Introduction & Review of literature
1.1 Introduction

Among all organisms, fungi are the second largest group in the world after insects (Hawksworth 1991) and key component of tropical ecosystems throughout world. These heterotrophic eukaryotes have a worldwide distribution, and grow in a wide range of habitats, ranging from psychrophilic to thermophilic hence, including extreme environments such as deserts, areas with high salt concentrations (Vaupotic et al. 2008), ionizing radiation (Dadachova et al. 2007) as well as in deep sea sediments (Raghukumar and Raghukumar 1998; Singh et al. 2010) and anoxic sediments (Jebaraj et al. 2010). Other examples of aquatic fungi include those living in hydrothermal areas of the ocean (Le Calvez et al. 2009). Although often inconspicuous, fungi occur in every environment on the earth and play vital roles in most ecosystems.

The distribution of fungi in the marine environment has not been studied well when compared with the studies on fungi in freshwater and terrestrial ecosystems. The marine environment includes oceans, estuaries, mangroves, salt marshes, solar salterns and lagoons where the salinity ranges from 5 to 35 psu (practical salinity unit). Marine fungi form an ecological, and not a taxonomic group (Hyde et al. 2000). Among these, the obligate marine fungi grow and sporulate exclusively in sea water, and their spores are capable of germinating in sea water. On the other hand, facultative marine fungi are those from fresh water or a terrestrial milieu that have undergone physiological adaptations that allow them to grow and possibly also sporulate in the marine environment (Kohlmeyer and Kohlmeyer 1979). About 800 species of obligate marine fungi belonging to the fungi have been reported so far (Hyde et al. 2000). Several recent papers have addressed the issue of the diversity and numbers of marine fungi (Jones 1995; Jones and Mitchell 1996; Jones and Alias 1997). Kis-Papo (2005) compiled all the publications and listed 467 higher marine fungi from 244 genera. They may be divided into a majority of Ascomycota (97%), a few Basidiomycota (~2%) and anamorphic fungi (<1%).

1.2 Saltern ecosystem

Thalassohaline hypersaline environments are generally considered to be those originating by evaporation of sea water and with halite (NaCl) concentrations of
greater than 10% (m/w) (Oren 2002). These provide some of the most extreme habitats in the World. They are common all around the globe, and include, for example, marine ponds and salt marshes that are subjected to evaporation, salt or soda lakes, and sea-salt and man-made salterns (Truper and Galinski 1986). Solar salterns are composed of multiple shallow ponds that are located in tropical, subtropical, and temperate parts of the World. In these ponds, the NaCl is gradually concentrated as the seawater evaporates. Salt production in salterns usually begins with seawater as the initial source of brine, which is evaporated through a series of ponds, to the final pond where the NaCl and other salts precipitate out of the saturated brine. The bittern that remains after this crystallization of the halite is rich in magnesium chloride (MgCl$_2$) and provides a special ecological habitat within the salterns. Variable water activities ($a_w$) because of the increasing concentrations of NaCl, as well as the low oxygen concentrations and high light intensity, present life-limiting parameters in salterns (Brock 1979).

Solar salterns located in India were established around seventh century as a man-made system of ponds, and they are located in the eastern Arabian Sea, in the various states of India viz. Gujarat, Goa, Maharashtra, Tamil Nadu and Rajasthan. In Indian subcontinent, tropical to temperate climatic conditions allow the production of salt during the summer and in winter, when climate is arid. The strong local winds enhance evaporation of water and keep the temperatures of the water in brines moderate 20$^\circ$C in winter to higher up to 50$^\circ$C during summer. When the water reaches a suitable concentration of salt in the reservoir pans, it is directed to the next the evaporative ponds, separated by canals and finally to the crystallizer ponds where, crude salt crystal heaps are prepared. The solar salterns in India with their successive evaporation ponds, the salt-pan mud, and the crystallizer ponds provide a relatively simple ecosystem that is popular for studies of halotolerant and halophilic microorganisms (Dave and Desai 2006; Nayak et al. 2012; Thomas et al. 2012). These hypersaline waters in salterns were at first believed to be populated almost exclusively by archaea, bacteria, and the eukaryotic alga Dunaliella salina (Rodriguez-Valera et al. 1981; Javor 1989; Oren 2005). However, the high diversity of eukaryotic microorganisms in hypersaline waters became evident soon after fungi were first reported as active inhabitants of solar salterns (Gunde-Cimerman et al. 2000). Numerous halotolerant and halophilic fungi were initially isolated from the Secovlje...
solar salterns (Gunde-Cimerman et al. 2000), and later also from salterns around the World: La Trinidad in the Ebro River Delta and Santa Pola on the Mediterranean coast of Spain, Camargue in France, and the salterns on the Atlantic coast in Portugal, in Namibia, the Dominican Republic, Puerto Rico and Goa in India (Butinar et al. 2005a, b, c; Cantrell et al. 2006; Nayak et al. 2012). Numerous halotolerant and extremely halotolerant fungi (Zalar et al. 1999a, b, c; Gunde-Cimerman et al. 2000; Butinar et al. 2005a, b, c; Zalar et al. 2005, 2007, 2008a, b; Butinar et al. 2011; Nayak et al. 2012) and a few halophilic representatives (Zalar et al. 2005a) have been isolated from these hypersaline waters.

1.3 Isolation, detection and identification of fungi in salterns

Fungi were initially isolated from hypersaline water samples taken from seasonal solar salterns Secovlje along the northern Adriatic coast, on the border between Slovenia and Croatia (Gunde-Cimerman et al. 2000). The hypersaline waters were sampled throughout the season, from May to October, and the fungi were isolated by filtration of the water, microbial baiting and spreading of biofilms covering the surface of the brine. Fungal population dynamics was then followed using filtration. Selective media with lowered $a_w$ as a result of high fractions of NaCl (from 17–32%) or sugars (from 50–70%) were incubated with added antibiotics (to prevent bacterial growth) from several days up to several weeks. All of the isolated fungi were examined for their ability to grow at 17% NaCl, as the criterion for halophily (Gunde-Cimerman et al. 2000). These fungi appeared in distinct seasonal peaks, and reached 40000 CFU/L on enumeration media. These peaks primarily correlated with the highest levels of phosphorus and nitrogen, followed by dissolved oxygen concentration, $a_w$, pH and year of sampling (Butinar et al. 2011).

These initial studies were later supplemented with the isolation of fungi from the crystallizers of different salterns that operate throughout the year on three continents, including the salterns along the Red Sea coast in Israel (Eilat), along the Mediterranean coast in Spain (Santa Pola, and the Ebro River Delta) and France (Camargue), along the Atlantic coast in Namibia (Skeleton Coast), and along the coasts of the Dominican Republic (Monte Cristy), Puerto Rico (Fraternidad), Mandovi estuary (Goa) and Portugal (Samouco). Fungi were also isolated from the Dead Sea (Ein Bokek, Ein Gedi), the Great Salt Lake (Utah, USA) and the Enriquillo Salt Lake.
The presence of fungi was not only documented in brine, but also on wood immersed in brine (Zalar et al. 2005b), on the surface of halophytic plants and most recently, in tropical microbial mats (Cantrell et al. 2013). Many of the halotolerant species commonly found in salterns were also found in Arctic glacial ice and other water based Arctic environments, due to low $a_w$ as the common critical parameter in both environments (Gostincar et al. 2010).

Since 1998, 22 species of fungi have been either described as new in science or newly accommodated from hypersaline environments. They include three species of the genus Wallemia, i.e. W. sebi, W. muriae and W. ichthyophaga, which were accommodated into the newly described class Wallemiomycetes and order Wallemiales (Zalar et al. 2005a), two new species of the genus Emericella, i.e. E. appendiculata and E. stellamaris (Zalar et al. 2008a), black yeast Trimmatostromasalinum (Zalar et al. 1999b) and twelve new species of the genus Cladosporium, i.e. C. dominicanum, C. fusiforme, C. halotolerans, C. herbarioides, C. psychrotolerans, C. ramotenellum, C. salinae, C. spinulosum, C. subinflatum, C. subtilissimum, C. tenellum and C. velox (Zalar et al. 2007; Schubert et al. 2007). The species Aureobasidium pullulans was redefined as four varieties, one of them, i.e. A. pullulans var. pullulans, was frequently detected in hypersaline water environments (Zalar et al. 2008b). Additionally, two new species of Candida, i.e. Candida galli and C. pseudorugosa (Butinar et al. 2005a), and Eurotium halotolerans were isolated from hypersaline waters of different salterns worldwide (Butinar et al. 2005b). The presence of fungi in the salterns was confirmed also by their chemical lipid signature in the environment. Examination of the lipid composition of the dominant melanized yeast-like fungi revealed sterols as the most distinct lipid fraction. Ergosterol dominated all of the distributions, but the major differences between species were related to the subordinate sterols. The characteristic 24-methylcholest-7-en-3β-ol and 24-methylcholesta-7,24(28)-dien-3β-ol were described in significant proportions in the water column particles and sediments of gypsum and halite precipitation ponds, providing a specific signature for the presence of these fungi (Mejanelle et al. 2000).

Although the diversity of fungi inhabiting hypersaline waters is surprisingly high, certain genera and species of fungi dominate almost all of the investigated environments. They are divided in three main groups: (i) halotolerant and halophilic
fungi that have been primarily isolated on selective media containing different concentrations of NaCl, (ii) xerotolerant fungi that grow on both sugar and saline media, and (iii) sporadic airborne isolates that 'contaminate' low aw media and are present in low numbers. Disregarding the medium from which they were selected, only fungi that can grow in vitro in 3 M NaCl have been further identified and analyzed. Some of the typical representatives of this group show adaptive xerotolerance/halotolerance, and these are represented by different species of the genera *Aspergillus*, *Penicillium* and *Eurotium*, and by some species of the genus *Cladosporium*. The dominant halotolerant and halophilic fungi were almost exclusively isolated on selective media with high concentrations of NaCl. This group of fungi displayed halophilic behavior that differed both from that of the majority of halophilic prokaryotes as well as from the xerotolerant/halotolerant fungi mentioned earlier. With few exceptions, the fungi in this group did not require NaCl for viability, as they were able to grow and adjust to the whole salinity range, from freshwater to almost saturated NaCl solutions (Plemenitas et al. 2008). Their growth in vitro was optimal across a broad salinity range, from 5 to 17% NaCl, and they have been regularly isolated from global environments at salinities of above 10% NaCl (Gunde-Cimerman et al. 2005b). To accommodate this particular type of adaptation, a new term of 'extremely halotolerant fungi' was introduced (Gostincar et al. 2010; Plemenitas et al. 2008). These were represented primarily by black yeast-like fungi and related species from the melanized filamentous genera *Cladosporium* and *Alternaria* (Plemenitas et al. 2005). The term halophilic is thus now used only for the few species of fungi that have an obligate requirement for NaCl in the growth medium, such as two species of the genus *Wallemia*, i.e. *W. muriae* and *W. ichthyophaga* (Zalar et al. 2005a).

1.4 The definition of halophily in fungi

Over the years, several definitions for the description of the diverse abilities of fungi to adapt to a wide range of salt concentrations have been proposed. For these fungi that prefer reduced aw, the most commonly used adjectives were xerophilic and osmophilic, as these fungi can grow at aw below 0.85; this corresponds to 17% NaCl or 50% glucose added to a growth medium (Gunde-Cimerman et al. 2005a). However, these fungi are not only able to grow at low aw; some also show preferences for certain chemical natures of the solutes that lower the aw (de Hoog et al. 2005; Gunde-Cimerman et al. 2005b).
Cimerman and Plemenitas 2006). Hence these osmotolerant/osmophilic fungi can live in environments that are rich in sugar, whereas those that are halotolerant/halophilic can live in environments that are rich in salt. In contrast to obligate halophilic archaea and bacteria, in the fungal kingdom, no evidence of obligate requirements for salt was reported until only recently. When fungi were found to constitute active communities in the hypersaline water of solar salterns, they were considered halophilic if they were regularly isolated from water at 17–32% NaCl, primarily on saline selective media, and if they were able to grow in vitro on 17% NaCl (Gunde-Cimerman et al. 2000). These fungi were then shown to sustain a range of different salt concentrations, right across the whole salinity range. Later, a few fungal species that show superior growth on media with NaCl as the controlling solute and necessarily require lowered aw for growth were first reported (Zalar et al. 2005a); hence the term halophilic fungi was challenged again. Thus, halotolerant and extreme halotolerant are now the terms used to describe fungi that can grow across a range of different salt concentrations, even from fresh water to NaCl saturation (Gunde-Cimerman and Plemenitas 2006), and the term halophily remains reserved for those that require salt for growth.

1.5 The main groups of halotolerant and halophilic fungi

Most halophilic and halotolerant fungi described to date from solar salterns have been identified either as known food-borne species with previously unrecognized natural niches, or as species that were not known to science, and consequently were newly described. Although, at present there are a total of 106 orders of fungi known (Kirk et al. 2001), tolerance for low aw is apparent in only 10 of these. In any of these particular orders, growth at decreased aw is in most cases limited to a few species or to a single genus of an order. However, in the orders Wallemiales (Basidiomycota), Capnodiales, Dothideales and Eurotiales (Ascomycota), halophily is expressed in several groups of the same order that are not the nearest phylogenetic neighbours to each other. When we compare the distributions of halotolerance/xerotolerance in the fungal kingdom with those of opportunistically pathogenic fungi, we find that they belong to the same orders of fungi. Pathogenicity and consistent opportunism are equally uncommon in the fungal kingdoms as consistent as xerotolerance. With only a few exceptions, species showing xerotolerance have no reported pathogenicities, and thus these two properties appear to be mutually exclusive at the level of individual species (de Hoog et al. 2005).
The mycobiota in hypersaline waters around the World comprise: meristematic melanized yeast-like fungi and different related species of the genus *Cladosporium* (Gunde-Cimerman et al. 2000; Butinar et al. 2005b; Zalar et al. 2007), non-melanized yeasts (Butinar et al. 2005a), filamentous genera *Wallemia, Scopulariopsis* and *Alternaria* (Gunde-Cimerman et al. 2005b; Zalar et al. 2005a) and different species of the genera *Aspergillus* and *Penicillium*, with their teleomorphic stages (*Eurotium, Emericella* and *Petromyces*) (Butinar et al. 2005c). Morphology and growth features of some of these representative fungi are shown in **Fig. 1.1(a-o)** (Zarj et al. 2012). Within Ascomycota, the main orders with halophilic and halotolerant representatives are *Capnodiales, Dothideales* and *Eurotiales*. Both orders *Capnodiales* and *Dothideales* have a xerotolerant tendency, as they contain a large number of extremotolerant species that can grow as epilithic or cryptoendolithic species at high or low temperatures (Wollenzien et al. 1995; Selbmann et al. 2005), and in Arctic glacier ice (Gunde-Cimerman et al. 2003) and hypersaline coastal salterns worldwide (Butinar et al. 2005b). These fungi are practically unknown in non-natural environments and they have not even been isolated from salted food products. The dominant halophilic species are represented by *Hortaea werneckii, Phaetotheca triangularis, Trimmatostroma salinum* and the halotolerant *Aureobasidium pullulans*.

Another important group of extremophilic fungi within *Capnodiales* are members of the genus *Cladosporium* (section *Cladosporium*). These fungi have a cosmopolitan distribution, as ubiquitous decomposers of dead plant material (David 1997), and therefore they are commonly found in indoor and outdoor air. Some species that have been described as new were consistently isolated from salterns and salty lakes worldwide, where they represented the most common and frequent fungal taxa (Gunde-Cimerman et al. 2000). Initially, these were considered as air-borne contaminants, but surprisingly, many of these *Cladosporium* isolates were later identified as *Cladosporium sphaerospermum*, which is known as one of the most common air-borne, cosmopolitan *Cladosporium* species (Park et al. 2004). Taxonomic analyses revealed a complex of eight new species that have either narrow or wide ecological distributions (Zalar et al. 2007).
Figure 1.1 Representatives of the saltern mycobiota. (a) Hortaea werneckii colonies; (b) Aureobasidium pullulans budding cells; (c) Hortaea werneckii budding cells; (d) Phaeotheca triangularis conidia and hyphae; (e) Trimmatastroma salinum meristematic clumps; (f) Eurotium amstelodami in culture; (g) Eurotium chevalieri anamorph; (h) Penicillium crustosum conidiophores; (i) Aspergillus niger conidiophores; (j) Emericellastellamaris ascospores; (k) Wallemia muriae in culture; (l, m) W. ichthyophaga meristematic clumps; (n) W. ichthyophaga colonies; (o) W. sebi colonies.
The other important order within Ascomycota is *Eurotiales*, in which xerotolerance and halotolerance are recurrent phenomena. These species are located in remote clades within the order, and thus it is likely that halotolerance is the main plesiomorphic trait shared by the entire order. The most important group of halotolerant species is represented by *Aspergillus niger*, *Aspergillus sydowii*, *Eurotium amstelodami* and *Penicillium chrysogenum*, which have been recovered from brine, baits and biofilms. *Aspergillus flavus*, *Aspergillus tubingensis*, *Aspergillus versicolor*, *Eurotium herbariorum*, *Penicillium citrinum* and *Penicillium steckii* are species that are detected at relatively high frequencies of occurrence, but at lower mean counts.

Species in *Emericella*, *A. versicolor* and *A. sydowii* have also been identified as part of fungal communities in hypersaline environments. Four species were assigned to the genus *Emericella*, including two new halotolerant species (*Emericella filifera* and *Emericella stellamaris*). Although many *Penicillium* species grow well on salted foods, only five species have been recognized as part of an indigenous fungal community. Two of these, *P. chrysogenum* and *P. brevicompactum*, appear pan-globally, while three have been defined as new species. Statistical analyses have indicated that the most consistent group of multisite species is represented by *A. niger*, *E. amstelodami* and *P. chrysogenum*. The Ascomycetous yeast order *Saccharomycetales* contains numerous species that are associated with sugary plant saps and exudates. Osmotolerant yeast taxa belong mainly to the families *Saccharomycetaceae* and *Metschnikowiaceae*. However, yeasts that have been isolated from hypersaline waters that have hemi-ascomycetous affinities only belong to the genera *Candida*, *Debaryomyces*, *Metschnikowia* and *Pichia*. Different species of the genus *Pichia* are frequent saline aquatic isolates, with *Pichia membranifaciens* being the most common (Soares et al. 1997). The species *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei* were isolated for the first time from the hypersaline water of the Dead Sea. Among these, only *C. parapsilosis* was known previously as food-borne halotolerant yeast, while the others were not known for their halotolerance (Butinar et al. 2005a).

The division Basidiomycota contains three orders with halophilic and halotolerant representatives. In the recently separated order *Trichonosporales* (Scorzetti et al.
2002), the yeast *Trichosporon mucoides* has been frequently isolated from hypersaline waters of salterns and from the Dead Sea. In Sporidiales, several *Rhodotorula* species have been isolated from salterns, including the repeatedly isolated *Rhodospiridium sphaerocarpum, Rhodospiridium babjevae* and *Rhodotorula laryngis* (Butinar et al. 2005a). The order Wallemiales was only recently introduced to accommodate the single genus *Wallemia*, a phylogenetic maverick in the Basidiomycota (Zalar et al. 2005a). Thus, until recently, this genus contained only the species *Wallemia sebi*. However, taxonomic analyses of isolates from sweet, salty and dried food (Samson et al. 2002) and from hypersaline evaporation ponds in the Mediterranean, Namibia, and the Dead Sea (Wasser et al. 2003) have resolved this genus into three species: *W. sebi*, *Wallemia muriae* and *Wallemia ichthyophaga* (Zalar et al. 2005a). It is remarkable that the entire genus *Wallemia*, and therefore the entire order *Wallemiales*, are either xerophilic/halophilic or xerotolerant.

### 1.6 Adaptive mechanisms of fungi at high salinity

#### 1.6.1 The compatible solute strategy

Exposure of fungi to high salinity represents two different environmental stimuli: one is osmotic stress, and the other is ionic stress. In non-adapted organisms, hyperosmotic stress triggers water efflux from the cell that results in the reduction of the turgor pressure and dehydration of the cytoplasm, thereby increasing the solute concentration in the cytosol. Ionic stress on the other hand causes ions (e.g. Na\(^+\)) to enter the cytoplasm, leading to a further increase in the ion concentration and the subsequent damage of the membrane systems, as well as of cellular proteins. Fungi adapt to life at low aw by accumulating the compatible solutes that do not interfere with the vital functions of their cellular proteins as their main survival strategy, to counteract changes in turgor pressure. They also need to maintain their intracellular concentrations of Na\(^+\) below toxic levels (Kogej et al. 2007). Most halophilic bacteria accumulate these compatible solutes, such as ectoine, glycine betaine, glutamate and proline, while the accumulation of glycerol is well known in the alga *Dunaliella salina* and in most fungi (Blomberg and Adler, 1992; Burg et al. 2007). In *D. salina*, the accumulated glycerol can represent up to 70% of their cell mass when they are growing in salt-saturated brine. Glycerol is also the main compatible solute in halotolerant *A. pullulans*, extremely halotolerant *H. werneckii*, and halophilic *W. ichthyophaga*, as well as in other halotolerant fungi. Due to the specific meristematic
growth in multicellular clumps of these fungi, estimation of their glycerol content is harder to obtain (Petrovic et al. 2002; Kogej et al. 2007; Lenassi et al. 2011). It has been shown that glycerol in combination with trehalose and other polyols can maintain positive turgor pressure at high salinity in moderately salt-tolerant yeast species, like Debaryomyces hansenii, Candida versatilis, Rhodotorula mucilaginosa or Pichia guilliermondii (Almagro et al. 2000). In the black yeast *H. werneckii*, this can be complemented at lower salinities by mycosporine-glutaminol-glucoside, a mycosporine that was primarily known as being involved in fungal sporulation and UV protection (Oren et al. 2007), and at higher salinities by other polyols, such as erythritol, arabitol and mannitol (Kogej et al. 2006). In *W. ichthyophaga* glycerol is complemented with minor amounts of arabitol and only trace amounts of mannitol (Kralj Kuncic et al. 2013).

### 1.6.2 Ion homeostasis

Physiological studies on *H. werneckii* and *W. ichthyophaga* showed that in contrast to the moderately salt-tolerant marine yeast *D. hansenii*, which accumulates more Na\(^+\) than *Saccharomyces cerevisiae* (Ramos 2005), they maintain very low intracellular K\(^+\) and Na\(^+\) levels even when grown in the presence of 4.5 M NaCl. This suggested that *H. werneckii* can effectively extrude and prevent the influx of Na\(^+\) ions. Indeed, identification of the two salt-responsive P-type (ENA-like) ATPases (Gorjan and Plemenitas 2006) and their salt-dependent activities is in line with this concept. Thus, in response to elevated NaCl concentration, *H. werneckii* can be classified as a Na\(^+\) extruder with an intricate compatible solute strategy, which has been revealed by genomic data (Lenassi et al. 2013). *Wallemia ichthyophaga*, on the other hand, lacks most cation transporters, present in the genome of *H. werneckii*, therefore its main strategy appears to be prevention of ion entry, as a result of extremely thickened cell walls (Zajc et al. 2013).

### 1.6.3 Cell wall structure and pigmentation

The cell wall of black yeasts is melanized. It has been shown that in *H. werneckii* this melanization depends on the salt concentration in the medium. When grown without NaCl, melanin granules are deposited in the outer layer of the cell wall, where they form a thin layer of melanin with separate larger granules. At optimal salinity, on the other hand, *H. werneckii* forms a dense shield-like layer of melanin granules on the
outer side of the cell wall, which can mechanically stabilize the cell wall, thus counterbalancing the increased turgor pressure (Kogej et al. 2006).

*Wallemia ichthyophaga* grows meristematically and forms characteristic compact multicellular clumps. When growing at higher salinity, a threefold thickening of the cell wall and an almost fourfold increase in the size of multicellular clumps occurs. The thick cell wall and compact cell clumps are thought to be important for the successful growth in extremely saline conditions (Kralj Kuncic et al. 2013).

1.6.4 Plasma membrane fluidity

As described above, the intracellular hyper-accumulation of glycerol as the main compatible solute at elevated concentrations of NaCl is an important physiological response of *H. werneckii*. However, glycerol has a high permeability coefficient for passage through lipid bilayers. As such, eukaryotic cells that use glycerol as a compatible solute need to combat the resulting loss of glycerol either by its accumulation using transport systems, which is energetically costly, or by changing the properties of their cell membrane, by increasing the sterol content or by reducing the membrane fluidity (Oren 1999).

In *H. werneckii*, the total sterol content of their plasma membrane remains largely unchanged with increased salinity (Turk et al. 2004), and thus their increased plasma membrane fluidity is the result of changes in the structure of the component phospholipids. It thus appears that by modifying the cell wall structure instead of directly lowering the membrane fluidity, *H. werneckii* can maintain high membrane fluidity even at high salinities. Thus, it was hypothesized that at optimal growth salinities, the melanized cell wall of *H. werneckii* also helps it to retain higher concentrations of glycerol in the cells, despite the highly fluid membrane (Gostincar et al. 2009).

1.7 Importance of the saltern mycobiota

Over the last few years, fungi thriving under conditions that are extreme from an anthropocentric point of view and which thus live at the so-called ecological periphery, have deserved and received increasing scientific attention. Some halotolerant and halophilic fungi have possible important biotechnological applications. These will arise from studies of their basic characteristics and adaptation
mechanisms, at the level of their secondary metabolites, cell membranes, intracellular and extracellular enzymes, genetic transfer systems and intracellular osmolytes, and especially of their compatible solutes, which have a wide range of applications because of their ability to stabilize proteins and nucleic acids (Arakawa and Timasheff 1985; Kurz 2008). The salt-tolerant black yeast *Aureobasidium pullulans* produces exopolymer pullulan that has a broad spectrum of use in the food and pharmaceutical industry (Leathers 2003; Singh and Saini 2008), and the extremely halotolerant black yeasts *Trimmatostroma salinum* and *H. werneckii* and halotolerant fungi *Aspergillus tubingensis* have been shown to produce extracellular hydrolytic enzymes that are active at high salt concentrations and that could therefore have important roles in different industries (Zalar et al. 2005b; Raol et al. 2014). *H. werneckii* also produces antibiotic compounds that remain to be commercially exploited (Brauers et al. 2001).

Indeed, many halophilic and halotolerant fungi synthesize specific bioactive metabolites under stress conditions, and particularly at increased salt concentrations, when higher hemolytic activities have been seen (Sevic et al. 2011). Progressive soil salinization represents a serious agricultural problem worldwide, as 10 million hectares of arable land is lost in this way annually. Genetic manipulations of crops that have increased salt tolerance have still not yielded satisfactory results. The use of halophilic and halotolerant fungal genetic sources might provide the desired improvement in the breeding of such crops in the future. The halotolerant *H. werneckii* has been shown to be a promising source of salt-tolerant transgenes for agriculture. In yeast, as well as in plants, Hal2 is a sodium and lithium-sensitive 3’-phosphoadenosine-5’-phosphatase, which is an important determinant for halotolerance (Glaser et al. 1993). Thus, over expression of novel isoenzymes or of Hal2-like proteins from *H. werneckii* can remarkably increase the halotolerance of *S. cerevisiae* (Vaupotic et al. 2007). Microorganisms and their metabolites can affect the salt production in the evaporating ponds of salterns, as they can physically affect the evaporation process and as their by-products can chemically modify or bind with the dissolved ions (Javor 2002). Moreover, the biological systems in the salterns can also “contaminate” the salt that is used for food preservation. It has been known for a long time that haloarchaea can be introduced into food via salt and can spoil heavily salted proteinaceous products (Norton and Grant 1988; Grant 2004). Recently, different fungi have been isolated from salt used for human consumption and for food salting (Butinar et al. 2011). As many fungi from salt and hypersaline water produce the
same mycotoxins as those found on the salted meat products, the salt is most probably the contamination source for some toxinogenic fungi (Andersen 1995; Larsen et al. 2001). Precautionary measures should therefore be considered seriously, such as heat treatment of salt prior to adding it to the food products, to kill the fungal conidia (Butinar et al. 2011).

1.8 Glycosyl hydrolases

Glycoside hydrolases or glycosidases (EC 3.2.1) are a widespread group of enzymes that hydrolyze the glycosidic bonds between two carbohydrates or between a carbohydrate and an aglycone moiety. A large multiplicity of these enzymes is a consequence of the extensive variety of their natural substrates: di-, oligo-, and polysaccharides. The existence of glycosidases, also known as carbohydrate, has been known for more than 100 years and they are the very first biological catalysts investigated. The study of molecular biology and biochemistry of these enzymes has resulted in the development of novel biotechnological tools of great importance and provided us with a broader understanding of biological structures and processes (Grosova et al. 2008; Elnashar and Yassin 2009). However, overall impact of enzymes on industry is still quite limited due to high cost of their production and purification. Potential advantages of enzymes like high specificity, improved activity under mild conditions, high turnover number and biodegradability are offset by the glaring disadvantage of intrinsic instability and hence difficulties in production, processing and storage leading to their high costs (Neri et al. 2008). Glycosidases are classified into two types, exo-glycosidases and endoglycosidases based on the nature of hydrolysis. Numerous glycosidases from different sources have been classified into 91 families which form different products and displayed a complete range of different bonds specificity (Hakamata et al. 2009).

O-Glycoside hydrolases are a widespread group of enzymes which hydrolyses α or β glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety (Vocadlo et al. 2001). These enzymes are also known as carbohydrate. Glycosyl hydrolases transfer the glycosyl bond to a water molecule while glycosyl transferases transfer this glycosyl bond to -OH group of another glycosyl residue. Enzymes which catalyze the hydrolysis of glycosidic linkages are widely distributed in nature and these include α and β galactosidases, cellulases,
invertase, maltase, α glucosidase, α and β amylases, pullulanase, chitinase, dextranase etc. (Neri et al. 2008; Maria et al. 2010).

1.9 β-Galactosidases

β-galactosidase (commonly known as lactase) is an enzyme (EC 3.2.1.23) that catalyzes the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactopyranose (Gasteiger et al. 2003). Conventionally, its main application has been in the hydrolysis of lactose in milk or derived products, particularly cheese whey. More recently, β-galactosidases with transgalactosylation activities (i.e. which can oligomerise galactosides) have been extensively exploited for the production of functional galactosylated products (Fig. 1.2).

Figure 1.2 Biotechnological applications of β-galactosidases: hydrolysis of lactose in milk and cheese whey and synthesis of GOS or other lactose/galactose derivatives by transgalactosylation reactions. The gray boxes show examples of microbial enzyme sources for each application. (c) Cold-active enzymes; (t) thermophilic enzyme (Oliveira et al. 2011).
Many organisms naturally synthesize β-galactosidase, including microorganisms, plant and animal cells (Husain 2010; Panesar et al. 2006). Traditionally, the β-galactosidases most widely used in industry were obtained from *Aspergillus* spp. and *Kluyveromyces* spp. (Husain, 2010; Panesar et al., 2006; Siso, 1996; Zadow, 1984), because these could be readily obtained with acceptable productivities and yields from cultivations of these microorganisms. Additionally, products obtained from these organisms are generally recognized as safe (GRAS status) for human consumption, which is critical for food related applications. In *Aspergillus* spp. β-galactosidase is secreted to the extracellular medium. These fungal enzymes have a pH optimum in the acidic range (2.5–5.4) and a high temperature optimum that allows their use at temperatures up to 50°C (Panesar et al. 2006; Zadow 1984). Their main application is in the hydrolysis of acid whey, which is derived from the production of fresh or soft cheeses (Yang and Silva, 1995). Conversely, in *Kluyveromyces* spp. the β-galactosidase is intracellular; lactose is first transported to the interior of the yeast cell by a permease and then hydrolyzed intracellularly to glucose and galactose, which follow the glycolytic pathway or the Leloir pathway, respectively (Domingues et al. 2010). The yeast enzyme has a near neutral optimum pH (6.0–7.0) and therefore has a broader range of applications, particularly in the hydrolysis of milk and sweet whey (derived from hard cheese manufacturing) (Panesar et al. 2006; Yang and Silva 1995; Zadow 1984). Because of its intracellular nature, the enzyme needs to be extracted from the yeast cells by disrupting or permeabilizing the cells using chemical and/or mechanical treatments. Panesar et al. (2006) compiled a list of β-galactosidase commercial preparations. Yeast sources are *Kluyveromyces lactis* and *Kluyveromyces marxianus* (species that now includes former species *Kluyveromyces fragilis* and *Saccharomyces fragilis* as well as its anamorph Candida pseudotropicalis; Lachance 1998), while fungal sources include *Aspergillus niger* and *Aspergillus oryzae*. The β-galactosidase from *Escherichia coli* is the most extensively studied but its industrial use is hampered by the fact that it is not considered safe for food applications. Nevertheless, it is commercially available for analytical purposes (Panesar et al. 2006; Siso 1996). Finally, a preparation obtained from *Bacillus* sp. is also commercialized (Panesar et al. 2006). Lactic acid bacteria (include a diverse group of lactococci, streptococci and lactobacilli) and bifidobacterium, which are recognized as safe organisms, have been regarded as good sources of β-galactosidases, especially for functional food applications (Husain 2010). More recently, there has been growing
interest in a considerable number of β-galactosidases from other sources that present
diverse properties of biotechnological interest (Panesar et al. 2006). Particular
attention has been rewarded to thermotolerant or cold-active enzymes from yeast and
bacterial sources (Husain 2010; Panesar et al. 2006; Park and Oh 2010). The X-ray
crystal structures of several microbial β-galactosidases have been unraveled, although
none of the enzymes with solved structures is known to be used in food processing
(Gosling et al. 2010). Nowadays, recombinant DNA technology can be used to
express and optimize the production of interesting β-galactosidases from the most
diverse sources in microbial hosts that are recognized for their highly efficient
heterologous protein production. This possibility greatly expands the range of
potential applications for β-galactosidases and their economically effective utilization
in industrial processes. Modern molecular biology tools combined with bioprocess
engineering strategies can be used to optimize protein production, resulting in
technically and economically effective enzyme production systems. Besides the wide
ranging properties offered by natural sources, new features such as reduced product
inhibition (Park and Oh 2010), higher product yields (Gosling et al. 2010) or secretion
signals (Becerra et al. 2001) may be built into specific β-galactosidases using state-of-the-art protein engineering tools.

Numerous methods have been identified for the determination of β-galactosidase
activity. However, its activity can be determined readily by lactose as a substrate and
measuring the resulting glucose or galactose. Because of transfer reaction, it is
advisable to determine both appearance of glucose and disappearance of lactose.
Alternatively, O-nitrophenyl-β-D-galactopyranoside (ONPG) can be used as a
substrate and progress of the reaction can be followed by estimating the formation of
a chromogen, O-nitrophenol. In fact, ONPG is considered as one of the best substrate
for the measurement of β galactosidase activity. The active site of enzyme has one
thiol and one imidazole group and the reaction corresponds to SN2 like displacement
mechanism (Mateo et al. 2001).

1.9 β-Galactosidases from extremophiles

Enzymes from thermophilic microorganisms are particularly attractive for their
thermostability and resistance to organic solvents thereby giving favorable
equilibrium for transglycosylation reactions. A number of thermostable β-
galactosidases have been isolated previously from both mesophilic eubacteria and archaeabacteria. They were characterized and employed to hydrolyze lactose and in producing GOS (Garcia-Garibay et al. 2000; Akiyama et al. 2001). However, most thermostable enzymes are synthesized at very low levels by thermophilic bacteria or archaeabacteria and are therefore cumbersome to purify. Thus, their large scale production was achieved at industrial scale by producing them in mesophilic hosts using recombinant techniques (Demirjian et al. 2001). The efficiency of recombinant thermostable β-galactosidases obtained from Thermus sp., Pyrococcus furiosus, Thermotoga maritima, Sulfolobus solfataricus and Geobacillus stearothermophilus had been reported in pursuit of GOS production at high temperatures. They exhibited several advantages over native enzymes including their ease of purification, large scale production and improvements in their activity (Bruins et al. 2001; Park et al. 2008; Placier et al. 2009). Thermostable β-galactosidase gene bgaB from B. stearothermophilus was cloned and expressed in B. subtilis WB600 by Chen et al. (2009). The recombinant enzyme exhibited molecular mass and isoelectric point of 70 kDa and 5.1, respectively, while pH and temperature optimum for this enzyme were at pH 7.0 and 70°C, respectively. Kinetics of thermal inactivation and half-life for recombinant thermostable β-galactosidase at 65 and 70°C were 50 and 9 h, respectively, and K_m and V_max values were 2.96 mM and 6.62 µM/min/mg. The results suggested that this recombinant thermostable enzyme may be suitable for both hydrolysis of lactose and production of GOS in milk processing.

Xia et al. (2010) reported the extracellular secretion of a cytoplasmic thermostable β-galactosidase from Geobacillus stearothermophilus IAM11001 in B. subtilis. Defined and rich culture media were used for recombinant enzyme production and the extracellular target enzymatic activity reached about 44% of the total enzymatic activity synthesized at 18 h of cultivation in LB medium. This study revealed that co-expression of the B. subtilis proteins, TatAd and TatCd, were indispensable for the secretion of target enzyme.

A novel heterodimeric β-galactosidase was obtained from Lactobacillus pentosus KUB-ST10-1 using ammonium sulfate fractionation followed by hydrophobic interaction and affinity chromatography. The electrophoretically homogenous enzyme showed molecular mass of 105 kDa, temperature optimum at 65°C and had a specific
activity of 97 U (ONPG)/mg protein. The $K_m$, $k_{cat}$ and $k_{cat}/K_m$ values for lactose and ONPG were 38 mM, 20/s, 530/M/s and 1.67 mM, 540/s, 325,000/M/s, respectively. A maximum yield of 31% GOS of total sugars was obtained at 78% lactose conversion by *Lactobacillus pentosus* β-galactosidase. The enzyme showed a strong preference for the formation of β-(1 → 3) and β-(1 → 6) linkages, and the main transgalactosylation products identified were the disaccharides β-D-Galp-(1 → 6)-D-Glc, β-D-Galp-(1 → 3)-D-Glc, β-D-Galp-(1 → 6)-D-Gal, β-D-Galp-(1 → 3)-D-Gal, and the trisaccharides β-D-Galp-(1 → 3)-D-Lac, β-D-Galp-(1 → 6)-D-Lac (Maischberger et al. 2010).

The biotechnological interest of mesophilic enzymes was motivated by their ability to function under conditions that normally denature thermophilic enzymes. *K. lactis* β-galactosidase lac4 gene was expressed in *E. coli* as soluble His-tagged recombinant enzyme under optimized culture conditions. The expressed protein was multimeric with a subunit molecular mass of 118 kDa. The purified enzyme required Mn$^{2+}$ ions for activity and was inactivated irreversibly by imidazole above 50 mM. The activity was optimal at 37°C and 40°C for ONPG and lactose, respectively. It exhibited $K_m$ and $V_{max}$ values of 1.5 mM and 560 µM/min/mg, and 20 mM and 570 µM/min/mg with ONPG and lactose as substrate, respectively (Kim et al. 2003). The utilization of such *K. lactis* β-galactosidase had increased lactose hydrolysis and prevented the formation of non-enzymatic browning products which are generally formed at lower temperatures. A flocculent *S. cerevisiae* strain secreting *A. niger* β-galactosidase activity was constructed by transforming *S. cerevisiae*NCYC869-A3 strain with plasmid pVK1 harboring *A. niger* β-galactosidase gene, LacA (Domingues et al. 2002). It exhibited higher level of extracellular β-galactosidase activity and allowed enzyme production with high productivity in continuous fermentation systems with downstream processing. Moreover, it provided an alternative method to produce fungal β-galactosidases since the enzyme was produced in pure form. In another study, cloning and expression of genes encoding heterodimeric β-galactosidase from *L. reuteri* L103, *Lactobacillus acidophilus* R22, *Lactobacillus plantarum* WCFS1 and *Lactobacillus sakei* Lb790 was investigated. These enzymes consist of two subunits of approximately 73 and 35 kDa which are encoded by two overlapping genes, lacL and lacM, respectively. The presented results lead the way to efficient overproduction of β-galactosidase in a food grade expression system which is of high interest for
applications in food industry (Halbmayr et al. 2008). In another study, recombinant β-galactosidase was produced from human isolate *Bifidobacterium breve* B24 and characterized for the glycoside transferase (GT) and glycoside hydrolase (GH) activities on lactose (Yi et al. 2011). The recombinant enzyme shown by activity staining and gel filtration chromatography was composed of a homodimer of 75 kDa with a total molecular mass of 150 kDa. The $K_m$ value for lactose (95.58 mM) was 52.5 fold higher than corresponding $K_m$ values for the synthetic substrate ONPG (1.82 mM). It showed its pH and temperature optimum at pH 7.0 and 45°C. About 97% of lactose in milk was hydrolyzed by this enzyme (50 units) at 45°C for 5 h to produce 46.30% of glucose, 46.60% of galactose and 7.10% of GOS. The results suggest that this recombinant β-galactosidase may be suitable for both the hydrolysis and synthesis of GOS in milk and lactose processing.

The production of cold-stable β-D-galactosidases and microorganisms that resourcefully ferment lactose is of high biotechnological interest, particularly for removal of lactose in milk and dairy products and cheese whey bioremediation at low temperatures. Recently, a gene encoding β-D-galactosidase was isolated from the genomic library of Antarctic bacterium *Arthrobacter* sp. 32c. Although, the highest activity of this purified enzyme was found at 50°C, 60% of the highest activity of this enzyme was determined at 25°C and 15% of the highest activity was detected at 0°C. The cold stable properties of *Arthrobacter* sp. 32c β-D-galactosidase could be useful for commercial/industrial conversion of lactose into galactose and glucose in milk products (Hildebrandt et al. 2009). The drive for cost cutting efficiency in heating and cooling steps of biotechnological processes in improving the recovery of the products of enzymatic reactions has led to an increasing interest in using enzymes isolated from psychrophilic microorganisms. Psychrophilic enzymes demonstrated huge biotechnological potential in detergent formulations (e.g. proteinases, lipases, amylases and cellulases), dairy industry (e.g. β-galactosidase), environmental biosensors (e.g. dehydrogenases), bioremediation (e.g. oxidases) and for biotransformations (e.g. methylases and aminotransferases). They have developed adaptive mechanisms in bringing efficient enzymatic reactions even at 0°C but the major drawback of these cold active enzymes is their thermolability, i.e. they are inactivated at moderate temperatures at which mesophiles and thermophiles remained functional (Layer and Fischer 2006; Splechtna et al. 2007a). Psychrophilic β-
galactosidases are potentially useful (i) for fast lactose digestion at low temperatures to produce lactose-free milk products, (ii) to avoid lactose crystallization in dairy products, and (iii) to degrade them as their bulk pollutants in dairy sewage thereby reducing environmental pollution. Thus, an attention was drawn in recent years to study cold active β-galactosidases from different sources like *Arthrobacter psychrolactophilus*, *Bacillus subtilis* KL88, *Carnobacterium piscicola* BA, *Planococcus* and *Pseudoalteromonas haloplanktis*, *Pseudoalteromonas* sp. 22b, etc. The optimal properties and kinetic parameters of β-galactosidases from these sources were investigated and employed for the production of GOS and lactose hydrolysis. It was observed that these enzymes exhibited greater activity in temperature range from 0 to 20°C (Sheridan and Brenchley 2000; Turkiewicz et al. 2003).

A gene encoding a β-galactosidase in *Planococcus* isolate was cloned in an *E. coli* host. Sequence analysis of this gene placed it in glycosidase family 42 most closely related to an enzyme from *Bacillus circulans*. Even though an increasing number of family 42 glycosidase sequences are appearing in databases, little information about the biochemical features of these enzymes is available. Therefore, Sheridan and Brenchley (2000) purified and characterized such family 42 β-galactosidase enzyme. The purified enzyme did not appear to have any metal requirement, had an optimum pH of 6.5 and an optimum temperature of activity at 42°C, and was irreversibly inactivated within 10 min when it was incubated at 55°C. The enzyme had an apparent $K_m$ of 4.9 µmol of O-nitrophenyl-β-D-galactopyranoside, and the $V_{max}$ was 467 µmol of O-nitrophenol produced/min/mg of protein at 39°C. Of special interest was the finding that the enzyme remained active at high salt concentrations, which makes it a possible reporter enzyme for halotolerant and halophilic organisms. A novel GH family 42 cold-active β-galactosidase identified from the psychrotrophic and halotolerant *Planococcus* sp. L4 (BgaP) was crystallized and a complete data set was collected from a single frozen crystal on an in-house X-ray source. The crystal diffracted to 2.8 Å resolution and belonged to space group $P1$, with unit-cell parameters $a = 104.29$, $b = 118.12$, $c = 121.12$ Å, $\alpha = 62.66$, $\beta = 69.48$, $\gamma = 70.74°$. A likely Matthews coefficient of 2.58 Å$^3$ Da$^{-1}$ and solvent content of 52.32% suggested the presence of six protein subunits in the asymmetric unit (Zhang et al. 2011).
1.10 Application of β-galactosidases

1.10.1 Milk lactose hydrolysis

Lactose is a disaccharide found in milk. It is formed by glucose and galactose. In humans, lactose intolerance or lactose malabsorption is a common problem. In fact, it is estimated that over 70% of the world's adult population have problems in digesting lactose, resulting from absent or reduced β-galactosidase activity in the small intestine. Indigested lactose therefore passes into the large intestine where it is fermented by colonic microflora causing symptoms such as abdominal pain, gas, nausea and diarrhea (Adam et al. 2004; Husain 2010). The extent of these symptoms is variable and indeed most individuals can tolerate a moderate amount of lactose in their diet (Lifran et al. 2000). Nevertheless, there is a considerable market for lactose-free milk and dairy products, which can be obtained by enzymatic hydrolysis using β-galactosidases. The solubility and sweetness of lactose is low compared to other sugars, including glucose, galactose, fructose and sucrose (Ganzle et al. 2008; Zadow 1984). Therefore, lactose hydrolysis diminishes precipitation problems and enhances the sweetening power, thus expanding the food applications of lactose solutions, for instance in the confectionary and ice-cream industries replacing sucrose or starch syrups (Siso 1996; Zadow 1984). The *Pseudoalteromonas* cold-adapted β-galactosidase enzyme, produced and purified from *E. coli* cells (Cieslinski et al. 2005), was applied in the hydrolysis of lactose in a milk assay where 90% of milk lactose was hydrolyzed for 6 h at 30°C. The advantage of this recombinant enzyme is that it can be used at lower temperatures hydrolyzing 90% of milk lactose in 28 h at 15°C. Recombinant cold-adapted *L. acidophilus* β-galactosidase produced and purified also from the *E. coli* expression system, hydrolyzed 73% of lactose in milk in 30 h at 10°C (Pan et al. 2010). The recombinant acidotolerant β-galactosidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842, produced from the *E. coli* expression system, has been explored for lactose hydrolysis (Rhimi et al. 2009). Biochemical characterization of the purified protein revealed optimal activity in a wide temperature range (35 to 50°C) and at pH values comprised between 5.0 and 5.5. Interestingly, primary structure alignment suggested that only acidotolerant enzymes including that of *L. bulgaricus* have glutamic acid at position 491. Making use of the *E. coli* recombinant system and site-directed mutagenesis a modified version of the recombinant enzyme was produced: the glutamic acid at position 491 was replaced by
alanine (E491A). The biochemical characterization revealed that the E491A mutation did not affect the kinetic parameters and temperature profile of the protein but the pH optima profile was undoubtedly shifted from 5.0–5.5 for the wild-type enzyme to 6.5–7.0 for the modified enzyme. When comparing lactose hydrolysis rates both in milk and whey, the original version was more efficient for whey hydrolysis (88% bioconversion at 42°C after 10 h with 1 mg/mL β-galactosidase against 60% for the mutated version in the same conditions) while the mutated version was more efficient in milk lactose hydrolysis (89% bioconversion at 42°C after 10 h with 1 mg/mL β-galactosidase against 80% for the original version).

1.10.2 Whey bioremediation

Besides milk, the main source of lactose is cheese whey, which is a primary byproduct of cheese manufacturing. About 9 L of whey stream are generated during the production of 1 kg of cheese, amounting to over 160 mt of whey produced worldwide each year (Guimaraes et al. 2010). Whey’s organic load is high (biochemical oxygen demand of 30–50 g/L and chemical oxygen demand of 60–80 g/L), mainly because of the lactose content, which together with the high volumes to which it is generated makes cheese whey a quite concerning environmental issue, and solutions for its valorization are strongly required (Guimaraes et al. 2010). In this regard, lactose hydrolysis by β-galactosidases again plays an important part by broadening whey’s applications. Particularly, whey fermentations can be expanded beyond the somewhat limited capacities of lactose consuming microorganisms, to the use of lactose-hydrolyzed whey as feedstock for the production of value added molecules or bulk commodities by lactose-negative microbes. For instance, *Saccharomyces cerevisiae* wild strains (lactose-negative) can be used to produce ethanol from hydrolyzed whey, although catabolite repression-resistant mutants must be applied in order to avoid glucose–galactose diaux (Bailey et al. 1982; Terrell et al. 1984). In the case of low-price bulk products, like ethanol, the cost of β-galactosidase for hydrolysis is often the bottleneck limiting the economical viability of the fermentation process.

Typically, fungal β-galactosidases have acidic pH-optima and thus they are most effective for the hydrolysis of lactose present in acidic products such as whey. The industrially relevant β-galactosidase from *A. niger* has been produced in flocculent *S.*
cerevisiae cells and applied in whey hydrolysis. In a preliminary assay, the produced recombinant enzyme was ultrafiltrated and applied directly for whey hydrolysis (0.5 mL enzyme sample to 10 mL of whey-permeate solution) achieving 90% lactose bioconversion in 20 h at 30°C. This recombinant system could be of interest for the dairy industry as the recombinant β-galactosidase was secreted from flocculent S. cerevisiae cells at the same time as ethanol was produced while using whey permeate as substrate (Domingues et al. 2003). Moreover, several investigators demonstrated the degradation of whey lactose by β-galactosidase enzyme for manufacturing GOS as well as useful sweet syrup, that can be used in dairy, confectionery, baking and soft drinks industries (Foda and Lopez-Leiva 2000; Novalin et al. 2005; Grosova et al. 2008).

1.10.3 Galacto-oligosaccharide (GOS) production by β-galactosidase

In recent years, much investigation has been carried out in the field of probiotics and prebiotics as functional foods. Galacto-oligosaccharides (GOS) are used as non-digestible, carbohydrate-based food ingredients in human and animal nutrition as prebiotic. Much of the research is focused upon microorganisms that produce β-galactosidases with improved quality for production of galacto-oligosaccharides (Table 1.1). The synthesis of GOS with a high yield of 55% from 275 g/L lactose at 50°C for 12 h was performed using transglycosylating β-galactosidase producing Enterobacter cloacae. The enzyme showed an extensive range of acceptor specificity for transglycosylation and catalyzed glycosyl transfer from ONPG to various chemicals resulting in novel saccharide yields from 0.8% to 23.5% (Lu et al. 2009).

Wu et al. (2006) have screened a mutant strain of Bacillus indica L3 for the production of heteropolysaccharide-7 (PS-7). The highest amount of PS-7 formed by the mutant was 2.88 g/L with a viscosity of 4530 cP in lactose-based MSM medium. The PS-7 manufacture was enhanced by the addition of 4 g/L glucose into lactose-based MSM medium, reaching 5.52 g/L with a viscosity of 39531 cP. PS-7 of 6.18 g/L with a viscosity of 45772 cP was produced from the mutant grown in whey medium. The PS-7 production from the mutant reached 7.04 g/L when 4 g/L glucose was added to the whey medium. Li et al. (2009) have cloned a novel gene encoding transglycosylating β-galactosidase (BGase) from Penicillium expansum F3 and subsequently expressed on the cell surface of S. cerevisiae EBY-100 by galactose
induction. The B Gase-anchored yeast could directly utilize lactose to produce GOS, as well as the by-products glucose and a small quantity of galactose.

**Table 1.1** Galacto-oligosaccharides production by β-galactosidase enzyme.

<table>
<thead>
<tr>
<th>β-galactosidase producers</th>
<th>Galacto-oligosaccharides (GOS) and by-products</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>GOS, glucose, galactose</td>
<td>Lu et al. 2009</td>
</tr>
<tr>
<td><em>Beijerinckia indica</em> L3</td>
<td>Heteropolysaccharide-7</td>
<td>Wu et al. 2006</td>
</tr>
<tr>
<td><em>Penicillium expansum</em> F3</td>
<td>GOS, glucose, galactose</td>
<td>Li et al. 2008; Li et al. 2009</td>
</tr>
<tr>
<td><em>Lactobacillus</em> sp.</td>
<td>s-D-Galp-(1→6)-D-Glc, s-D-Galp-(1→6)-D-Lac, s-D-Galp-(1→6)-D-Gal, β-D-Galp-(1→3)-D-Lac, β-D-Galp-(1→3)-D-Gal</td>
<td>Splechtna et al. 2007a</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em> BCRC 15708</td>
<td>tri-, tetra-saccharides, lactose, galactose</td>
<td>Hsu et al. 2007</td>
</tr>
<tr>
<td><em>Geobacillus stearothermophilus</em> KVE39</td>
<td>Lactosucrose, s-D galactopyranosyl-(1→3)-s-Dgalactopyranosyl-(1→4)-D-glucopyranoside (3'-galactosyl-lactose)</td>
<td>Placier et al. 2009</td>
</tr>
<tr>
<td><em>Lactobacillus reuteri</em></td>
<td>s-D-Galp-(1→6)-D-Glc, s-D-Galp-(1→6)-D-Gal, β-D-Galp-(1→3)-D-Gal, β-D-Galp-(1→6)-D-Lac, β-D-Galp-(1→3)-D-Lac</td>
<td>Splechtna et al. 2007b</td>
</tr>
<tr>
<td><em>Lactobacillus bulgaricus</em></td>
<td>Sialyllactose, 14 other oligosaccharides</td>
<td>Barile et al. 2009</td>
</tr>
<tr>
<td><em>Lactobacillus delbrueckii subsp. bulgaricus</em></td>
<td>Galactose, lactic acid, acetic acid, ethanol</td>
<td>Shene and Bravo 2007</td>
</tr>
<tr>
<td><em>Bifidobacterium infantis</em></td>
<td>GOS, lactose, monosaccharides</td>
<td>Jung and Lee 2007</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>s-D-Galp-(1→6)-D-Lac, s-D-Galp-(1→6)-D-Glc</td>
<td>Iqbal et al. 2010</td>
</tr>
<tr>
<td><em>Bacillus circulans</em></td>
<td>N-acetyllactosamine, N-acetylglucosamine</td>
<td>Kaftzik et al. 2002</td>
</tr>
</tbody>
</table>
Li et al. (2009) have cloned a novel gene encoding transglycosylating \( \beta \)-galactosidase (BGase) from \( P. \) expansum F3 and subsequently expressed on the cell surface of \( S. \) cerevisiae EBY-100 by galactose induction. The BGase-anchored yeast could directly utilize lactose to produce GOS, as well as the by-products glucose and a small quantity of galactose. The glucose was consumed by the yeast, and the galactose was used for enzyme expression, thus to a great extent facilitating GOS synthesis. The GOS yield reached 43.64\% when the recombinant yeast was cultivated in yeast nitrogen base-casamino acids medium containing 100 g/liter initial lactose at 25\(^\circ\)C for 5 days. In 2007, the process by which GOS formed from lactose was optimized using \( \beta \)-galactosidase from \( Lactobacillus \) sp. It proved to be beneficial to directly apply the crude cell-free enzyme extract for the conversion, since similar GOS yields and composition were obtained as when using the pure enzyme preparation, but expensive purification could be avoided (Splechtna et al. 2007a). Hsu et al. (2007) have studied the production of GOS by transgalactosylation using \( \beta \)-galactosidase of \( B. \) longum BCRC 15708. Two types of GOSs, tri- and tetra-saccharides were formed after \( \beta \)-galactosidase action on 40\% lactose. Tri-saccharides were the major type of GOS formed. In general, an increase of the initial lactose concentration in the reaction mixture resulted in a higher GOS production. A highest yield of 32.5\% (w/w) GOSs could be achieved from 40\% lactose solution at 45\(^\circ\)C, pH 6.8, when the lactose conversion was 59.4\%. The corresponding productivity of GOSs was 13.0 g/L\(^{-1}\) h\(^{-1}\).

A mutagenesis advance was applied to the \( \beta \)-galactosidase (BgaB) of \( G. \) stearothermophilus KVE39 to improve its enzymatic transglycosylation of lactose into oligosaccharides. A straightforward screening strategy based on the reduction of the hydrolysis of a potential transglycosylation product (lactosucrose), provided mutant enzymes possessing enhanced synthetic properties for the auto condensation product from nitrophenyl-galactoside and GOS from lactose. Alteration of one arginine residue to lysine (R109K) augmented the oligosaccharide yield compared to that of the wild-type BgaB. Consequently, Site-saturation mutagenesis at this position demonstrated that valine and tryptophan further enlarged the transglycosylation performance of BgaB. During the transglycosylation reaction with lactose of the evolved \( s \)-galactosidases, a key tri-saccharide \([s-D\text{-galactopyranosyl-(1→3)}-s-D\text{-galactopyranosyl-(1→4)}-D\text{-glucopyranoside} (3'-galactosyl-lactose)]\) was formed (Placier et al. 2009). Splechtna et al. (2007b) have investigated GOS formation from
lactose in discontinuous and constant modes of conversion using β-galactosidase (s-gal) from *L. reuteri*. In the continuous stirred tank reactor, s-gal from *L. reuteri* showed up to 2-fold higher specificity toward the formation of s- (1→6)-linked GOS, with s-D-Galp-(1→6)-D-Glc and s-D-Galp-(1→6)-D-Gal being the main GOS components formed under these conditions.

Cheng et al. (2006) have used *Bacillus* sp. for the production of low-content GOS from lactose that resulted in the highest yield of tri-saccharides and tetra-saccharides. GOS production was improved by mixing β-galactosidase with glucose oxidase. The low content GOS syrups, produced by β-galactosidase was subjected to the fermentation by *K. marxianus*, whereby glucose, galactose, lactose and other disaccharides were at a low level, resulting in up to 97% and 98% on a dry weight basis of high-content GOS with the yields of 31% and 32%, respectively.

In 2008, a procedure was proposed for producing non-monosaccharide and high-purity GOS from lactose by *P. expansum* F3 β-galactosidase immobilized in calcium alginate. A purity of 28.7% (w/w) GOS was obtained from 380 g/L lactose solution at pH 5.4 and 50°C. The immobilized enzyme was used for repeated GOS synthesis and showed good working stability. Digestible sugars in the GOS were dwindling after fermentation with *S. cerevisiae* L1 or *K. lactis* L3 entrapped in the calcium alginate. Purity greater than 37% with yields greater than 27% of non monosaccharide GOS were obtained by *S. cerevisiae* L1 for 19 batches and purity greater than 97% with a yield greater than 20% of high-purity GOS was produced using *K. lactis* L3 for two batches (Li et al. 2008). Layer and Fischer (2006) have performed *in vitro* glycosylation of peptides and proteins by trans-galactosylation of protected serine and threonine by β-D-galactosidase. The trans-mono-galactosylation of serine with a surplus of lactose produced 28% of *N*-tertbutoxycarbonyl- 1-O-β-D-galactopyranosyl-L-serinemethylester. The same transformational conditions, when applied to threonine, produced *N*-tertbutoxycarbonyl-1-O-β-D-galactopyranosyl-L-threoninemethylester in lower quantities. Mono-galactosylated serine and threonine are further galactosylated in the examined experimental setup to yield bi-galactosylated products also, especially at 50°C with completely dissolved lactose.

In 2009, oligosaccharides in bovine cheese whey permeate was characterized by a combination of nanoelectrospray Fourier transform ion cyclotron resonance mass
spectrometry and matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry. In adding together to sialyllactose (the most abundant oligosaccharide in bovine colostrum), 14 other oligosaccharides were identified, half of which have the same composition of human milk oligosaccharides. These oligosaccharides could potentially be used as additives in infant formula and products for the pharmaceutical industry (Barile et al. 2009). Splechna et al. (2006) have formed prebiotic GOS from lactose using the β-D-galactosidases (β -Gals) of L. reuteri L103 and L461. Greatest GOS yields were 38% when using an initial lactose concentration of 205 g/L and at 80% lactose conversion. Disaccharides other than lactose and trisaccharides made up the enormous majority of GOS formed. The main products were identified as β-D-Galp-(1→6)-D-Glc (allolactose), β-D-Galp-(1→6)-D-Gal, β-D-Galp-(1→3)-D-Gal, β-D-Galp-(1→6)-Lac, and β-D-Galp-(1→3)-Lac. There were no key products with β1→4 linkages. Both intermolecular and intramolecular transgalactosylation were observed. D-Galactose proved to be a very competent galactosyl acceptor; thus, a relatively large amount of galactobioses was formed.

Commercially available GOS, in powder or liquid form, are mixtures of several types of GOS (more than 50%), lactose (20%), glucose (20%) and a small amount of galactose. GOS are quite stable during long-term storage at room temperature even in acidic conditions. Therefore, GOS can be applied without decomposition in variety of foods. Major companies dealing with oligosaccharides production (including GOS) are in Japan (Urgell and Orleans 2001). Recently, there is also increasing trend of GOS production in Europe. Besides lactulose and soybean oligosaccharides, all oligosaccharides are prepared by transglycosylation from mono- and disaccharides or by controlled hydrolysis of polysaccharides (Rastall and Maitin 2002).

1.10.4 Biosensors and fusion proteins

Biosensors are important tools in a variety of fields including immunoassays, toxicology analysis, forensics, drug screening, gene expression analysis, gene identification, agro diagnostics and pharmacogenetics (Huang et al. 2002). They possess advantages such as reliability, sensitivity, accuracy, ease of handling and low cost compared with conventional detection methods which rendered them ideal for biomedical applications and environmental analysis (Kumbhat et al. 2007). It
combines the selectivity of the molecular recognition of biomolecules and sensitivity of signal transducers (Cooper 2002; Patel 2002). A wide variety of biosensors have been developed using different bio-recognition elements such as enzymes, antibodies, peptides, whole cell and nucleic acids.

Recombinant β-galactosidase (normally, *E. coli* β-galactosidase) has been extensively used as a fusion protein in different fields due to its easy detection (Caubin et al. 2001; Huang et al. 2009). One of the most relevant applications is in biosensors. The expression and production of recombinant β-galactosidases have been determinants for the progress of this area. Several works can be found in literature concerning the use of recombinant β-galactosidases in biosensors construction and functionalization. One example is the use of a chimera protein made by the fusion of β-galactosidase from *E. coli* and the choline-binding domain of the (acetylmuramoyl)-L-alanine amidase from *Streptococcus pneumoniae* in the development of a specific immobilization method of proteins onto a gold electrode (Madoz et al. 1997). Also worth of noting is the application of recombinant fusion β-galactosidases in the development of allosteric biosensors. These specific biosensors allow detection of antibodies against different viruses by accommodating peptide sequences from surface viral proteins, acting as antibody receptors, into permissive sites of allosterically responsive recombinant β-galactosidases. Among the advantages of such biosensors as diagnostic tools is the homogeneous nature of the assay, the short time required for the enzymatic reaction and antibody detection, and the potential for handling large number of samples and for automatic processing, as shown for human immunodeficiency virus (Ferraz et al. 2006; Ferraz et al. 2008). More recently, allosteric biosensors for detection of the animal foot-and-mouth disease virus (FMDV) have been developed, allowing the differentiation between sera FMDV-infected animals and those of naïve and conventionally vaccinated ones (Sanchez-Aparicio et al. 2009).

As new enzymes are discovered and characterized by recombinant DNA technology, new biosensors are also described in literature. One recent example is a β-galactosidase having a modular structure with a choline-binding domain that has been identified for the first time in *Streptococcus mitis*. The recombinant enzyme, produced from the *E. coli* expression system, presented negligible product inhibition by higher
concentrations of glucose, weak inhibition by galactose in comparison with other β-galactosidases, high substrate specificity towards lactose and superior stability at room temperature. A promising integrated amperometric lactose biosensor is under development for application in dairy industry which contains the recombinant *S. mitis* β-galactosidase, glucose oxidase, peroxidase, and tetrathiafulvalene co-immobilized by cross-linking with glutaraldehyde in a gold disk electrode modified with a 3-mercaptopropionic acid self-assembled monolayer (Campuzano et al. 2009).

1.11 Objectives of the study

β-galactosidase hydrolyzes D-galactosyl residues from polymers, oligosaccharides and secondary metabolites. Biotechnological application of this enzyme involves the removal of lactose from milk and whey for lactose intolerant people and production of galactosylated product. It is widely used in food industry to improve sweetness, solubility, flavor and digestibility of the dairy products. However, presence of salt content in whey restricts its utilization in lactose hydrolysis. β-galactosidase production by halotolerant *Aspergillus* spp. is not well studied. By keeping the above points in our mind present study was framed to achieve following objectives.

- Isolation, identification and phylogenetic analysis of halotolerant fungi from man-made solar salterns located at Kambhat, Gulf of Cambay, Gujarat, India and their screening for production of hydrolytic enzymes with emphasis on β-galactosidase.
- Formulation of low-cost, lactose-free production medium for enhanced production of β-galactosidase enzyme using halotolerant *Aspergillus tubingensis* GR1 and optimization of process parameters for production of β-galactosidase at laboratory scale bench-top bioreactor.
- Utilization of agro-industrial waste for β-galactosidase production under solid state fermentation (SSF) using halotolerant *A. tubingensis* GR1 isolate and its purification by two step Three Phase Partitioning (TPP) process.
- Kinetic and thermodynamic characterization of a halotolerant β-galactosidase enzyme produced by halotolerant *A. tubingensis* GR1 and its application in hydrolysis of lactose from milk and whey as well as Galactooligosaccharide (GOS) formation.
1.12 References


Chapter 1


