, the sugar contained in NYDB is readily utilised by yeasts while sucrose has to be broken down first. The fact that *P. anomala* gave higher yields when grown in NYDB media, where glucose was the only sugar, may indicate a 'glucophilic' behaviour of the yeast. The higher yields obtained when glucose was used as a solute to modify a_w justified this point.

In general, chemically defined media tend to result in poorer fermentation performances than those achieved with complex media where compounds such as amino acids and vitamin are present as constituents. However, there are cases where the opposite happens as a result of accumulation of various toxic compounds such as ethanol, acetic acid and cations at growth inhibitory levels or higher foaming tendency and lower oxygen transfer (Zhang & Greasham, 1999).

Sugar cane molasses is commonly used as a raw material in the manufacture of baker's yeast (*S. cerevisiae*). However, in batch yeast cultivation, high sugar concentrations in the culture can result in the "Crabtree" effect (Win *et al.,* 1996). The Crabtree effect, together with the Pasteur effect, are two regulatory modes affecting the relative contributions of respiration and fermentation to metabolism. The Pasteur effect describes the repression of fermentation by aerobiosis. The Crabtree effect is defined as the occurrence of alcoholic fermentation under fully oxidative conditions. It is the result of respiratory enzyme inhibition and increases ethanol production. Ethanol production was probably the main reason for the lowering of the *S. cerevisiae* maximum cellular yield (Win *et al.,* 1996). The Crabtree effect has been described for only a few types of yeast (Breunig *et al.,* 2000); however there are cases where so-called 'Crabtree-negative' yeasts appeared to perform alcoholic fermentation (Kiers *et al.,* 1998) under certain conditions. Alcoholic fermentation, and thus lower yield production, is not easy to attribute to the Crabtree effect alone as medium composition, sugar type used, sugar concentration and yeast strain predisposition were found to be also involved (Kiers *et al.*, 1998; Leyva *et al.*, 1999). The use of shake cultures makes things more complex as they readily become oxygen limited and oxygen availability is not easy to control in this case (Kiers *et al.,* 1998). Moreover, Costa et *al.* (2001) pointed out that 40 g l^{-1} molasses concentration (the same used in making up the molasses-based media in this study) did not significantly improve

growth of the biocontrol bacterium *Pantoea agglomerans* CPA-2 implying that a high concentration of this carbon source could be toxic to cells of this antagonist. Leyva *et al.* (1999) showed that alcoholic fermentation in *Zygosaccharomyces bailii*, a 'fructophilic' yeast, when grown on fructose was not affected by oxygen concentration while when grown on glucose, ethanol production increased in the absence of oxygen. *P. anomala* was found to be able to ferment on both glucose and sucrose (Barnett *et al.*, 2000).

The combination of shake culture use and the presence of sucrose as the main sugar in the molasses-based media and their implication to the Pasteur and Crabtree effects may account for the lower yeast yield when compared with yields on NYDB. In any case, the higher quality of the cells produced, as expressed by the high viable counts and the endogenous reserve profiles, compensate for the lower yield. Moreover, conditions under which fungal spore production is optimal usually differ from those that result in a desirable polyol or trehalose content (Hallsworth & Magan, 1995).

4.2   ENDOGENOUS Ψ_c OF *P. anomala* CELLS

This study investigated for the first time the Ψ_c levels of the *P. anomala* cell treatments. The results obtained showed that cell Ψ_c decreased with decreasing medium Ψ_w, with Ψ_c almost always equivalent to that of the medium. This is in accordance with Griffins' (1981) finding that when thermodynamic equilibrium between cells and external medium is reached, the intracellular medium is always at a slightly lower Ψ_c than the external medium; this difference is balanced by the turgor pressure of the cell and is more important for osmotolerant yeasts.

With *P. anomala* cells grown in molasses-based medium, Ψ_c was equal to Ψ_w, indicating the ability of yeast cells to synthesise compatible solutes for maintaining cell functioning. When NaCl was used as a modifying solute in both media treatments the Ψ_c was lower than the Ψ_w of the external medium, indicating that the yeast strongly responded to ionically imposed water stress. Marechal *et al.* (1995) reported that when Ψ_w is decreased, there is initially a passive stage of water exiting which can

lead to the death of the cell. This response is characterised by a rapid decrease in cell volume in a few sonds. The passive response is soon followed by an active, biological one which occurs more slowly (minutes or hours) and is due to the active osmoregulation system of the cell (Brown & Edgley, 1980). This system enables the cell to restore its internal volume only if the passive response does not result in irreversible damage. My results are in accordance with Magan (1997) who underlined the importance of the cells ability to be in osmotic equilibrium with the surrounding environment in order to prevent swelling of the cytoplasm. Recent studies by Abadias et al. (2000) and Ramirez et al. (2004) showed the same effect of medium Ψ_w on Ψ_c of C. sake cells and F. graminearum mycelial colonies, respectively.

4.3 ENDOGENOUS ACCUMULATION OF SUGARS AND POLYOLS IN *P. anomala* CELLS AND THEIR RELEASE AFTER EXPOSURE TO HYPO-OSMOTIC SHOCK

P. anomala cells responded to water potential stress by increasing the intracellular proportion of the polyols glycerol and/or arabitol depending on the stress solute used and the a_w of the medium. Trehalose accumulation in yeast cells was found to be dependent on the medium used to produce the cells and on the solute used to modify the medium. The importance of cell post-harvest isotonic washing treatment was also shown as hypo-osmotic shock (water washing treatment) resulted in osmolyte (glycerol, erythritol, arabitol and mannitol) and sugar (trehalose and glucose) release.

More specifically, for cells produced in both NYDB and molasses-based media modified with the non-ionic solute glycerol, glycerol itself was the main solute accumulated in significantly higher amounts than with other treatments, indicating that it was probably directly taken up from the media. Previously, glycerol was found to be directly taken up from the media in other yeasts (Blomberg & Adler, 1992; Teixidó et al., 1998b; Abadias et al., 2000; Abadias et al., 2001b). Glycerol was also the main compatible solute in spores of other fungal BCAs such as *Epicoccum nigrum* (Pascual et al., 1996) and *Penicillium frequentans* (Pascual et al., 2000), as well as in many yeasts such as *Saccharomyces cerevisiae* (Brown, 1978) and *Candida sake* (Teixidó et al., 1998a; Abadias et al., 2000; Abadias et al., 2001b) when

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microorganisms were exposed to water stress. It was notable that there was a switch from mannitol/erythritol accumulation to glycerol/arabitol accumulation when a_w decreased. This finding coincides with other studies (Hallsworth & Magan, 1994a;c; Abadias *et al.,* 2000)**;** however, this was not the case with *Ulocladium atrum* spores where the opposite occurred (Sancisi-Frey, 2000).

Arabitol (in cells produced in both media types) and glycerol (in cells produced in molasses-based media) were the main solutes accumulated when yeast cells were exposed to water stress. In NaCl treatments, for cells produced in both media, arabitol was the main solute accumulated followed by glycerol at 0.98 a_w while at 0.96 a_w there was a change with a significant glycerol accumulation and a 50% decrease in arabitol. In sorbitol treatments, for cells produced in both NYDB and molasses-based media, arabitol and glycerol were mainly responsible for yeast cell osmoregulation. In proline treatments, in cells produced in both media, the dissacharide trehalose was accumulated in the largest quantities of all treatments and arabitol was the polyol mainly accumulated in the yeast cells. The present study showed that glycerol and arabitol accumulated to significantly higher levels in *P. anomala* cells produced under water-stress, indicating their important role in osmoregulation in this yeast. Glycerol and arabitol accumulation was affected by temperature and incubation time and was maximal when *P. anomala* cells were produced at 25 ^{o}C after 3 days incubation (S. Ferretti, personal communication). Culture age was also found to affect polyol accumulation in the filamentous entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces farinosus* (Hallsworth & Magan, 1996), *Ulocladium atrum* (Sancisi-Frey, 2000) and the yeast *Candida sake* (Teixidó *et al.,* 1998a). Mannitol was not found to play any role in *P. anomala* cell osmoregulation, while eryhtritol accumulated only in cells produced on sorbitol-modified 0.96 a_w media.

Overall, there was no clear accumulation pattern of glucose in *P. anomala* cells; indeed it was present in only a few treatments. In glucose treatments in both liquid media, glucose accumulation was the largest of all the other treatments used; similar results were obtained with *Candida sake* (Abadias *et al.*, 2000). Abadias *et al.* (2000, 2001b) found no correlation of *Candida sake* glucose intracellular accumulation and decrease of a_w of production media and thus no clear role of glucose in osmoregulation. However, in *Ulocladium atrum* spores glucose was found to be an

important precursor in osmoregulation by providing material for synthesis of other compounds important in osmoprotection as it was present in unstressed conditions but disappeared when water stress was imposed (Sancisi-Frey, 2000).

In general, cells produced in NYDB did not accumulate trehalose, with the exceptions of the control and proline treatments. It should be noted that proline's catabolism results in α-ketoglutarate formation, an intermediate of the tricarboxylic acid (TCA) cycle which may slow down or block the glycolytic metabolic pathway, resulting in trehalose accumulation (Prescott *et al*, 1999). However, a detailed and clear explanation is difficult to give as proline's catabolism is a reversal of its synthetic process and both are closely related and dependent on carbohydrate metabolism. Teixidó *et al.* (1998b) found that *Candida sake* cells produced in unmodified NYDB or modified with glycerol and NaCl after 48 h incubation, accumulated very little trehalose. Other studies (Teixidó *et al.*, 1998b) with *Candida sake* cells produced in NYDB modified with glucose and trehalose after 48 h incubation found low (2 mg g^{-1} f.w. yeast cell) trehalose accumulation. However, Plourde-Owobi *et al.* (2000) pointed out that *Saccharomyces cerevisiae* strains deleted for the TPS1 gene, encoding trehalose-6-phosphate synthase, were able to actively uptake trehalose. This direct uptake was mediated by the α-glucoside transporter encoded by the AGT1 gene and took place when yeast extract was used. Yeast extract is a component of NYDB and other enriched media (thus widely used in yeast research) and is prepared with lysed extracts of yeast that may contain trehalose. However, this may not be the case for all yeasts as Miyazaki *et al*. (1996) reported that a strain of *C. sake* grown in YMPG medium (Yeast Extract Peptone Glucose medium) did not accumulate any trehalose.

On the other hand, cells produced in molasses-based media accumulated trehalose in all treatments, with proline and NaCl treatments resulting in the largest trehalose accumulation followed by glucose and sorbitol treatments. Abadias *et al.* (2000) did not find any trehalose accumulation in *C. sake* cells produced in molasses-based media but found glucose accumulation instead. This may be due to the fact that the two yeasts have different patterns of sugars accumulation with *P. anomala* characterised by strong trehalose accumulation/synthesis, potentially conferring useful traits in formulation and shelf-life. Hallsworth and Magan (1994c) pointed out that

conidia with elevated trehalose concentrations remained viable for a 17-week storage period for longer that those from control treatments.

Trehalose was found to accumulate in *S. cerevisiae* during nutrient starvation, stationary phase of growth and under stress conditions such as heat, osmotic and ethanol shock (Parrou *et al.,* 1997; Plourde-Owobi *et al.,* 2000). Its protective role regarding biological membranes and proteins (Crowe *et al.,* 1984; Singer & Linquist, 1998) suggest that this molecule could play a dual role as a carbon and energy reserve and as a stress-protectant (Singer & Linquist, 1998). Beker & Rapoport (1987) pointed out that organisms that are able to survive desiccation accumulate trehalose and that the quality of dried biomass is directly dependent on trehalose content. It has also been reported that exposure to one stress generally increases the acquisition of tolerance against challenge by another stress (Parrou *et al.,* 1997). However, biosynthesis [(catalysed by trehalose-6-phosphate synthase (Tre6P) and Tre6P phosphatase] and degradation of trehalose (catalysed by two trehalases, the one cytosolic neutral and the other vacuolar acid) as metabolic pathways, interfere with glycolysis and the molecule was found to degrade rapidly after stress (Singer & Linquist, 1998); therefore the central role of trehalose in the resistance of yeast cells to several stresses is still a matter of debate in the literature (Van Dijck *et al.,* 1995; Plourde-Owobi *et al.,* 2000). The alternative pathway of trehalose accumulation is the direct uptake from the medium (Hallsworth & Magan, 1994b; Teixidó *et al.,* 1998b; Plourde-Owobi *et al.,* 2000) but this occurs only up to a 13% g g^{-1} dry weight level due to spatial constraint or by the osmotic pressure (Plourde-Owobi *et al.,* 2000). Previous studies have shown that trehalose concentrations tended to decrease with increasing water-stress, in three species of entomopathogenic fungi (Hallsworth & Magan, 1994b). In contrast, the reverse was true for water-stressed spores and mycelium of the BCA, *Trichoderma harzianum* (Harman *et al.,* 1991). Pascual *et al.* (1996) found no distinct pattern of trehalose accumulation in relation to water-stress in *E. nigrum* spores. Sancisi-Frey (2000) pointed out that trehalose was not involved in osmoregulation in *U. atrum* spores as, once more, there was no pattern in trehalose accumulation when a$_w$ of production media decreased. Abadias *et al.* (2000, 2001b) reached the same conclusion when studying *C. sake*.

Therefore, cells produced in molasses-based media have a higher concentration of total sugars; this is possibly due to the different and higher sugar content composition of molasses-based media. Moreover, cells produced in molasses-based media accumulated/synthesised more total polyols than those produced in NYDB media. However, cells produced in both media resulted in the same pattern of compatible solute accumulation/synthesis, a fact showing that differences in medium composition account for the quantitative differences in total sugar and total polyol content of the cells, regardless of the solute used to modify a_w. The fact that *P. anomala* cells, regardless of the production medium used, had similar levels of Ψ_c, equilibrated with the surrounding environment, may be an indication that other compounds besides the compatible solutes detected in this study may be responsible for osmoregulation in yeast cells produced in NYDB. The presence of solutes like ions, amino acids or other metabolites and sugars could possibly account for the contribution to the total osmotic potential of the yeast cells. However, osmoregulation in yeast cells can be attributed to a very small degree to inorganic solutes (Hobot & Jennings, 1981).

In the present study, it was also shown that post-harvest isotonic washing treatment had significant effects on endogenous polyol and trehalose intracellular retention. Regardless of type of solute used to modify a_w of the molasses-based media, arabitol and glycerol were found to be retained in higher amounts in *P. anomala* cells when post-harvest isotonic washing took place; in proline and NaCl treatments the arabitol intracellular amount was almost doubled. The same pattern was also noted for trehalose intracellular retention which was doubled in glucose and proline treatments and 4x greater in the NaCl 0.98 a_w treatment. Cell washings of respective treatments were found to contain high amounts of trehalose, arabitol and mannitol. Therefore, *P. anomala* cells responded to hypo-osmotic stress by releasing osmolytes and trehalose in the washing medium (water). Washings of glucose and glycerol treatments contained very high amounts of glucose and glycerol respectively; this was due to the high glucose and glycerol presence in the liquid media and did not represent any kind of cell response to hypo-osmotic stress conditions. Kayingo *et al.* (2001) found that the osmotolerant yeasts *Pichia sorbitophila* and *Zygosaccharomyces rouxii* released arabitol and glycerol when exposed to hypo-osmotic stress. The amount of osmolytes released was directly proportional to the shock intensity and occurred very rapidly

(less than 5 min); cell survival as well as subsequent growth were largely unaffected. The same authors suggest that osmolyte release shows the presence of a channel mediated transport system similar to that of Fps1p in *S. cerevisiae*. However, whether different osmolytes share the same transporter or whether separate channels for their release exist remains to be established. Pascual *et al.* (1999) also found that significant amounts of endogenous solutes (polyols and sugars) were washed out of conidia when water was used as a post-harvest washing medium.

4.4   EFFECT OF FERMENTATION MANIPULATION ON VIABILITY OF *P. anomala* CELLS

In general, the viable count of *P. anomala* cells obtained from the unmodified and modified molasses-based media was higher than that obtained from the unmodified and modified NYDB media for 24 and 48 h-old cells. However, 72 h-old cells obtained from unmodified medium and the modified with all the solutes (the only exception being the proline treatment) from NYDB media gave a much higher viable count than that obtained from cells produced in molasses-based media. Moreover, the viable count of 72 h-old cells produced in NYDB media was much higher than that obtained from 48 h-old cells, while 72 h-old cells produced in molasses-based media gave roughly the same or slightly lower viable counts than that obtained from the 48 h-old cells.

A possible explanation may be that the large quantities of intracellular trehalose accumulated in yeast cells grown in molasses-based media may account for the differences in growth and viability (in 72 h-old cells) between the latter and yeast cells grown in NYDB media. Hallsworth & Magan (1995) pointed out that conidia of three entomopathogenic fungi containing high glycerol and erythritol concentrations germinated more quickly. However, substantial quantities of trehalose in conidia appeared to reduce the beneficial effect of glycerol and erythritol during germination at reduced a_w. The difference in viability is more apparent in 72 h-old cells as trehalose accumulation increases as cultures age (Parrou *et al.,* 1997). High intracellular trehalose accumulation appears to have implications in formulation and shelf-life of BCAs. Therefore, its strong accumulation in *P. anomala* cells grown in

molasses-based media makes the further study of the latter quite intriguing. Studies with the yeast *C. sake* where cells were produced in unmodified NYDB (Teixidó *et al.,* 1998b) and molasses-based media (Abadias *et al.*, 2001b) showed that 24 and 48 h-old cells produced in molasses-based media gave a higher viable count; however, no study has been done with 72 h-old *C. sake* cells grown in NYDB medium. The present study has shown that viable counts of cells grown at reduced water activity (0.98 and 0.96 a_w) on NYDB media were equal to or higher (especially in glycerol and glucose treatments) than cells grown on unmodified media, with freely available water (0.996 a_w). However, growth of yeast cells was shown to decrease as a_w of the medium decreased, especially at 0.96 a_w; therefore the high viable count of the cells produced at reduced a_w (especially in the cases of yeast cells produced in glycerol and NaCl modified media) can be attributed to the presence of intracellular solutes, in particular glycerol and arabitol. Cells produced in NaCl 0.96 a_w treatment had very slow growth but gave remarkably high viable counts and accumulated a high amounts of glycerol after 72 h incubation. Unfortunately, toxicity of *P. anomala* cells to high concentration of PEG 200 did not allow assessment of environmental stress tolerance of the yeast cells.

Studies with *P. anomala* cells obtained from molasses-based media showed that viable counts of cells grown at reduced a_w (0.98 a_w) media were the same or higher than cells grown on unmodified media, with freely available water (0.996 a_w). Viable counts of cells of 24-d-old cultures grown in the low water activity (0.96 a_w) medium was much lower than cells grown on unmodified media. However, over time viable counts of cells from 0.98 and 0.96 a_w treatment media had little differences. In some treatments (glycerol, proline and NaCl) viable counts of cells obtained from 0.96 a_w treatments were higher than those obtained from cells grown at 0.98 a_w. This could be due to the fact that glycerol and arabitol accumulation increased with cultures age (Hallsworth & Magan, 1996). Viability of *P. anomala* cells plated on unstressed (0.995 a_w) and water stressed NYDA media (0.96 a_w) were similar, for the control treatment, while in other treatments differed remarkably. In 24 h-old cultures, cells obtained from sorbitol 0.98 a_w, glycerol 0.96 a_w, and NaCl 0.96 a_w treatments gave higher viable counts when plated on water stressed NYDA medium (0.96 a_w), indicating a higher water stress tolerance. Overall, viable counts of *P. anomala* cells plated on unstressed (0.995 a_w) and water-stressed NYDA media (0.96 a_w) were

similar after 48 h incubation. Abadias *et al.* (2001b) examined water stress tolerance of the biocontrol yeast *C. sake* using a water stress environment (NYDA) of 0.95 a_w. The differences in viable counts of cells on unstressed (0.995 a_w) and water stressed NYDA media (0.96 a_w) were much more pronounced and only after 72 h incubation were they comparable to the control, glucose 0.98 a_w, NaCl 0.98 a_w, proline 0.98 a_w, glycerol 0.96 a_w and NaCl a_w 0.96 treatments. In general, cells from NaCl and proline 0.98 and 0.96 a_w treatments were found to have good viability (>10^{10} CFU ml^{-1}) and were more viable than the control treatment, when plated on both unstressed (0.995 a_w) and water stressed NYDA media (0.96 a_w), followed by the glycerol and glucose treatments. Both *C. sake* and *P. anomala* cells were found to be more stress tolerant when grown for 72 h in NaCl and proline modified media. There was no clear trend indicating that cells grown at 0.96 a_w level were more stress tolerant than those grown at 0.98 a_w. Although there was a trend indicating that viability of *P. anomala* cells plated on water stressed NYDA media (0.96 a_w) increased with the age of the culture. These findings agree with those of Abadias *et al.* (2001b). Moreover, Mackenzie *et al.* (1988) have pointed out that the generation of resistant cells develops in the sond half of the exponential phase.

High viable counts of the NaCl and proline treatments, especially in a water stressed environment, were associated with arabitol and glycerol intracellular concentrations. It was notable that in cells grown at 0.98 a_w, arabitol was by the far the main polyol accumulated while in those grown at 0.96 a_w glycerol increased at the expense of arabitol, and in proline treatment, of arabitol and trehalose. This is similar to what Hallswoth & Magan (1994a) found. They pointed out the fact that lowered a_w of production media favoured intracellular accumulation of lower M_r polyols. Abadias *et al.* (2001b) found that glycerol and arabitol were the main solutes accumulated by *C. sake* cells grown in the molasses-based medium in response to lowered a_w. Other ecophysiological studies of *C. sake* demonstrated that glycerol and arabitol were the main solutes accumulated in cells when the latter were grown in glycerol, glucose and NaCl modified media (Teixidó *et al.,* 1998a, 1998b). Once more the slow growth of yeast cells is compensated by high viable counts associated with high intracellular accumulation of glycerol and arabitol.

4.5   STORAGE STABILITY OF *P. anomala* CELLS FORMULATED AS WET PASTE

This study is the first investigation of potential of a wet paste system as an efficient *P. anomala* formulation. Wet paste is a concentrated pellet of cells that is not liquid and, not dry and can be easily dispensed for utilisation. Storage stability of *P. anomala* cells formulated as wet pastes was greatly influenced by the cultural conditions of the yeast cells and by storage temperature. Selection of cell treatments was based on previous studies on viability and intracellular accumulation of sugars and polyols.

Regardless of growth conditions, basal media and addition of solutes, storage at ambient (22°C) temperature resulted in a very rapid cell viability reduction, 1-2 log factor reduction during the first 30 days of storage. At the end of the storage period (1 year) cell viability was reduced by up to 4-6 log factor. When cells were stored at 4°C, viability losses were up to 0.7 log factor during the first month; at the end of the storage period viability had been reduced by 3.5-4.5 log factor. The same effect was also observed with dry *P. anomala* formulation systems and their storage stability. Selmer-Olsen *et al.,* (1999) reported that low temperature keeps the metabolic activity at a low level thus increasing storage stability. It is to be noted that, principally, there were no differences in cell viability plated on fully available NYDA medium and that under water stress.

Yeast cells grown in NYDB, containing less amounts of trehalose and arabitol, were found to be less stable than cells grown in molasses media at both storage temperatures for a period of 90 days. For longer storage periods, cells from the different basal media showed similar stability. A possible explanation for this might be the enhanced intracellular trehalose content found in the latter. Trehalose is accumulated in the cytosol of yeasts (Keller *et al.,* 1982), and besides protecting membranes and proteins during drying (Crowe *et al.,* 1984), also slows down the vital metabolism and thereby promotes the transition to a resting state of cells. Thus, trehalose might act as a general inhibitor of metabolic activities to prevent function and preserve the structure of the cells in the stationary phase (Wiemken, 1990). It seems that trehalose intracellular reserves are lost after 90 days of storage and any beneficial effect of its presence is then lost. Abadias *et al.* (2003) worked with yeast

C. sake liquid formulations and found that when adding trehalose in preservation media, cells remained stable for 7 months when stored at 4°C. In the same study use of isotonic solutions was also employed but it was shown that the idea of preserving *C. sake* cells with isotonic solutions did not principally work because the use of different compounds in preservation medium in an isotonic manner had no differences from water treatment alone. A similar study (Ypsilos, 2004) with blastospores of *M. anisiopliae* formulated as wet pastes showed that storage stability was very low and no further consideration of this type of formulation on a commercial basis was done.

In conclusion, wet paste was found to be a particularly unstable formulation system for *P. anomala* cells, even though there are no special requirements for its preparation. Further investigations using several isotonic adjuvants could be interesting but is not recommended since the formulation system itself is very unstable and any increases in storage stability would still result in unacceptably low viability levels that would probably not allow commercial development of such a system.

4.6   FLUIDISED BED DRYING AND STORAGE STABILITY OF *P. anomala* CELLS

This study was the first investigation of effectively formulating *P. anomala* cells using a fluidised bed dryer and the impact that cultural conditions, endogenous solutes, isotonic solutions and additives have on the viability and shelf-life of the formulated cells.

A fluid product usually has a limited shelf-life and occupies a large volume, and thus cannot be easily transported to distinct markets at low cost. Furthermore, the microorganism is less stable in a liquid than a dried product. Therefore, attempts to dehydrate microorganisms are more commonly employed in industry (Wang *et al.,* 2004). Fluidised bed drying has been extensively used to manufacture active dry yeast on a large scale **(**Bayrock and Ingledew, 1997a). The equipment used in our study works by producing a high velocity air stream, at a temperature range of 30-90°C, resulting in the yeast cell paste material entering a fluidised state and thus, promoting

drying. Unfortunately, the hot air temperature does not represent the yeast temperature during the course of fluidised bed drying, so all bed drying temperatures recorded correspond to the temperature of the drying air just prior to contact with the yeast material (Bayrock and Ingledew, 1997a).

For the whole procedure to be standardised at optimum conditions, *P.anomala* cells grown in unmodified molasses-based medium, which was the standard-control treatment used throughout the fluidised bed drying experimental work, were dried at different temperatures and for different times. It should be noted that when cells were dried for 20 min, they were removed from the fluidised bed dryer after 10 min drying and slightly crushed with a pestel and mortar. Beker & Rapoport (1987) found that during drying the yeasts undergo the so-called "sticky stage", during which they have a marked tendency for agglomeration. Bayrock & Ingledew (1997a) also found that crust formation on the exterior of yeast particles was one factor limiting water mobilisation and may offer a partial explanation for the variability in end yeast viabilities found. Hence, by drying yeast in a fluidised bed dryer, the literature advises the use of disintegration forces, sufficient to prevent any substantial increase in particle size but insufficient to break up the yeast cells.

This first optimisation step showed that yeast cells were desiccation tolerant when fluidised bed drying occurred at temperatures up to 60°C; at 70°C cells died. Moisture content of the cells decreased with drying time to values < 10% (5.8-8%) at the temperature range of 40-70°C. However, 7% moisture content is a cut-off point for *P. anomala* cell survival, since cells dried at 70°C were found to have a moisture content well below 7%. Bayrock & Ingledew (1997a) found that at between 10 and 20% moisture content, water within the cell cannot serve as a continuous solvent for biochemical reactions involving conformational changes of cellular polymers. Below 10% moisture content, the remaining water is likely to be strongly bound to organic molecules and considerable energy would be required to remove it. Beker & Rapoport (1987) earlier pointed out that on drying of yeasts to 7-10% moisture content, almost all free and part of the bound water were removed. It is at this final stage of dehydration, when a part of bound water is removed from the cells that the great damage of biopolymers and cell membranes takes place. Leakage of intracellular components from yeast cells may be significantly decreased during rehydration at

increased (about 40°C) temperatures. In another study with *S. cerevisiae* ADY (active dry yeast), it was reported that the temperature and relative humidity of the drying air are two factors that determine the drying time needed to prepare yeasts at a desired moisture level. Yeast viability has been shown to be markedly decreased only when cell moisture content reached 15%. (Bayrock & Ingledew, 1997b). At least four possible causes for lowered yeast viability after drying in a fluidised bed dryer were considered: moist heat, dry heat, dehydration and oxidation. Dehydration appeared to be the most likely mechanism affecting the viability of yeasts in the fluidised bed dryer through all stages of drying at temperatures below 50°C. As temperatures increase above 50°C moist heat is more effective in killing the yeast than dehydration. Bayrock & Ingledew, (1997b) concluded that at moisture contents above 15%, the selected temperature (between 30 and 70°C) does not have an impact on active dry yeast (ADY) viability. At moisture contents below 15%, drying temperatures determine the final ADY viability. This seems to be true for *P. anomala* cells, as well. The optimum drying conditions for *P. anomala* cells that were subsequently and routinely used were drying at 50°C for 20 min; cells were 66.7% viable with 7.2% moisture content.

Resistance of yeasts to drying was found to be considerably raised by increasing the trehalose content in cells (Beker & Rapoport, 1987). When trehalose was externally added to compressed yeast there was an increased protection effect during drying (Bayrock & Ingledew, 1997b). *P. anomala* cells were found to have increased trehalose retention when osmoprotection, by means of post-harvest cell washing with isotonic solutions, was employed. Therefore, usage of different isotonic solutions prior to cell fluidised bed drying appeared to be an important aspect to be checked. Usage of NaCl isotonic solutions resulted in increased *P. anomala* cell viability after fluidised bed drying. This resulted in increased trehalose intracellular retention which offered increased desiccation tolerance during drying. Another, clear effect of isotonic solution use was an increase in final cell moisture content ranging from 1-7%. This was related to different *P. anomala* modified treatments and to the different type of isotonic solutions used. A possible explanation for this might be that the different compounds surrounded yeast cells and did not allow easy water removal, especially at moisture content below 10% which marks the beginning of bound water removal. PEG 200, is a diol derivative and was found to have a deleterious effect on *P.*

anomala cells when added before drying. Even though polyols, especially glycerol (a triol), have been successfully and extensively used as cryoprotecants (Hubálek, 2003), they seem to be inappropriate and result in high cell mortality when used at a drying method (Larena *et al.*, 2003a; Larena *et al.*, 2003b). Use of isotonic NaCl solutions as a means of retaining intracellular trehalose and thus offering increased yeast cell desiccation tolerance was found to be of great importance.

From a commercial point of view, use of several additives prior to fluidised bed drying (carriers and/or adjuvants), which might result in high viability of microbial products and may be highly desirable. To this end, different compounds at different doses were added to *P. anomala* cells prior to fluidised bed drying. It was found that increase of dose resulted in increased cell viability and the best results occurred when compounds were added at 1/1 (w/w). However, use of talc as an additive had a deleterious effect on cells and was inappropriate for use as a carrier and/or adjuvant. Moreover, addition of several compounds at lower dosages resulted in decreased cell viability when compared to control (untreated) cells. Moisture content of cells was also affected by use of several additives at different doses. In general, additives increased final cell moisture content and only at higher doses was this decreased with corn meal (CM), cotton seed flour (CSF) and wheat starch (WS) resulting in very low values (5.2-6.85%). Skimmed milk (SM) resulted in increased cell protection but increased moisture content well above 10%. When the aforementioned additives were used as carriers with SM used as an adjuvant (with WS and CSF) and glycerol (with CM) at 10% (w/w) dosage, there was an increase in viability; CSF + 10% SM resulted in the highest cell protection during the drying process, with a final moisture content of 4.7%. Addition of additives partly dried *P. anomala* cells prior to insertion into the fluidised bed dryer and had a positive effect on final cell drying tolerance. However, lower doses of additives did not have the same effect and caused stress shock cells resulting in increased mortality. SM and glycerol served as further membrane protectants. It should be noted that the CM + 10%glycerol formulation was the easiest to resuspend with CSF +1 0%SM being sond best and WS+10%SM being the most difficult to resuspend. Previously, Sandoval-Coronado *et al.* (2001) pointed out that medium composition and different formulation treatments had an impact on desiccation tolerance and storage stability of *Paecilomyces fumosoroseus* blastospores. Furthermore, the moisture content of the formulations was generally

similar to the moisture-carrying capacity of the additives used. Use of various additives or carriers prior to drying is recommended since, among others, it results in a decrease of yeast-additives material moisture content (Beker & Rapoport, 1987).

P. anomala cells grown in molasses media modified with proline and NaCl at 0.98 a_w, contained higher intracellular trehalose levels. They were subsequently fluidised bed dried with the 3 best resulting formulations; use of NaCl isotonic solution as a post-harvest cell washing was employed to check whether increased trehalose retention resulted in better formulation performance. In the NaCl 0.98 a_w treatment, use of isotonic post-harvest washing significantly increased *P. anomala* cell viability in all formulated treatments Viable CFUs mg^{-1} formulation was the same (5-6x10^6) on both unstressed and water stressed NYDA media. In the proline 0.98 a_w treatment, use of isotonic post-harvest washing did not affect cell viability or CFUs mg^{-1} formulation on NYDA medium with fully available water. Surprisingly post-harvest washing treatment with water resulted in increased CFUs mg^{-1} formulation on water –stressed NYDA medium. However, viable CFUs mg^{-1} formulation was 6-8 x 10^6, higher than the unmodified control *P. anomala* cells which gave a viable CFUs mg^{-1} formulation count of 4 x 10^6 with CSF + 10%SM formulation.

Use of isotonic post-harvest washing affected moisture content resulting in an increase. The formulation of *P. anomala* masked the effect of isotonic post-harvest washing in the proline treatment. However, this was not so for the NaCl treatment. A possible explanation for this is that in the proline treatment use of a isotonic solution resulted in increased trehalose retention by 100% while in NaCl treatment this increase was 400%. Beker & Rapoport (1987) reported that culture conditions greatly influence the microorganism's resistance to drying. The resistance of one and the same strain may vary within very wide limits, depending on the composition of the nutrient medium used for cultivation. For example, viability of *S. cerevisiae*, grown on a rich molasses medium may reach 90% while that of the same strain grown on a synthetic medium is only 20-40%. A high viability of dried yeast can be reached if grown on concentrated molasses media with a limited nitrogen content. It was also noted that yeasts sampled during the exponential growth phase are considerably more labile against drying than those of the stationary growth phase. The phenomenon is typical for cell material from complex as well as synthetic media. Thus, the

importance of appropriate ecophysiological manipulation, such as pretreament of *P. anomala* cells, prior to drying was clearly demonstrated. The final CFU mg^{-1} formulation count was satisfactory on a commercial basis since 5 x 10^6-1 x 10^7 CFUs mg^{-1} formulation is what the biotechnology industry aims for (Teixidó, personal communication).

Storage stability of formulated fluidised bed dried *P. anomala* cells was greatly influenced by the type of post-harvest washing treatment (isotonic, water), fresh cell treatments, different type of formulation and storage temperature. Regardless of all other parameters, storage stability was greatly influenced by storage temperature. At ambient temperature, cell treatments lost viability more quickly than the respective treatments when stored at 4°C. Of the different treatments used for a storage period of 150 days, *P. anomala* cells grown in proline modified molasses media formulated as WS + 10%SM were found to decrease viability from 73.5% to 69.2% when stored at 4°C. Storage at ambient temperature resulted in 50% viability after 150 days of storage. The relationship between mortality and storage temperature is well established (Lievense & Van't Riet, 1994) and many authors have reported the effect of temperature and package atmosphere on stability of dried microorganisms after storage (Champagne *et al.,* 1991; Costa *et al.,* 2000). Sandoval-Coronado *et al.* (2001) also showed that fluidised bed dried *Paecilomyces fumosoroseus* blastospore storage stability was significantly affected by storage temperature and was higher at 4°C than 28°C.

Different types of formulation markedly affected storage stability. Of all the formulations used, CM + 10%glycerol resulted in rapid loss of viability at both storage temperatures (20%). CSF + 10%SM provided the most stable formulation at both temperatures and WS + 10%SM resulted in high storage stability at 4°C. The moisture content of the CSF + 10% treatment was found to be very low (4.3%). Moisture content is an important parameter for the stability of dried microorganisms (Wang *et al.,* 2004). In general, microorganisms survive better at low water activity. However, overdrying may diminish the viability and stability of microorganisms (Font de Valdez *et al.,* 1985). The choice of an optimal water content for storage of cells is dependent on whether the goal is high survival rates immediately after drying or low inactivation during storage. In all cases, the moisture content level, below

which most metabolic processes in desiccated organisms dramatically slow down or are hardly measurable, is 10% (Aguilera & Karel, 1997). Beker & Rapoport (1987) found that dried baker's yeast with 8-10% moisture content lost about 50% of their initial viability during 5 months storage. Dried yeasts of 7.5-8.5% moisture, when stored in semi-hermetic packs at room temperature, lost about 7% of viability during the first months of storage. It is suggested that the lower the moisture content of preparations, the higher the viability of microbial cells during storage. For example drying spores of *Metarhizium anisopliae* and *Beauveria brongniartii*, formulated with lactose as carrier and 5% of a lubricant as an adjuvant, at 60 and 40°C resulted in 4% and 16% viability, respectively, and a moisture content level of 5% (Horaczek & Viernstein, 2004). The resulting products quickly lost viability after 2 weeks of storage at 10 °C. Interestingly, *Epicoccum nigrum* conidia fluidised bed dried without any additives maintained 100% viability for 90 days at room temperature and then decreased to 75 and 37% after 120 and 150 days of storage respectively (Larena *et. al.*, 2003a). Conidia moisture content was not measured but drying at relatively low temperature (30-40°C for 20 min) without addition of protectants, indicated that it would be rather high. However, the remarkably high storage stability of dried *E. nigrum* conidia argues against this. The observed loss of viability of dried *E. nigrum* conidia after storage at room temperature could be due to temperature and/or presence of air in contact with the stored conidia. Larena *et al.* (2003b) showed that fluidised bed drying of *Penicillium oxalicum* conidia without any additives at 30-40°C for 20 min retained 100% viability after drying for 30 days at room temperature. Viability decreased to reach 40% after 60 days and remained unchanged up to the end of the storage period (180 days). Moisture content of dried conidia was not measured. Becker & Rapoport (1987) reported that, in order to increase stability of dried yeasts during storage, it is advisable to add anti-oxidants, thiourea, sorbitane ether and some other additives to cells prior to drying.

Another interesting point to address is the increased storage stability of *P. anomala* cells grown in NaCl, when washed post-harvest with an isotonic solution. This stability was noted for all formulation and storage temperature treatments. The increased intracellular trehalose retention, observed when post-harvest isotonic washing was implicated, possibly accounts for the increased *P. anomala* storage

stability. Previous studies by Hallsworth and Magan (1994c) pointed out that conidia with elevated trehalose concentrations remained viable for longer.

In conclusion, the importance of *P. anomala* ecophysiological manipulation, different formulation and post-harvest washing treatments on *P. anomala* desiccation tolerance during fluidised bed drying has been clearly demonstrated. The importance of the aforementioned parameters and the effect of storage temperature were also shown. Subsequent studies should consider this effect before subjected fungal inocula to different drying regimes.

4.7   FREEZE DRYING AND STORAGE STABILITY OF *P. anomala* CELLS

Freeze drying has been studied as a dehydration process for *P.anomala* in order to achieve an alternative solid formulation. This study showed the impact of protective additives, rehydration media, *P. anomala* cell modification and cell post-harvest washing with isotonic solution on yeast viability and storage stability after freeze drying. It was also demonstrated that *P. anomala* is highly resistant to freezing, thawing and dehydration during the processes of freeze and freeze drying, which is useful from a commercial point of view. Storage stability at 4°C of freeze dried *P. anomala* cells was also particularly high over a period of 150 days while storage at 22°C resulted in more rapid decrease in cell viability over a period of 30 days. Even though freeze drying procedure was found to be a suitable method for several bacteria (Rudge, 1991; Costa *et al.,* 2000; Wang *et al.,* 2004, Zayed & Roos, 2004*)* and fungi (Larena *et al.,* 2003a; Larena *et al.,* 2003b), it was less successful for yeasts such as *Candida sake* (Abadias *et al*., 2001a) and *Saccharomyces cerevisiae* (Miyamoto-Shinohara *et al*., 2000). Viability of *S. cerevisiae* freeze dried in a 10% skimmed milk and 1% sodium glutamate medium, was about 10% immediately after freeze drying and storage stability under vacuum at 5°C remained significantly unchanged during a 10-year storage period (Miyamoto-Shinohara *et al.*, 2000). Zayed & Roos (2004) underlined the importance of establishing production and maintenance techniques which maximize the storage stability, viability and activity of the microorganisms used.

A multitude of factors affect the effectiveness of cryopreservation of microorganisms. For example, species, strain, cell size and form, growth phase and rate, incubation temperature, growth medium composition, pH, osmolarity and aeration, cell moisture content, lipid content and composition of the cells, initial cell concentration, composition of the freezing medium, cooling rate, warming rate and recovery medium all affect survival (Kirshop & Doyle, 1991; Rudge, 1991; Costa *et al.*, 2000; Hubálek, 2003; Palmfeldt *et al.*, 2003). Miyamoto-Shinohara *et al.* (2000) found that survival rates of different yeasts and bacteria differed between microorganisms and that storage stability at 10 years time was species-specific. Survival of freeze drying is commonly found to be higher for stationary phase cells (Souzo, 1992). One of the most important conditions is the composition of the medium used to suspend the organisms for freezing. Although a good survival of deep-frozen microbes (bacterial and microbial spores) has occasionally been observed without a protective additive, the presence of a suitable additive usually increases the survival considerably (Hubálek, 2003).

The viability of a strain can be maintained for more than 20 years if the cell concentrations are 10^6-10^{10} cell ml^{-1} before freeze drying and the cells survive the drying process (Rudge, 1991). However, Plamfeldt *et al.* (2003) found that exaggeration of the cell concentrating procedure may lead to decreased yield of viable cells after freeze-drying. Considering these, 1 x 10^9 *P. anomala* cells ml^{-1} as initial cell concentration was used in our study

Initial optimisation step of whole freeze drying procedure by use of different protective and rehydration media demonstrated that *P. anomala* cells are tolerant of freezing and thawing cycles even without use of protectants. However, survival after freezing was further increased when a mixture of skimmed milk (SM)+sucrose at 10% (w/v) each were used. The survival rate of *P. anomala* cells dramatically decreased (<20%) in the absence of both protective and rehydration media. It seems that the drying step had a lethal effect on *P. anomala* cell viability. The major causes of loss of cell viability in freeze drying are probably ice crystal formation, high osmolarity due to high concentrations of internal solutes with membrane damage, macromolecule denaturation and the removal of water, which affects properties of many hydrophilic macromolecules in cells (Zayed & Roos**,** 2004). Horaczek & Viernstein (2004) also

found that high tolerance to freezing and thawing of *Beauveria brongniartii* and *Metarhizium anisopliae* conidia was much reduced when they were subsequently subjected to freeze drying (viability reduction of 30% and 91% respectively), indicating that the process of drying is responsible for a high proportion of the conidial/cell death. Larena *et al.,* (2003b) found that freeze dried *Penicillium oxalicum* conidia in SM + sucrose suspension medium retained almost 100% viability. Viability of freeze dried conidia without protectants resulted in 37% reduced viability; the same conidia frozen with no protectants, lost 33% viability after thawing which was unchanged when protectants were used.

In the absence of a protective medium and when rehydration in 10% SM medium took place the survival rate of *P. anomala* cells increased to 50%. However, cell survival rate was further increased to 95-97% with the right combination of protective and rehydration media. A protective solution of SM+sucrose resulted in a high final cell protection which was not further increased when cells were rehydrated in 10% SM medium. This finding indicates that the presence SM is important for *P. anomala* cell desiccation tolerance and that its use at the beginning of the process efficiently protected the cells until the final rehydration step. It also provided the final product with a porous structure that made rehydration easier. Consequently, SM was used as a support material in a mixture with sucrose. The beneficial effect of SM when present both before and after freeze drying, (in protective and rehydration media respectively) was counteracted by difficulties in handling the material during autoclaving since it resulted in the formation of lumps. The differences exhibited in yeast survival indicated that certain suspension media were more effective than others in protecting *P. anomala* cells subjected to freeze drying.

Use of water+agar+tween as a rehydration medium was shown to be effective for freeze dried *P. anomala* cells only when the latter were suspended in SM+sucrose medium. Rehydration is an important step in the recovery of microorganisms from dried products. An organism which survives the various steps, such as freezing, drying and storage, may lose its viability during rehydration. Poor recovery of cells may be attributed to an inadequate rehydration procedure (Wang *et al.,* 2004).

Addition of several compounds may have a protective effect by preventing or reducing the adverse effect of the freeze drying process (Zayed & Roos, 2004). A good protectant should provide cryoprotection to the cells during the freezing process, be easily dried, and provide a good matrix to allow stability and ease of rehydration. Various groups of compounds have been tested for their protective action, including polyols, polysaccharides, disaccharides, amino acids and protein hydrolasates, proteins, minerals, salts of organic acids and vitamin-complex media (Berny & Hennebbert, 1991; Champagne *et al.,* 1991). However, protection offered by a given additive during the processes will vary with the species of microorganism (Font de Valdez *et al.,* 1983).

Additives like polymers with a high molecular weight (MW), e.g. proteins, polysaccharides, cannot penetrate cell wall and thus cytoplasmic membrane (Hubálek, 2003). They absorb on the microbial surface where they form a viscous layer, cause partial efflux of water from the cell, inhibit the growth of ice crystals by increasing solution viscosity and keep the structure of ice amorphous in the close proximity of the cell. Additives can interact with each other in mixtures thereby producing effects other than those that would occur with individual additives. One compound in the mixture may dominate the other or they may combine to produce additive or synergic effects: it has been observed that the protective effect of combinations of additives can be greater than one would expect if the action of each agent were simply additive (Hubálek, 2003).

The protective effect of sugars, especially disaccharides, on freeze drying survival, has been attributed to their capacity to hydrate biological structures, such as proteins and membranes, referred to as the water replacement hypothesis (Crowe *et al*., 1984). They also reduce the amount of bound water on the surface of proteins (Hanafusa, 1985). To protect a cell membrane, sugars must be present on both sides of the membrane (Crowe *et al*., 1985). *P. anomala* cells were found, as previously shown, to date intrcellularly accumulate high levels of the disaccharide trehalose. Aguilera & Karel (1997) also pointed out that polyols and sugars seem to require the presence of at least five properly arranged hydroxyl groups to provide protection.

Sucrose can also be successful when used as a freeze drying protectant (Aguilera & Karel, 1997). It has also bee shown to stabilize enzymes during freeze-thawing, freeze drying and air drying and was found to stabilize lipid bilayers during freezing (Berny & Hennebbert, 1991). Palmmfeldt *et al*. (2003) used sucrose as a protective solute for *Pseudomonas chlororaphis* freeze drying and found that sucrose is taken up in the disaccharide form possibly acting as a compatible solute). SM + sucrose gave a better protection when they were used together (Larena *et al.,* 2003b, Zayed & Roos, 2004).

Skimmed milk solids are expected to prevent cellular injury by stabilizing cell membrane constituents. In addition, milk proteins may form a protective coating on the cell wall proteins and calcium in milk increases survival after freezing or freeze drying (Aguilera & Karel, 1997). Abadias *et al.* (2001a) found that 10% SM resulted in high *Candida sake* viability after freeze drying. Protective solutions also contain buffering solutes that stabilize pH; SM contains many solutes such as phosphates and citrate that would provide this buffering capacity. SM also resulted in higher total solids content of suspension media (Zayed & Roos, 2004) and can act as a UV-protectant (Horaczek & Viernstein, 2004).

Use of different isotonic solutions greatly affected *P. anomala* cell freeze drying tolerance. When no protective medium was used, the effect on the cell survival rate was particularly pronounced in glucose and NaCl post-harvest isotonic washing treatments. This comes is no surprise in the glucose treatment since sugars are known as drying protectants. The NaCl isotonic treatment, however, most likely offered enhanced desiccation tolerance since a greater retention of intracellular compatible solutes and trehalose was involved. However, when SM + sucrose was used as a protective medium, it completely masked any positive effect of post-harvest isotonic washing treatment had on yeast cell viability. Post-harvest washing with isotonic solutions had no or an adverse effect on cell freezing and thawing tolerance, clearly indicating that their positive effect in the drying stage was due to the increased retention of the well known desiccation protectant trehalose. NaCl was chosen as the best post-harvest isotonic solution since its presence appeared to offer good freezing and thawing protection without further intervening in freeze drying tolerance. Any possible positive effect would be mostly due to increased trehalose and arabitol retention.

Isotonic post-harvest washing treatment resulted in modified *P. anomala* cells with increased freeze drying tolerance, particularly in the absence of a protective medium. Modified *P. anomala* cells, with either the presence or absence of isotonic washing, were more freeze drying tolerant than the unmodified control treatment. Increased trehalose intracellular accumulation might account for this phenomenon. When a protective medium was used the effect of isotonic washing was masked by the latter. However, in proline and NaCl grown yeast cell treatments, isotonic washing resulted in 99% survival after freeze drying.

Storage stability of freeze dried *P. anomala* cells was greatly influenced by the type of different fresh cell treatments and storage temperature. Regardless of different fresh cell treatments, storage stability was greatly influenced by storage temperature. At ambient temperature, cell treatments dramatically lost viability after 30 days of storage; control cells decreased their viability from 96 to 24%. All of the other treatments followed the same pattern. With the water post-harvest washing treatment, cell viability decreased from 94-99 to 27-35%, with proline 0.98 a_w exhibiting the highest viability and sorbitol 0.98 a_w treatment the lowest. The pattern was similar for isotonic NaCl post-harvest washing treatment. It seems that SM+sucrose suspension medium was the key factor which significantly affected storage stability masking any effect isotonic washing treatment may have had on freeze-dried *P. anomala* cell storage stability. Increased intracellular trehalose seems to be implicated in enhanced *P. anomala* cell storage stability. Several studies showed that when trehalose was involved, there was increased cell stability of bacteria (Zayed & Roos, 2004) and spores of filamentous fungi (Hallsworth & Magan, 1994b). The disaccharide trehalose acts as a critical membrane-protecting agent for yeast cells during environmental stress conditions such as heat treatment, dehydration and freezing (Zayed & Roos, 2004). Synthesis and accumulation of trehalose by yeast cells correlates with increased survival following dehydration or freezing (Hino *et al.,* 1990).

Moisture content is an important parameter for storage stability of dried microorganisms since they are more sensitive to environmental stress at high-water activities (Wang *et al.,* 2004). Furthermore, the optimum residual moisture content varies with the composition of the fluid in which the microorganisms are dried, with the storage atmosphere and the species of microorganism (Wang *et al.,* 2004). In the

same study, it was also reported that lactic acid bacteria and bifidobacteria freeze dried in fermented soymilk had a final moisture content ranging from 2.9% to 3.5%. Zayed & Roos (2004) showed that increasing the moisture content level from 2.8 to 5.6% did not affect the final viable cell number during 7 weeks of storage. However, when moisture content was further increased to 8.8% there was a dramatic decrease in storage stability. In our study, the final moisture content of the freeze dried *P.anomala* cells was not measured as the fluid volumes employed before freeze drying and the dry matter resulting afterwards were far too small to allow any measurements to be made.

Lactic acid bacteria and bifidobacteria freeze-dried in fermented soymilk and stored at $4^{o}C$ in a laminated pouch had a storage stability of 51 and 69% respectively after 4 months of storage (Wang *et al.,* 2004)**.** Moreover, regardless of packaging materials and storage temperatures the viable cells of lactic acid bacteria and bifidobacteria decreased as the storage time increased. However, a higher viable population was noted in the freeze-dried material held at 4 than $25^{o}C$ (viability difference 10-25%). Moreover, suspension of *L. salivarius* in water without any protective agent before freeze drying gave a very low survival rate (4%) which significantly increased when dried in skimmed milk and sugars (trehalose and sucrose) solution (Zayed & Roos, 2004). Freeze dried conidia of *Penicillium oxalicum*, with or without additives, did not maintain viability over time at room temperature: a significant reduction was observed 30 days after freeze drying (Larena *et al,* 2003b). However, freeze dried *P. oxalicum* conidia with SM + sucrose had about 20% viability after 150 days storage. The relationship between mortality and storage temperature is well established (Lievense & Van't Riet, 1994) and many authors have reported the effect of temperature and package atmosphere on stability of dried microorganisms after storage (Champagne *et al.,* 1991; Costa *et al.,* 2000). Freeze drying was found to be the most stable formulation for *Beauveria brongniartii* as storage tests resulted in survival rates of 78% when stored at $10^{o}C$ for 2 months. Complete loss of activity occurred after 8 weeks at $20^{o}C$ (Horaczek & Viernstein, 2004).

4.8 ENDOGENOUS PROTEIN AND ENZYME PRODUCTION BY *P. anomala* CELLS

The aim of this study was to evaluate whether the different profiles in endogenous protein of different modified *P. anomala* cell treatments, and the retention of higher amounts of trehalose and arabitol by osmoprotection of modified cell treatments, had any effect on the production of the extracellular enzymes β-(1-3)-glucanase, chitinase and β-glucosidase. Quick screening of enzyme production by use of a semi-quantitative API ZYM kit and induction of three aforementioned hydrolases by use of CWP+glucose as a carbon source in minimal salt medium, were first set up before carrying out more detailed enzyme production investigation.

Use of the API ZYM kit showed that different carbon sources had an effect on enzyme production. Use of *P. verrucosum* CWP+glucose, compared to *P. verrucosum* and *P. roqueforti* CWP alone, resulted in higher butyrate esterase and caprylate esterase lipase and lower napthol-AS-BI-phospshohydralase and acid phosphatase activity. In all treatments, β-glucosidase activity was low and reached a peak after 5 days incubation. When the effect of different modified *P anomala* cell treatments and osmoprotection, by use of isotonic post-harvest washing, were checked only small differences were found for activity of caprylate esterase lipase, napthol-AS-BI-phospshohydralase and acid phosphatase activity and a slightly higher enzyme activity when osmoprotection was implicated. Whether these enzymes play any role in biocontrol activity exerted by the yeast is difficult to confirm. However, we do have an indication of *P. anomala* enzymatic activity profile.

Modification of *P. anomala* cells by addition of several solutes to molasses media resulted in reduced endogenous protein accumulation; lower a_w of media resulted in lower protein accumulation. It seemed that yeast cells reduced their protein content in order to osmoregulate by accumulating polyols, in particular arabitol and glycerol, and trehalose. Osmoprotection did not increase endogenous protein; exceptions to this were NaCl 0.98 and 0.96 a_w and glucose 0.96 a_w treatments.

The API ZYM system was primarily used to determine the activity of hydrolytic enzymes produced by bacteria but more recent studies have confirmed its usefulness

also in mycology (Kurnatowska, 1998). Kurnatowska (1998) showed that the highest activity of acid phosphatase and napthol-AS-BI-phosphohydrolase was detected from *Candida albicans* strains isolated from patients with various diseases of the oral cavity. Youngchim *et al.* (1999) studied enzyme production of the AIDS related dimorphic fungus *Penicillium marneffei* and found that its yeast phase produced extracellular acid phosphatase, an enzyme implicated in other pathogens' virulence, such as *Cryptococcus neoformans* (Chen *et al.,* 1997). Butyrase esterase and caprylate esterase lipase activity, were also found to be sreted by *Penicillium merneffei*, possibly playing a role in pathogenesis; the activity of both enzymes were implicated in *Candida albicans* virulence (Tsuboi *et al.,* 1996). Hydrolytic activity of yeasts is considered to be an important feature of cow mastitis fungal infections (Malinowski *et al.,* 2001). However, the same study suggested that enzymatic activity characterizes the species rather than yeast pathogenicity; this is the opposite of Kurnatowska's (1998) findings.

It is important to stress that whilst the measurement of enzyme activities using synthetic substrates as described is relatively straightforward, such data does not identify the specific nature of the enzyme involved. Therefore, attempts should be made to use alternative more quantitative measurement of enzyme activity.

When minimal salt media were supplemented with synthetic carbon and chitin sources only low β-(1-3)-glucanase and chitinase activity were induced. For both enzymes, activity was increased over time (72 h) to reach 1.2 units (laminarin and chitin+glucose supplementation for respective enzymes). CWP production for use as a carbon source had to be employed. Synthetic carbon sources were, in some cases, found to be quite useful in induction of enzyme activity. *P. guilliermondii* was found to produce high extracellular β-(1-3)-glucanase levels in minimal medium supplemented with the sugars fructose and sucrose but not with chitin (Winsiewski *et al.,* 1991). However, CWP alone or combined with glucose were found to result in significant enzyme activity induction in several studies (Castoria *et al.,* 1997; Jijakli & Lepoivre, 1998; Castoria *et al.,* 2001; Fan *et al.,* 2002).

Use of *Penicillium verrucosum* CWP + glucose as a carbon source resulted in significantly higher β-(1-3)-glucanase (2.22 units) activity and increased over time.

Different modified *P. anomala* cells resulted in significantly different enzyme activity and osmoprotection did not play any role in this effect. There was no clear correlation with endogenous protein. However, enhanced trehalose retention seemed to have an adverse effect on β-(1-3)-glucanase activity. It seems that this carbohydrate reserves repressed the cells' need to produce β-(1-3)-glucanase which in turn hydrolyses cell wall glucans and provides cells with readily available carbohydrates. Extracellular β-(1-3)-glucanase (EC 3.2.1.39) was also found to be excreted by yeast cells and have been suggested to play a key role in the lytic process of fungal cell walls (Castoria *et al.,* 1997; Jijakli & Lepoivre, 1998; Castoria *et al.,* 2001; Fan *et al.,* 2002). Regardless of the carbon source used, amount of extracellular β-(1-3)-glucanase produced by *Pichia guilliermondii* was five times more than the one produced by *Debaromyces hansenii.* The former yeast was also found to be the most effective antagonist. Winsiewski *et al.* (1991) suggested that high levels of this cell wall hydrolase, along with *Pichia guilliermondii* tenaciously attached to *Botrytis cinerea* hyphae, may play a role in yeast biocontrol activity. High β-(1-3)-glucanase activity was detected in minimal salt medium supplemented with glucose in combination with CWP of *Rhizopus stolonifer* as a carbon source. The maximum level of activity reached was 114 SU (specific activity unit) for *Pichia membraniefaciens* and 103.2 SU for *Candida guilliermondii.* (Fan *et al*., 2002). Castoria *et al.,* (2001) induced the highest β-(1-3)-glucanase activity produced by *Aureobasidium pullulans* by supplementing media with CWP of *B. cinerea* alone. Castoria *et al.* (1997) found that β-(1-3)-glucanase activity produced by yeasts depends on the fungal source of CWP used. Jijakli & Lepoivre (1998) found that a minimal salts medium supplemented with glucose and laminarin induced very little or little, β-(1-3)-glucanase respectively, while CWP resulted in the highest enzyme activity induction which increased over time (5 days). When purified β-(1-3)-glucanase was applied to germinated spores and germ tubes of *Botrytis cinerea in vitro*, this caused hyphal swelling and leakage of cytoplasm, indicating cell wall-degrading ability.

Very little chitinase activity was induced and only reached 0.89 units after 120h incubation. However, osmoprotection in different modified yeast treatments resulted in increased chitinase activity. Increased glycerol and trehalose retention in glucose and proline 0.98 a_w treatments, respectively, may partly account for enzyme activity induction since no differences in endogenous protein were noticed between

treatments. However, no clear conclusion can be drawn since the chitinase activity was rather low. The results suggest that the role of chitinase in yeast biocontrol activity is very limited. Those findings agree with those from Fan *et al.* (2002) who demonstrated that *Pichia membraniefaciens* was able to produce exochitinase but at only low quantities (3.13 SU maximum).

Chitin is an unbranched homopolymer of N-acetylglucosamine (GlcNAc) residues linked by β-1,4 bonds. Chitin microfibrils are major structural components of arthropods cuticle cells, cell walls of most filamentous fungi and many other types of microorganisms; however, it is absent from plant cells (Carstens *et al.,* 2003). β-(1-3)-glucan is another main structural component of the fungal cell wall. Chitinases (EC 3.2.1.14), which hydrolyse the β-1,4 linkages in the insoluble chitin microfibril, are found in a variety of microorganisms, including plants, most fungi, yeasts and some prokaryotes (Cohen, 1993). Yeasts are unable to separate normally if they lack chitinase activity in the logarithmic growth phase (Kuranda & Robbins, 1991). Although chitin does not exist in plant cells, chitinases have been purified from a large number of plants from diverse taxonomic groups. In plants, invasion by a pathogen induces the production of pathogenesis-related (PR) proteins, such as proteinases, β-(1-3)-glucanases and chitinases (Kombrink & Somssich, 1995). It has been shown that chitinases degrade fungal cell walls, inhibit fungal growth by lysing the hyphal tips as well as being associated with hyphal walls *in planta* (Carstens *et al.,* 2003). Chitinases not only play an important role in the defence mechanism of plants, but also in the mycoparasitic processes of fungi. The antifungal mechanism of *Trichoderma*, an extensively studied and widely used biocontrol fungus, relies on chitinase cell wall-degrading enzymes (Lorito *et al.,* 1998).

Mycoparasitism of antagonists is associated with the production of cell wall-degrading enzymes and inducement of host defence. Urquhart & Punja (2002) also found that isolates of five species of *Tilletiopsis*, powdery mildew antagonist yeasts, produced low levels of β-(1-3)-glucanase and chitinase that may play a role in antagonism. The antifungal activity of those two enzymes strongly suggested the involvement of extracellular enzymes in the biocontrol mechanism of *Pichia membraniefaciens* and *Candida guilliermondii* (Fan *et al.,* 2002).

The hydrolytic enzyme β-glucosidase was found to be produced by *P. anomala* cells and its specific activity was rather low. Osmoprotection had no effect on total and specific enzyme activity but different modified yeast cells were found to srete different amounts of β-glucosidase. The proline 0.98 a_w cell treatment had the lowest enzyme specific activity representing only a small part of the total enzyme activity. In general, β-glucosidase production by yeast cells was low suggesting no important role in biocontrol activity. However Lorito *et al.* (1994) proved that the synergistic antifungal effect of chitinase in combination with glucosidase of *Trichoderma harzianum* was effective in inhibiting B*otrytis cinerea.* Endoglucanases, exoglucanases and β-glucosidases are the three major types of enzyme required for the hydrolysis of crystalline cellulose to glucose (Marin *et al.,* 1998a). Therefore, they could play a role in fungal cell wall degradation. Its production, along with other enzymes is important. For example, for colonization of ripening or harvested maize seeds by *Fusarium* species. (Marin *et al.,* 1998a). Keshri & Magan (2000) found that non-mycotoxigenic strains of *Fusarium* spp. produced higher β-glucosidase amounts than toxigenic ones, an indication that strain type may also influence hydrolytic enzyme production.

In conclusion, *P. anomala* J121 was found to produce considerable β-(1-3)-glucanase and low chitinase and β-glucosidase amounts. The available literature, suggests the involvement of the first hydrolase in biocontrol activity is important. The role of the other two is not clear for *P. anomala*. Osmoprotection does not have any direct effect on endogenous protein and enzyme production; however, an indirect effect could be that enhanced intracellular trehalose retention seemed to repress β-(1-3)-glucanase production.

4.9   BIOASSAYS

Any improvements in yeast inocula by ecophysiological manipulation should be assessed in a controlled laboratory bioassay before being tested in small-scale and large-scale storage conditions. However, differences in BCAs biocontrol efficacy *in vitro* and in natural conditions are well documented (Chelack *et al.,* 1991; Petersson *et al.,* 1998). Fresh *P. anomala* cells were checked against fluidised bed dried cells

grown in unmodified molasses and media modified with proline; cell osmoprotection using isotonic solutions was also compared.

P. anomala cells reduced populations of *Penicillium verrucosum* strain 22625 with no significant differences between treatments and a_ws after 30 days of co-culture in wheat. Some differences were noticed after 10 days possibly due to better yeast colonisation and growth on wheat during the early stages in the bioassay. *Penicillium verrucosum* 22625 inhibition was less pronounced than would be expected according to previous studies using the same pathogen-antagonist system (Petersson *et al.,* 1998). However, different conditions, sterile grain (used in this study) against nonsterile grain, non-limiting O_2 conditions against limiting O_2 conditions, different a_w and a different *Penicillium verrusocum* strain possibly accounted for this. The populations of *P. verrucosum* were lower at reduced a_w levels. It also seems that, on this occasion, 10^5 CFUs g^{-1} *P. anomala* was not a high enough initial inoculum concentration to inhibit 10^3 CFUs g^{-1} *P. verrucosum* 22625. The temperature used (21^oC) might have also affected biocontrol efficacy as it favours *P. verrucosum* 22625 growth and OTA production (Cairns-Fuller, 2004).

Petersson & Schnürer (1995) found that *P. anomala* had strong antagonistic activity in wheat in a dose dependent manner, with 10^5 and 10^6 completely inhibiting the growth of *Penicillium roqueforti*. However, inhibition was least pronounced at the optimum temperature (21^oC) and water activity (0.95) for *P. roqueforti* growth. Björnberg & Schnürer (1993) also demonstrated that *P. anomala* cells inhibited *P. roqueforti* and *Aspergillus candidus* in a pronounced way at suboptimal temperatures for their growth and sporulation i.e. below 15^oC and above 30^oC. Biological control is probably most effective in cases in which the degree of adaptation to a specific environment is higher for the biocontrol micro-organism than for the potential pathogen (Droby *et al.,* 1991).

Populations of *P. anomala* when co-cultured with *Penicillium verrucosum* were significantly lower than when the yeast was alone. Yeast populations at 0.93 a_w were less pronounced in all treatments (even when yeast was cultured alone). At all storage times, population increase of fresh cells was always significantly higher than dried treatments. Interestingly, lower yeast population did not result in less *P. verrusocum*

inhibition. It could be that abiotic factors, notably water availability, interacted with mould growth, thus enhancing inhibition. *P. anomala* was previously shown to be effective in low O_2 conditions (Petersson *et al.,* 1998). In the present study, air O_2 levels were used in order to have an indication of yeast biocontrol efficacy under these conditions. However, in air, *P. verrrucosum* was much more competitive.

OTA production was significantly affected by a_w alone; this was made clear when *Penicillium verrucosum* 22625, a high OTA producer (Cairns-Fuller, 2004) grew on wheat alone. Interestingly, at 0.95 a_w, fresh cells and non-osmoprotected dried cells grown in proline modified molasses, stimulated OTA production. Although the two other dried cell treatments, did reduce populations of *P. verrucosum* 22625 but not OTA production. At a lower a_w, 0.93, a decrease in mould populations coincided with a decrease in OTA production. Only non-osmoprotected (washed post-harvest with water) dried yeast cells did not significantly reduce OTA production. Osmoprotected dried cells at both a_ws reduced OTA production. The isotonic solution itself could have had an influence on OTA production but it had no differential influence on fungal populations. It seems that *P. anomala* J121, a xerotolerant yeast, was more efficient at reducing OTA production at low water availabilities. In wetter treatments, there was either no effect or yeast stimulated OTA production.

Generally, competition could be expected to have a profound effect on sondary metabolism since microorganisms in co-culture compete for all essential environmental factors, including space and nutrients (Magan *et al.,* 2004). The nutrient supply strongly affects sondary metabolism, including mycotoxin producton (Luchese & Harrigan, 1993). Co-culturing microorganisms can result in either stimulation or inhibition of mycotoxin production. Presence of the yeast *Hyphopichia burtonii* significantly increased aflatoxin production by *Aspergillus flavus* on maize compared with pure cultures (Cuero *et al.,* 1987). Marín *et al.* (1998b) also found that fumonisin production by *Fusarium* species was maintained despite growth inhibition by competing fungi.

Biocontrol microorganisms should not enhance mycotoxin production. Sub-inhibitory levels of antagonistic microorganisms might, however, stimulate sondary metabolism, and thus mycotoxin production. It is well known that sub-inhibitory concentrations of

antimycotic chemicals, such as potassium sorbate, propionic acid, Benomyl and Bavistin can stimulate mycotoxin production (Petersson *et al.,* 1998). Petersson *et al.* (1998) also found that, even though *P. anomala* reduced the growth of *Penicillium verrucosum* IBT5010 and IBT12803 and respective OTA production *in vitro, in vivo* (in wheat) it only reduced growth and OTA production of *P. verrucosum* IBT12803 but not IBT5010.

Both ochratoxin A and aflatoxin were found to be produced in higher amounts when toxigenic fungi were inoculated in sterile wheat (Vandegraft *et al.,* 1973a; b; Chelack *et al.,* 1991). Mycotoxin production can thus decrease in response to competition between the natural microbiota and inoculated mycotoxin producers. Petersson & Schnürer (1995) showed that *P. anomala* inhibited *P. roqueforti* in wheat but also stimulated growth of lactic acid bacteria, possibly enhancing biological control. However, Petersson *et al.* (1999) reported later that enhanced growth of lactic acid bacteria might be a result of a complex interaction between the latter and *P. roqueforti.*

In this study, it was also shown that formulation additives had no adverse effect on mould growth and OTA production. Furthermore, modified yeast cells with increased levels of trehalose and arabitol were not better at inhibiting mould growth and/or OTA production. Even though bioassays were at reduced water availabilities, there was no significant advantage of modified yeast inocula. Recently, Ypsilos (2004) reported no improved biocontrol efficacy between modified and unmodified *Metarhizium anisopliae* blastospores at 100% humidity. However, Hallsworth & Magan's (1994c) reported that conidia of *Beauveria bassiana*, *Paecilomyces farinosus* and *Metarhizium anisopliae,* modified to contain higher amounts of glycerol and erythritol, were more efficient in killing wax moth larvae (*Galleria mellonella*) than unmodified ones. Pascual *et al.* (1996) also reported that modified *Epicoccum nigrum* conidia were better in controlling peach twig blight (*Monilinia laxa*) in field trials. However, modified *P. anomala* inocula were found to be produced in significantly higher concentrations and were significantly more tolerant to drying; therefore further investigation in large-scale biocontrol assays should be carried out. Petersson *et al.* (1999), demonstrated that large-scale application of *P. anomala* to airthight stored high moisture condition feed wheat could protect against post-harvest spoilage. The

combined effect of yeast with low O_2 and very high CO_2 might have inhibited *Penicillium roqueforti*. Pilot scale bioassays with best *P. anomala* modified inocula are in progress by Swedish E.U. project partners.

5   Chapter 5 CONCLUSION AND FUTURE WORK

5.1   CONCLUSION

Use of fungal biological control agents (BCAs) as an alternative method to chemical treatments appears, nowdays, more favourable than ever since public concern about environmental matters and pressure for healthier food products are combined with the ban or withdrawal of several pesticides (Butt *et al.,* 2001). However, BCAs are living organisms and their production, formulation, shelf-life, distribution and application have special requirements. Moreover, interaction with biotic factors of environment, in particular non-target organisms including vertebrates and humans should be carefully considered and studied in full prior to moving to the production step.

The present study considered *P. anomala* J121 a candidate BCA to be used as a commercial product. Careful consideration to improved quality and ecological fitness of inoculum was given and the following parameters were examined:

- ➢ Use of different production media (synthetic and complex) in order to choose a cheap and readily available one that ensures high numbers of good quality yeast inocula
- ➢ High viability of yeast cells under low water availability to give a competitive advantage in the target environmental niche
- ➢ Appropriate formulation system: I. wet pastes, II. fluidised bed drying and III. freeze drying systems
- ➢ Storage stability of products at ambient and 4°C
- ➢ Studies on possible modes of action-enzyme production
- ➢ Lab-scale bioassays

The ability of yeast cells to adapt to water stress conditions by accumulating several compatible solutes and trehalose was exploited by manipulating the growth medium. The impact on inocula viability, drying resistance, storage stability, enzyme production and biocontrol effectiveness were then examined and the main findings are summarized below.

Synthetic versus complex (industry byproduct) media

The yield and the quality of *P. anomala* cells were greatly influenced by the media, the water availability and the different types of solute used to modify media a_w. Use of post-harvest isotonic washing treatment also significantly affected quality by means of enhanced endogenous polyols and trehalose intracellular retention.

➤ On both types of basal media, a decrease of a_w resulted in a decrease in *P. anomala* cell yield

➤ The type of solute used to decrease media a_w had a differential effect on final yield due to its metabolic value and use by the yeast, e.g. readily utilised glucose increased yield at 0.98 a_w and glycerol was more tolerated since the molecule can be directly taken up by cells

➤ The rich defined medium (NYDB) was found to result in higher cell production than the complex molasses-based media. Different sugar compositions (glucose for NYDB, sucrose for molasses) and enhanced trehalose intracellular accumulation in molasses media might account for this finding

➤ At both media used, yeast cells responded to reduced medium Ψ_w by decreasing cell Ψ_c a such that $\Psi_c \leq \Psi_w$. This response was concomitant with increased intracellular proportion of the polyols glycerol and/or arabitol depending on the stress solute used and the a_w of the medium. Maximum intracellular polyols and trehalose accumulation occurred after 72 h incubation

➤ Trehalose accumulation was pronounced in molasses-based media, particularly with proline addition

➤ In proline and NaCl treatments, post-harvest isotonic washing resulted in the arabitol intracellular amount being doubled. Similarly, trehalose intracellular retention doubled in glucose and proline treatments and was 4x greater in the NaCl 0.98 a_w treatment

➤ 72 h-old cells grown in NYDB media gave a much higher viable count than those grown in molasses-based media. A possible explanation for his may be the large quantities of intracellular trehalose accumulated in yeast cells grown in molasses-based media

➤ Yeast cells gave high viable counts under water-stress conditions particularly as cultures aged

High polyol and trehalose intracellular accumulation and retention was carefully considered when studies on formulation were carried out.

Wet paste formulation system

➤ Storage stability of *P. anomala* cells formulated as wet pastes was greatly influenced by the cultural conditions of the yeast cells and by storage temperature

➤ Storage at ambient (22°C) temperature resulted in a rapid reduction in cell viability

➤ Wet paste formulation of *P. anomala* system is very unstable and should not be considered for use on a commercial basis

Fluidised bed drying and storage stability

➤ For *P. anomala* cells, optima drying conditions identified as 50°C for 20 min resulting in 67% viable with 7% moisture content.

➤ Use of NaCl isotonic solutions resulted in increased *P. anomala* cell viability after drying. Increased trehalose retention when osmoprotection took place possibly accounts for this.

➤ Not all isotonic solutions tested had a positive effect on *P. anomala* cell survival and the % moisture content. PEG 200 resulted in high cell mortality; a glucose isotonic solution resulted in high cell % moisture content and a sticky appearance

➤ Use of several additives as carriers and adjuvants at different doses prior to fluidised bed drying had differential effects on *P. anomala* cell survival and % moisture content

➤ Use of talc as an additive had a deleterious effect on cells and was inappropriate for use as a carrier and/or adjuvant

➤ The best fluidised bed drying formulations were corn meal (CM):+10% glycerol, cotton seed flour (CSF): 1 + 10% skimmed milk (SM) and wheat starch (WS): 1 + 10% SM

➤ CSF + 10% SM resulted in the highest cell protection during the drying process, with a final moisture content about 5% and was easy to resuspend

➤ In the NaCl 0.98 a_w treatment, use of isotonic post-harvest washing significantly increased *P. anomala* cell viability in all formulated treatments

➢ In the proline 0.98 a_w treatment, use of isotonic post-harvest washing did not have any effect on cell viability or CFUs mg^{-1} formulation on NYDA medium with fully available water

➢ Storage stability was greatly influenced by storage temperature. At ambient temperature, cell treatments lost viability more quickly than the respective treatments when stored at 4^oC

➢ CSF + 10% SM provided the most stable formulation at both temperatures

➢ *P. anomala* cells grown in NaCl, and washed post-harvest with an isotonic solution increased storage stability for all formulation and storage temperature treatments

In conclusion, the importance of *P. anomala* ecophysiological manipulation, different formulation and post-harvest washing treatments on *P. anomala* desiccation tolerance during fluidised bed drying was demonstrated. The importance of the aforementioned parameters and the effect of storage temperature were also shown. Subsequent studies should consider this effect before subjecting fungal inocula to different drying regimes.

Freeze drying and storage stability

➢ *P. anomala* cells were tolerant of freezing and thawing cycles even without the use of protectants. However, survival after freezing was further increased when a mixture of skimmed milk (SM) + sucrose at 10% (w/v) were used

➢ The survival rate of freeze dried *P. anomala* cells dramatically decreased (<20%) in the absence of protective and rehydration media

➢ A protective solution of SM + sucrose resulted in a high final cell protection which was not further increased when cells were rehydrated in 10% SM medium. This indicates that the presence of SM is important for desiccation tolerance of *P. anomala* cells and that its use at the beginning of the process protected the cells until the final rehydration step. It also provided the final product with a porous structure that made rehydration easier. Consequently, SM was used as a support material in a mixture with sucrose

➢ Isotonic post-harvest washing treatment resulted in modified *P. anomala* cells with increased freeze drying tolerance, particularly in the absence of a protective medium. Increased trehalose intracellular accumulation might

account for this phenomenon. When a protective medium was used the effect of isotonic washing was masked by the latter.

➢ Storage stability of freeze dried *P. anomala* cells was greatly influenced by the type of fresh cell treatments and storage temperature

➢ At ambient temperature, cell treatments dramatically lost viability after 30 days storage; control cells decreased their viability from 96 to 24%

➢ Osmoprotection using post-harvest isotonic washing treatment had no effect on storage stability. It seems that the SM + sucrose suspension medium was the key factor which significantly affected storage stability masking any effect isotonic washing treatment may have had on freeze-dried *P. anomala* cell storage stability

This study showed the impact of protective additives, rehydration media, *P. anomala* cell modification and cell post-harvest washing with isotonic solution on yeast viability and storage stability after freeze drying. It was also demonstrated that *P. anomala* is highly resistant to freezing, thawing and dehydration during the processes of freeze and freeze drying, which is useful from a commercial point of view. Storage stability at 4°C of freeze dried *P. anomala* cells was also particularly high over a period of 150 days while storage at 22°C resulted in more rapid decrease in cell viability over a period of 30 days.

Endogenous protein and enzyme production by *P. anomala* cells

➢ Use of the API ZYM kit showed that different carbon sources had an effect on enzyme production. Use of *Penicillium verrucosum* CWP + glucose, compared to *P. verrucosum* and *P. roqueforti* CWP alone, resulted in higher butyrate esterase and caprylate esterase lipase and lower napthol-AS-BI-phospshohydralase and acid phosphatase activity. In all treatments, β-glucosidase activity was low and reached a peak after 5 days incubation. Whether these enzymes play any role in biocontrol activity exerted by the yeast is difficult to confirm. However, we do have an indication of *P. anomala* enzymatic activity profile

➢ Modification of *P. anomala* cells by addition of several solutes to molasses media resulted in reduced endogenous protein accumulation; lower a_w of media resulted in lower protein accumulation. It seemed that yeast cells

> reduced their protein content in order to osmoregulate by accumulating polyols, in particular arabitol and glycerol, and trehalose

> When minimal salt media were supplemented with synthetic carbon and chitin sources only low β-(1-3)-glucanase and chitinase activity were induced

> Use of *P. verrucosum* CWP+glucose as a carbon source resulted in significantly higher β-(1-3)-glucanase (2.22 units) activity and increased over time

> Different modified *P. anomala* cells resulted in significantly different enzyme activity and osmoprotection did not play any role in this effect

> Enhanced trehalose retention seemed to have an adverse effect on β-(1-3)-glucanase activity. It seems that this carbohydrate reserve repressed the cells' need to produce β-(1-3)-glucanase which in turn hydrolyses cell wall glucans and provides cells with readily available carbohydrates

> Very little chitinase activity was induced and only reached 0.89 units after 120h incubation. The results suggest that the role of chitinase in yeast biocontrol activity is very limited

> The hydrolytic enzyme β-glucosidase was found to be produced by *P. anomala* cells and its specific activity was rather low. Osmoprotection had no effect on total and specific enzyme activity but different modified yeast cells were found to srete different amounts of β-glucosidase

In conclusion, *P. anomala* J121 was found to produce considerable β-(1-3)-glucanase and low chitinase and β-glucosidase amounts. The available literature, suggests the involvement of the first hydrolase in biocontrol activity is important. The role of the other two is not clear for *P. anomala*. Osmoprotection does not have any direct effect on endogenous protein and enzyme production; however, an indirect effect could be that enhanced intracellular trehalose retention seemed to repress β-(1-3)-glucanase production.

Bioassays

> *P. anomala* cells reduced populations of *P. verrucosum* strain 22625 with no significant differences between treatments and a_ws after 30 days of co-culture in wheat. Some differences noticed after 10 days were possibly due to better wheat colonization by the yeast at these early stages in the experiment

➢ *P. verrucosum* 22625 inhibition was less pronounced than would be expected according to previous studies using the same pathogen-antagonist system. However, different conditions, sterile grain (used in this study) against non-sterile grain, non-limiting O_2 conditions against limiting O_2 conditions, different a_w levels and different strain possibly accounted for this. Under aerobic conditions *P. verrrucosum* 22625 may be more competitive, while microaerophilic *P. anomala* was more competitive in airtight storage

➢ An initial inoculum of 10^5 CFUs g^{-1} *P. anomala* was shown to be not a high to inhibit 10^3 CFUs g^{-1} *P. verrucosum* 22625

➢ OTA production by *P. verrucosum* 22625 was significantly affected by a_w alone. At 0.95 a_w, *P. anomala* did not decrease OTA production by *P. verrucosum* 22625. At a lower a_w, 0.93, a decrease in mould populations coincided with decrease in OTA production

In this study, it was also shown that formulation additives had no effect on mould populations and OTA production. Furthermore, modified yeast cells with increased levels of trehalose and arabitol gave similar efficacy as fresh cells.

Overall finding

An optimized, lab-scale, liquid fermentation system using a cheap industry byproduct as a basal medium supported high *P. anomala* yield of high quality cells , in terms of desiccation tolerance. Osmoprotection of modified yeast cells was a key factor for intracellularly retaining the endogenous reserves. Fluidised bed drying, when appropriate additives were used, is a formulation system which maintained high inoculum quality during storage without contamination.

5.2   FUTURE WORK

➢ Studies on optimization of fermentation scale up

➢ Spray drying of modified *P. anomala* cells containing high trehalose content, non- and osmoprotected

➢ Liquid formulations with determination of best adjuvants to maintain internal endogenous reserves

➢ Pilot scale bioassays of fluidised bed drying *P. anomala* formulations, non- and osmoprotected, with higher initial inoculum concentration 10^6-10^7 and in airtight conditions

➢ Elucidation of whether the higher *P. anomala* biocontrol efficacy in airtight conditions is associated with increased β-glucosidase activity

➢ Use of green fluorescent protein (GFP) mutant *P. anomala* cells to follow colonisation

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APPENDIX I

Table 1. Analysis of variance of the effect of ± solute (control), water activity (a_w) and different type of solute (solute) on viability of *P. anomala* cells grown in unmodified (0.998 a_w) and modified (0.98 and 0.96 a_w) NYDB media after incubation at 25°C for 24 h. Cells were plated on unstressed media. The analysis was performed using $\log_{10}$ transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
control	1	0.01445	0.01445	0.48	0.492	NS
control.solute	4	11.41760	2.85440	94.06	<.001	**
control.aw	1	0.00553	0.00553	0.18	0.671	NS
control.solute.aw	4	4.35037	1.08759	35.84	<.001	**
Residual	88	2.67063	0.03035			
Total	98	18.45858				

Note: d.f., degrees of freedom; s.s., sum of squares; m.s., mean square; v.r., variance ratio; ** significant P<0.01; * P<0.05; NS not significant

Table 2. Table of means of variance of $\log_{10}$ viability of *P. anomala* cells grown in unmodified (0.998 a_w) and modified (0.98 and 0.96 a_w) NYDB media after incubation at 25°C for 24 h. Cells were plated on unstressed media. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

		aw		
	solute	0.98	0.96	control
treated	glycerol	9.128	9.062	
	glucose	9.284	8.965	
	sorbitol	8.932	8.705	
	proline	8.813	8.623	
	NaCl	7.699	8.579	
control	control			8.821
LSD				0.1632

Table 3. Analysis of variance of the effect of ± solute (control), water activity (a_w) and different type of solute (solute) on viability of *P. anomala* cells grown in unmodified (0.998 a_w) and modified (0.98 and 0.96 a_w) NYDB media after incubation at 25°C for 48 h. Cells were plated on unstressed media. The analysis was performed using $\log_{10}$ transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
control	1	0.04511	0.04511	0.99	0.323	NS
control.solute	4	6.41953	1.60488	35.07	<.001	**
control.aw	1	0.12716	0.12716	2.78	0.099	NS
control.solute.aw	4	2.15769	0.53942	11.79	<.001	**
Residual	88	4.02725	0.04576			
Total	98	12.77675				

Table 4. Table of means of variance of $\log_{10}$ viability of *P. anomala* cells grown in unmodified (0.998 a_w) and modified (0.98 and 0.96 a_w) NYDB media after incubation at 25^oC for 48 h. Cells were plated on unstressed media. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

		aw		
	solute	0.98	0.96	control
treated	glycerol	9.585	9.455	
	glucose	9.813	9.876	
	sorbitol	10.074	10.066	
	proline	9.551	9.337	
	NaCl	9.030	9.694	
control	control			9.722
LSD				0.2004

Table 5. Analysis of variance of the effect of drying temperature and duration on *P. anomala* cell viability and using a fluidised bed dryer. The analysis was performed using logit transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
Time	1	0.11436	0.11436	1.93	0.180	**
Temper	4	57.74231	14.43558	243.67	<.001	**
Time.Temper	4	1.55469	0.38867	6.56	0.002	**
Residual	20	1.18484	0.05924			
Total	29	60.59620				

Table 6. Table of means of variance of logit viability of *P. anomala* cells fluidised bed dried at different temperature and time levels. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

	Time	10	20
temperature	30	1.731	0.834
	40	1.150	1.294
	50	0.287	0.708
	60	0.5680	0.609
	70	-2.314	-2.642
LSD			0.1987

Table 7. Analysis of variance of the effect of drying temperature and duration on *P. anomala* cell final moisture content and using a fluidised bed dryer. The analysis was performed using logit transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
Temper	4	7.15439	1.78860	177.51	<.001	**
Time	1	3.72607	3.72607	369.80	<.001	**
Temper.Time	4	1.46914	0.36729	36.45	<.001	**
Residual	20	0.20152	0.01008			
Total	29	12.55112				

Table 8. Table of means of variance of logit final cell moisture of *P. anomala* cells fluidised bed dried at different temperature and time levels. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

	Time	10	20
temperature	30	-0.519	-1.903
	40	-1.564	-2.442
	50	-1.716	-2.539
	60	-2.361	-2.539
	70	-2.490	-2.752
LSD			0.1710

Table 9. Analysis of variance of the effect of post-harvest washing treatment on *P. anomala* different treatment cell viability after drying at 50°C for 20 min using a fluidised bed dryer. Post-harvest washing treatment was done with either water or solutions isotonic to the growth media those being PEG 200, glucose or NaCl. The analysis was performed using logit transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
Treatments	4	14.24590	3.56148	98.66	<.001	**
ISO	3	121.99195	40.66398	1126.52	<.001	**
Treatments.ISO	12	24.15721	2.01310	55.77	<.001	**
Residual	40	1.44387	0.03610			
Total	59	161.83894				

Table 10. Table of means of variance of logit *P. anomala* different treatment cell viability after drying at 50°C for 20 min using a fluidised bed dryer. Post-harvest washing treatment was done with either water or solutions isotonic to the growth media those being PEG 200, glucose or NaCl. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

	ISO			
Treatments	water	PEG200	glucose	NaCl
NaCl98	0.724	-2.642	-2.642	0.664
SOrb96	-0.462	-3.753	0.411	-0.148
Prol98	0.833	-2.777	0.494	0.818
glyc98	0.339	-2.330	0.635	0.694
gluc98	-0.013	-4.126	-1.413	0.053
LSD				0.3135

Table 11. Analysis of variance of the effect of additives used as carriers (100% or 1:1 w/w) like corn meal (CM), cottonseed flour (CSF), and wheat starch (WS) alone or with adjuvants (10% skimmed milk for CSF and WS and glycerol for CM) on *P. anomala* cell viability after drying at 50°C for 20 min using a fluidised bed dryer. Control is untreated *P .anomala* cells. The analysis was performed using logit transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
treatment	6	0.981823	0.163637	52.33	<.001	**
Residual	14	0.043776	0.003127			
Total	20	1.025599				

Table 12. Table of means of variance of logit viability of *P. anomala* cells when additives were used as carriers (100% or 1:1 w/w) like corn meal (CM), cottonseed flour (CSF), and wheat starch (WS) alone or with adjuvants (10% skimmed milk for CSF and WS and glycerol for CM). Cells were fluidised bed dried at 50°C for 20 min. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown

Treatments	
Control	0.708
CSF:1+10%SM	0.687
CM:1+10%Gly	0.144
WS:1+10%SM	0.553
CSF:1	0.487
WS:1	0.614
CM:1	0.171
LSD	0.0979

Table 13. Analysis of variance of the effect of control *P. anomala* cell formulations on viable CFUs mg^{-1} formulation on water stressed (0.96 a_w) NYDA medium. Formulations are A: corn meal:1+10% glycerol (w/w), B:cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreated (unformulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. The analysis was performed using log$_{10}$ transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr
additivesNaCl	3	0.0057903	0.0019301	18.73	<.001
Residual	8	0.0008243	0.0001030		
Total	11	0.0066146			

Table 14. Table of means of variance of LOG10 viable CFUs mg^{-1} formulation on water stressed (0.96 a$_w$) NYDA. Cells were formulated as A: corn meal:1+10% glycerol (w/w), B:cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreated (unformulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown

Treatments	
Control	6.6078
CSF:1+10%SM	6.6433
CM:1+10%Gly	6.5816
WS:1+10%SM	6.6149
LSD	0.01911

Table 15. Analysis of variance of the effect of proline 0.98 a$_w$ *P. anomala* formulations on cell viability. Formulations are A: corn meal:1+10% glycerol (w/w), B: cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreated (unformulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. The analysis was performed using logit transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
Prolineformulation	3	0.42853	0.14284	6.90	0.003	**
ISO	1	0.05111	0.05111	2.47	0.136	NS
Prolineformulation.ISO	3	0.14830	0.04943	2.39	0.107	NS
Residual	16	0.33119	0.02070			
Total	23	0.95912				

Table 16. Table of means of variance of logit viability of *P. anomala* formulations of cells grown in molasses-based media modified with proline 0.98 a$_w$. Formulations are A: corn meal:1+10% glycerol (w/w), B: cottonseed flour:1+10% skimmed milk (w/w) and C: wheat starch:1+10% skimmed milk (w/w). Control is untreated (unformulated) *P. anomala* cells. Cells were dried at 50°C for 20 min using a fluidised bed dryer. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

	Washing		
Prolineformulation	water	Iso	Pooled
No	0.928	1.100	1.014
CM+Gly	0.578	0.789	0.683
CSF+SM	0.902	0.724	0.813
WS+SM	0.900	1.064	0.982
LSD			0.1761

Table 17. Analysis of variance of the effect of protective solutions on *P. anomala* cell freeze tolerance. SM: skimmed milk, Glu: glucose, Sucr: sucrose. The analysis was performed using logit transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
Protectivesolution	6	7.2154	1.2026	9.94	<.001	**
Residual	14	1.6945	0.1210			
Total	20	8.9099				

Table 18. Table of means of variance of logit viability of **P. anomala** cells after freezing and thawing. Different protective solutions (SM: skimmed milk, Glu: glucose, Sucr: sucrose) were used**.** All data are means of three replicates per treatment. Least significant difference (LSD) is also shown

Treatments	
water	2.894
10%SM	4.361
10%glucose	3.753
10%sucrose	3.849
10%SM+10%glucose	3.515
10%SM+10%sucrose	4.595
20%SM	3.022
LSD	0.6092

Table 19. Analysis of variance of the effect of post-harvest washing of *P. anomala* cells grown in glucose 0.98 a_w molasses-modified media with PEG 200, glucose and NaCl and different protective media (water and 10%SM+10% sucrose) on cell tolerance after freeze drying. The analysis was performed using logit transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
rehydrationmedia	1	72.80599	72.80599	867.80	<.001	**
isotonicsolution	3	13.05348	4.35116	51.86	<.001	**
rehydmedia.isosolution	3	5.57252	1.85751	22.14	<.001	**
Residual	16	1.34235	0.08390			
Total	23	92.77434				

Table 20. Table of means of variance of logit viability of *P. anomala* cells grown in glucose 0.98 a_w molasses-modified media after freeze drying. Post-harvest washing treatments were PEG 200, glucose and NaCl and different protective media used were water and 10%SM+10% sucrose. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

	rehydrationmedia	
Isotonicsolution	water	10%SM+10%sucrose
Water	-1.155	3.476
NaCl	0.093	2.944
Glucose	1.490	3.750
PEG200	-1.495	2.697
LSD		0.5014

Table 21. Analysis of variance of the effect of *P. anomala* cells grown in molasses-modified media with proline (Pro98), glucose (Glu98), sorbitol (Sorb98), NaCl and glycerol (Gly98) at $0.98a_w$ and use of isotonic NaCl solution on freeze drying tolerance when water was used as rehydration medium. The analysis was performed using logit transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
control	1	5.41051	5.41051	158.56	<.001	**
control.celltreatment	4	3.37160	0.84290	24.70	<.001	**
control.iso	1	3.12441	3.12441	91.56	<.001	**
control.celltreat.iso	4	0.69816	0.17454	5.11	0.005	**
Residual	22	0.75072	0.03412			
Total	32	13.35540				

Table 22. Table of means of variance of logit viability of *P. anomala* cells grown in molasses-modified media with proline (Pro98), glucose (Glu98), sorbitol (Sorb98), NaCl and glycerol (Gly98) at $0.98a_w$ and use of isotonic NaCl solution on freeze drying tolerance when water was used as rehydration medium.. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

	washing		
celltreatment	water	iso	control
proline	0.284	0.578	
glucose	-1.064	0.121	
sorbitol	-0.351	0.363	
NaCl	-0.490	0.107	
glycerol	-0.678	-0.241	
control			-1.546
LSD			0.3128

Table 22. Analysis of variance of LOGIT of *P.anomala* freese dried cell viability when post-harvest washed with water and stored at 4°C. Cell treatments are proline (pro98), glucose (glu98), sorbitol (sorb98), NaCl (NaCl98) and glycerol (gly98); control cells were grown in unmodified molasses media. Storage times were 0 and 150 days. All data are means of three replicates per treatment.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
treat	5	5.48052	1.09610	15.02	<.001	**
time	1	15.96634	15.96634	218.77	<.001	**
treat.time	5	0.89355	0.17871	2.45	0.063	**
Residual	24	1.75154	0.07298			
Total	35	181.68069				

Table 23. Table of means of variance of LOGIT of *P. anomala* freeze dried cell viability when post-harvest washed with water and stored at 4°C. Cell treatments are proline (pro98), glucose (glu98), sorbitol (sorb98), NaCl (NaCl98) and glycerol (gly98); control cells were grown in unmodified molasses media. All cell were post-harvest washed with water. Storage times were 0 and 150 days. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

Time(days) Treatment	0	150
control	3.277	1.761
pro98	3.753	2.239
glu98	4.126	3.022
sorb98	3.515	2.129
NaCl98	3.988	2.275
gly98	2.784	2.025
LSD		0.4552

Table 24. Analysis of variance of LOGIT of *P. anomala* freeze dried cell viability when post-harvest washed with NaCl isotonic solution and stored at 4°C. Cell treatments are proline (pro98), glucose (glu98), sorbitol (sorb98), NaCl (NaCl98) and glycerol (gly98); control cells were grown in unmodified molasses media. Storage times were 0 and 150 days. All data are means of three replicates per treatment.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
treat	5	13.85353	2.77071	68.44	<.001	**
time	1	11.82226	11.82226	1145.38	<.001	**
treat.time	5	1.66468	0.33294	32.26	<.001	**
Residual	24	0.24772	0.01032			
Total	35	181.68069				

Table 25. Table of means of variance of LOGIT of *P. anomala* freeze dried cell viability when post-harvest washed with NaCl isotonic solution and stored at 4°C. Cell treatments are proline (pro98), glucose (glu98), sorbitol (sorb98), NaCl (NaCl98) and glycerol (gly98); control cells were grown in unmodified molasses media. All cell were post-harvest washed with water. Storage times were 0 and 150 days. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

Time(days) Treatment	0	150
control	3.277	1.761
pro98	4.595	3.100
glu98	3.377	3.022
sorb98	3.178	1.874
NaCl98	4.595	3.178
gly98	2.816	2.025
LSD	0.1712	

Table 26. Analysis of variance of exo β-1,3-glucanase activity of *P. anomala* grown in Czapek minimal salt medium in the presence of laminarin (Lam) 2 mg ml^{-1}, glucose (Glu) 2mg ml^{-1}, chitin 10 mg ml^{-1} and their combinations. Cells were incubated at 25°C.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
substrate	4	0.659711	0.164928	16.70	<.001	**
time	2	1.102893	0.551447	55.83	<.001	**
substrate.time	8	1.115262	0.139408	14.11	<.001	**
Residual	30	0.296333	0.009878			
Total	44	3.174200				

Table 27. Table of means of variance of. exo β-1,3-glucanase activity of *P. anomala* grown in Czapek minimal salt medium in the presence of laminarin (Lam) 2 mg ml^{-1}, glucose (Glu) 2mg ml^{-1}, chitin 10 mg ml^{-1} and their combinations. Cells were incubated at 25°C. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

	Time		
substrate	1	2	3
Lam	1.213	0.627	0.547
Glu	0.967	0.660	0.840
Chitin	1.027	0.330	0.747
Chitin+Lam	0.807	0.363	0.493
Chitin+Glu	0.360	0.477	0.743
LSD			0.1657

Table 28 . Analysis of variance of exo β-1,3-glucanase activity of *P. anomala* cells from modified molasses media with proline (Pro98),glucose (Glu98) and NaCl (NaCl98), all at 0.98 a$_w$ level. Control cells grew in unmodified molasses media and washed post-harvest with water.Cells were post-harvest washed with water and NaCl isotonic solution and subsequently grown in Czapek salt minimal medium in the presence of CWP of *P. verrucosum*+glucose (each 2mg ml^{-1}). Cells were incubated at 25°C.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
controlvsall	1	0.73601	0.73601	11.77	0.004	**
controlvsall.treatments	2	2.74253	1.37127	21.93	<.001	**
controlvsall.washing	1	0.01805	0.01805	0.29	0.600	NS
contvsall.treats.washing	2	0.36760	0.18380	2.94	0.086	NS
Residual	14	0.87547	0.06253			
Total	20	4.73966				

Table 29. Table of means of variance of exo β-1,3-glucanase activity of *P. anomala* cells from modified molasses media with proline (Pro98),glucose (Glu98) and NaCl (NaCl98), all at 0.98 a$_w$ level. Control cells grew in unmodified molasses media and washed post-harvest with water.Cells were post-harvest washed with water and NaCl isotonic solution and subsequently grown in Czapek salt minimal medium in the presence of CWP of *P. verrucosum*+glucose (each 2mg ml^{-1}). Cells were incubated at 25°C.All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

		washing	
CWPsub	water	iso	control
NaCl	2.727	2.437	
glu	2.130	2.540	
pro	3.223	3.293	
control			3.260
LSD			0.3792

Table 30. Analysis of variance of exochitinase activity of *P. anomala* grown in Czapek minimal salt medium in the presence of laminarin (Lam) 2 mg ml^{-1}, glucose (Glu) 2mg ml^{-1}, chitin 10 mg ml^{-1} and their combinations. Cells were incubated at 25°C.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
substrate	4	0.89938	0.22484	20.37	<.001	**
time	2	2.40727	1.20364	109.05	<.001	**
substrate.time	8	0.77233	0.09654	8.75	<.001	**
Residual	30	0.33113	0.01104			
Total	44	4.41011				

Table 31. Table of means of variance of. exochitinase activity of *P. anomala* grown in Czapek minimal salt medium in the presence of laminarin (Lam) 2 mg ml^{-1}, glucose (Glu) 2mg ml^{-1}, chitin 10 mg ml^{-1} and their combinations. Cells were incubated at 25°C. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

	Time		
substrate	1	2	3
Lam	0.167	0.217	0.387
Glu	0.363	0.283	0.797
Chitin	0.233	0.543	0.633
Chitin+Lam	0.330	0.417	1.040
Chitin+Glu	0.200	0.633	1.190
LSD			0.1752

Table 32. Analysis of variance of total (μmol 4-nitrophenol min^{-1}) enzyme activity of β-glucosidase produced by *P. anomala*. Treatment cells grew in molasses media modified with proline (Pro), glucose (Glu) and NaCl (NaCl), all at 0.98 a$_w$ level. Control cells grew in unmodified molasses media and washed post-harvest with water.Cells were post-harvest washed with water and NaCl isotonic solution and subsequently grown in Czapek salt minimal medium in the presence of *P.verrucosum*CWP+glucose (each 2mg ml^{-1}). Cells were incubated at 25°C for 120h.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
control	1	0.004706	0.004706	0.55	0.470	NS
control.treatment	2	0.282978	0.141489	16.55	<.001	**
control.washing	1	0.005339	0.005339	0.62	0.443	NS
control.treatment.washing	2	0.053911	0.026956	3.15	0.074	NS
Residual	14	0.119667	0.008548			
Total	20	0.466600				

Table 33. Table of means of variance of total (μmol 4-nitrophenol min^{-1}) enzyme activity of β-glucosidase produced by *P. anomala*. Treatment cells grew in molasses media modified with proline (Pro), glucose (Glu) and NaCl (NaCl), all at 0.98 a$_w$ level. Control cells grew in unmodified molasses media and washed post-harvest with water.Cells were post-harvest washed with water and NaCl isotonic solution and subsequently grown in Czapek salt minimal medium in the presence of *P.verrucosum*CWP+glucose (each 2mg ml^{-1}). Cells were incubated at 25°C for 120h.. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

NaCl	glu	pro	control	
0.548	0.658	0.852	0.643	
LSD				0.1402

Table 34. Analysis of variance of effect of endogenous total protein content in *P. anomala* cells grown in unmodified (control) and modified with different solutes (glycerol-gly, glucose-glu, NaCl, sorbitol-sorb and proline-pro), at 0.98 and 0.96 a$_w$ levels and different post-harvest treatment [water (a) and NaCl isotonic solution. Cells were grown at 25°C for 72 h

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
control	1	21.618	21.618	19.78	<.001	**
control.treat	4	359.605	89.901	82.24	<.001	**
control.aw	1	1.104	1.104	1.01	0.321	**
control.washing	1	102.547	102.547	93.81	<.001	**
control.treat.aw	4	32.723	8.181	7.48	<.001	**
control.treat.washing	4	77.666	19.416	17.76	<.001	**
control.aw.washing	1	32.003	32.003	29.28	<.001	**
control.treat.aw.washing	4	52.677	13.169	12.05	<.001	**
Residual	42	45.912	1.093			
Total	62	725.856				

Table 35. Table of means of variance of endogenous total protein content in *P. anomala* cells grown in unmodified (control) and modified with different solutes (glycerol-gly, glucose-glu, NaCl, sorbitol-sorb and proline-pro), at 0.98 and 0.96 a_w levels and different post-harvest treatment [water (a) and NaCl isotonic solution. Cells were grown at 25°C for 72 h. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

		washing		
Treat	aw	water	iso	control
gly	0.98	8.96	8.41	
	0.96	8.97	6.28	
glu	0.98	11.22	9.49	
	0.96	19.36	7.59	
NaCl	0.98	8.07	6.31	
	0.96	8.94	4.45	
sorb	0.98	5.25	5.06	
	0.96	5.35	5.30	
pro	0.98	6.31	4.77	
	0.96	5.84	4.47	
control				10.27
LSD			1.723	

Table 36. Analysis of variance of the effect of *P. anomala* presence on the growth and sporulation of *P. verrucosum* strain 22625 measured as c.f.u in co-cultures in petri dishes with γ-irradiated wheat grain (a) 0.95 and (b) 0.93 a_w at 25°C for 1, 10, 20 and 30 days. Different treatments were *P. verrucosum* alone (control), *P.anomala* unmodified fresh cells alone, *P. anomala* proline modified fresh cells washed with NaCl isotonic solution (A), *P. anomala* unmodified cells fluidised bed dried as cottonseed flour:1+10% skimmed milk formulation (B), *P. anomala* proline modified cells fluidised bed dried as cottonseed flour:1+10% skimmed milk formulation washed with water (C), *P. anomala* proline modified cells fluidised bed dried as cottonseed flour:1+10% skimmed milk formulation washed with NaCl isotonic solution (D). A, B, C, D treatments were co-cultures with *P. verrucosum*. The analysis was performed using log_{10} transformed data.

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr	
treatment	5	562.700833	112.540167	37023.32	<.001	**
aw	1	0.025051	0.025051	8.24	0.005	**
time	3	126.014608	42.004869	13818.71	<.001	**
treatment.aw	5	0.473358	0.094672	31.14	<.001	**
treatment.time	15	30.417086	2.027806	667.10	<.001	**
aw.time	3	0.151417	0.050472	16.60	<.001	**
treatment.aw.time	15	1.191562	0.079437	26.13	<.001	**
Residual	96	0.291812	0.003040			
Total	143	721.265727				

Table 37. Table of means of variance of LOG10 of viability of *P. verrucosum* strain 22625 measured as c.f.u in co-cultures with *P. anomala* in petri dishes with γ-irradiated wheat grain at (a) 0.95 and (b) 0.93 a_w at 25°C for 1, 10, 20 and 30 days. Different treatments were *P. verrucosum* alone (control), *P.anomala* unmodified fresh cells alone, *P. anomala* proline modified fresh cells washed with NaCl isotonic solution (A), *P. anomala* unmodified cells fluidised bed dried as cottonseed flour:1+10% skimmed milk formulation (B), *P. anomala* proline modified cells fluidised bed dried as cottonseed flour:1+10% skimmed milk formulation washed with water (C), *P. anomala* proline modified cells fluidised bed dried as cottonseed flour:1+10% skimmed milk formulation washed with NaCl isotonic solution (D). A, B, C, D treatments were co-cultures with *P. verrucosum. P. anomala* cells grown in molasses-modified media with proline (Pro98), glucose (Glu98), sorbitol (Sorb98), NaCl and glycerol (Gly98) at 0.98 a_w and use of isotonic NaCl solution on freeze drying tolerance when 10%SM+10% sucrose was used as rehydration medium.. All data are means of three replicates per treatment. Least significant difference (LSD) is also shown.

treatment	aw	Time			
		1	10	20	30
Pv	0.95	3.0014	5.7041	6.4023	6.7897
	0.93	3.1844	5.5934	6.2946	6.6506
Pa	0.95	-0.3010	-0.3010	-0.3010	-0.3010
	0.93	-0.3010	-0.3010	-0.3010	-0.3010
A	0.95	2.9907	5.3345	5.3730	5.5267
	0.93	3.1483	5.3262	5.3421	5.5050
B	0.95	3.0251	5.3640	5.4392	5.5740
	0.93	2.9907	5.2206	5.3977	5.5440
C	0.95	3.0323	5.3977	5.3972	5.5437
	0.93	2.9527	5.3483	5.3915	5.5396
D	0.95	2.9624	5.2854	5.3856	5.5354
	0.93	3.0404	6.3271	5.3735	5.5269
LSD			0.09817		

APPENDIX II

II.I PUBLICATIONS

Mokiou, S. & Magan, N. (2002). Ecophysiological manipulation of fermentation improves viability of the biocontrol yeast *Pichia anomala*. *Biological Control of Fungal and Bacterial Pathogens, IOBC wrps Bulletin,* Vol. 25 (10) pp.395-398.

III. II ORAL PRESENTATIONS

April 2003, Society for General Microbiology, 152nd meeting, Edinburgh, UK

Improving ecological competence of the biocontrol yeast *Pichia anomala* and fluidised bed drying formulations.

March 2004, Development of Biocontrol Agents of Diseases for Commercial Applications in Food Production Systems, Sevilla, Spain.

Production/formulation of the biocontrol yeast *Pichia anomala*: improving shelf-life and quality using endogenous solutes, isotonic solutions and additives during fluidised-bed drying

June 2004, Management of plant diseases and arthropod pests by BCAs and their integration in agricultural systems, Trento, Italy

Formulation and shelf-life studies of the biocontrol yeast *Pichia anomala*: positive effect of endogenous solutes, isotonic solutions and additives during fluidised-bed drying

II. III POSTER PRESENTATIONS

May 2002, Influence of A-Biotic and Biotic Factors on Biocontrol Agents, Kusadasi, TURKEY

Ecophysiological manipulation of fermentation improves viability of the biocontrol yeast *Pichia anomala*.

October 2002, 11th Panhellenic Symposium of Plant Pathology, Preveza, GREECE

Biological control of *Penicillium* spp. by use of the biocontrol yeast *Pichia anomala*: Ecophysiological studies and their impact on inoculum quality and shelf-life.