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Application of non-conventional yeasts in bioprocesses

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INDEX

1 - ABSTRACT

Sustainability is one of the most pressing challenge of our century, this term is becoming a main keyword of political agendas and more in general of mass media. To increase the “greenness of bioprocesses”, academia and industry, especially in the biotechnological and chemical fields, are focusing their studies with the scope to shift from traditional organic synthesis to new processes with reduced ecological foot-print. A good way to increase sustainability could be set up bioprocesses exploiting microorganisms. Nowadays, companies are searching new organisms that, differently from the well characterized *Saccharomyces cerevisiae*, show to be more resistant to the harsh conditions commonly occurring in industrial fermentations (high salt concentration, temperature and pressure). Due to their peculiar features, non-conventional yeasts (NCYs) seem to be a promising solution. On the other hand, the disadvantage to use these new organisms is related to the few studies and literature data available, especially compared to *S. cervisiae*. To fill this gap researchers have started to characterize these new species.

My PhD work had dual aim:

- First to identify good candidates, with specific physiological properties, that could be exploited in bioprocesses.
- Second to characterize new promising enzymatic activities useful for industrial applications.

In the first studies, I focused my attention on marine yeasts. I chose yeasts isolated from this environment, because their use gives the possibility to perform a seawater-based bioprocess saving large amount of fresh waters, reducing both cost and environmental impact. From our laboratory yeasts collection, I selected, for their halotolerance, two different *Debaryomyces hansenii* strains. Hence mechanisms involved in osmotic stress response have been investigated employing flow cytometry. I showed that hyper-osmotic stress elicits membrane depolarization and decreases membrane permeability to cationic compounds. This phenomenon reduces ions permeability and can negatively affect the uptake of charged substrate

during bioprocesses. My research proceeded with the set up of new fermentation protocols in seawater-based media composed by a mixture of hexose and pentose sugar and cheap nitrogen sources. In these conditions we obtained high biomass yield (0.627) in 40 h of bioprocess.

In the second part of my PhD project, I studied NCYs as sources of enzymes. With this aim I identified a nitrilase of marine strain of *Meyerozyma guilliermondii*, that displayed high activities on aromatic substrate, but also on arylaliphatic and aliphatic ones. These activities were maintained also in presence of high salts concentration. In particular M. *guilliermondii* nitrilase was able to perform complete dynamic resolution of mandelonitrile in seawaters within in 8 h.

In the last part of my PhD, I identified a novel extracellular and cell-bound phytase activity in *Cyberlindnera jadinii*. This enzyme is suitable as feed additive, indeed activities at pH 4.5 and 37°C (animals gastric pH and temperature) were 26.25 $mU/mg_{d.w.}$ and 58.36 $mU/mg_{d.w.}$, detected as extracellular and cell-bound respectively. Phytase activities had their optimum at 50°C, reaching 37.2 $mU/mg_{d.w.}$ (extracellular) and 146 $mU/mg_{d.w.}$ (cell-bound).

Data reported in my PhD work suggest that could be interest to proceed with further characterization on NCYs. New “green” bioprocesses characterized by high productivity could be a key for reach sustainability reducing the ecological impact of industrial production.

La sostenibilità è una delle sfide più importanti del nostro secolo ed il termine sostenibilità è diventato una delle principali parole chiave dei programmi politici e più in generale della comunicazione mediata dai *mass media*. Nei settori chimico e biotecnologico per aumentare l'ecocompatibilità dei processi, il mondo accademico e industriale sta concentrando i propri studi con lo scopo di ridurre l'utilizzo dei processi di sintesi chimica tradizionale sostituendola con nuovi a ridotto impatto ambientale. Una delle soluzioni percorribili è quella di mettere a punto bioprocessi industriali utilizzando i microrganismi. Attualmente le aziende sono alla ricerca di nuovi organismi che, a differenza del ben caratterizzato *Saccharomyces cerevisiae*, si dimostrino più resistenti alle condizioni che si verificano comunemente nelle fermentazioni industriali (come alta concentrazione di sale, temperatura e pressione). I lieviti non convenzionali (NCY) grazie alle loro caratteristiche peculiari, sembrano essere una soluzione promettente. Lo svantaggio di utilizzare questi nuovi organismi è però legato alla presenza di pochi studi e pochi dati di letteratura, rispetto a quelli pubblicati su *S. cervisiae*. Per colmare questa lacuna, i ricercatori hanno iniziato a caratterizzare queste nuove specie.

l mio lavoro di dottorato ha avuto un duplice obiettivo:

- Identificare candidati, con proprietà fisiologiche specifiche per mettere a punto nuovi bioprocessi.
- Caratterizzare nuove e promettenti attività enzimatiche utili per applicazioni industriali.

Nella prima parte del mio lavoro ho concentrato la mia attenzione sui lieviti marini. Ho scelto lieviti isolati in questo ambiente, perché il loro utilizzo permette di mettere a punto processi industriali con terreni preparati in acqua di mare, evitando così un elevato consumo di acqua potabile. Questa possibilità riduce infatti sia i costi che l'impatto ambientale dell'intero bioprocesso.

Dalla collezione di lieviti presente nel nostro laboratorio ho selezionato due diversi ceppi di *Debaryomyces hansenii* perché mostravano la migliore tolleranza al sale. In quest'ultimi ho analizzato i meccanismi coinvolti nella risposta allo stress osmotico utilizzando la citometria a flusso. Ho dimostrato che l'esposizione allo stress iperosmotico provoca la depolarizzazione della membrana cellulare, riducendone la sua permeabilità ai composti cationici. La minor permeabilità agli ioni potrebbe però influenzare negativamente i bioprocessi rendendo più difficoltoso l'*uptake* di substrati carichi positivamente. La mia ricerca è proseguita con la messa a punto di nuovi protocolli di fermentazione in terreni simil-industriali contenenti zuccheri quali esosi e pentosi e fonti economiche di azoto. I terreni sono stati preparati utilizzando acqua di mare. In queste condizioni abbiamo ottenuto un'elevata resa in biomassa (0,627) in 40 h di bioprocesso.

Nella seconda parte del mio progetto di dottorato, ho studiato i NCYs come fonti di enzimi. Ho identificato una nitrilasi appartenente ad un ceppo marino di *Meyerozyma guilliermondii*. La sua attività è interessante perché rimane elevata e stabile anche in presenza di un'alta concentrazione di sali nel *buffer* di reazione. In particolare questo enzima è grado di eseguire la risoluzione dinamica completa del mandelonitrile in acqua di mare in 8 ore.

Nell'ultima parte del mio dottorato di ricerca, ho identificato una nuova fitasi di *Cyberlindnera jadinii*. Quest'enzima può essere utilizzato come additivo alimentare, infatti le sue attività extracellulari e *cell-bound* rilevate a pH 4.5 e 37 °C (pH e temperatura che si ritrovano nello stomaco degli animali) sono rispettivamente 26.25 $mU/mg_{d.w.}$ e 58.36 $mU/mg_{d.w.}$ L'optimum di temperatura è stato individuato a 50 °C dove l'attività intracellulare raggiunge 146 $mU/mg_{d.w.}$.

I dati riportati nel mio lavoro di dottorato suggeriscono come possa essere interessante procedere con un'ulteriore caratterizzazione dei NCYs, poiché i nuovi bioprocessi "verdi" caratterizzati da alte produttività potrebbero essere la chiave per raggiungere la sostenibilità riducendo l'impatto ambientale della produzione industriale.

2 – INTRODUCTION

2.1 – Non-conventional yeasts in the modern biotechnology

The potential of yeasts in biotechnology is well known and documented. The most studied and utilized yeast is *S. cerevisiae* due to its capability to produce ethanol and because genetic manipulation is relatively easy in this species. Furthermore, *S. cerevisiae* shows a good tolerance to harsh conditions that occur during industrial bioprocess, and several research groups manipulated/engeneered it in order to obtain good cell factories (Nielsen, 2019). Common opinion retains that *S. cerevisiae* manipulation is reaching the limit, hence the scientific community is focusing on new emerging yeasts. These yeasts present peculiar features which enable them as better candidate for industrial/food bioprocess. To date, science community has modified strategy of process optimization, focusing its attention on new yeast species, classified as non-conventional yeasts (NCYs) (Rebello et al. 2018).

NCYs is synonymous to “non-*Saccharomyces*” yeasts, but beyond any definition, they represent the vast majority of yeast species so far described. The most non-conventional yeasts so far found in the industrial bioprocesses are *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Ogataea polymorpha* and *Pichia pastoris*. Nevertheless, more than 4000 species have been described (Kurtzmann, 2011; Rebello et al. 2018) and about 200 species have founded applications in biotechnology processes or have potential for such applications (Boekhout et al. 2011). These new yeast species have the ability to withstand various extreme conditions and show remarkable levels of thermotolerance, osmotolerance, pressure tolerance, inhibitors tolerance, tolerance to adverse pH and presence of cosolvent. Togheter these resiliences confer to NCYs the role of “perfect candidate” that could exploit as cells factory. These features allow to set up new bioprocesses, since during industrial process these harsh conditions usually occur. In addition, NCYs are could be consider a source of genes and proteins and can be found during

proteins production process in various industries (Stöckmann et al. 2009; Madhavan et al. 2018). Yeasts, but in particular NCYs, are widely used in bioprocesses because they are able to grow on a wide range of carbon sources. This skill is useful to set up bioprocess using agri-food wastes, since these substrates are rich in several sugar species and the capability to grow employing all of them help to increases final productivity (Do et al. 2019).

With the term agri-food are classified a huge variety of substrates (Figure 1 pag 6), but the most common in bioprocesses are:

- Molasses: by-products of the refining of sugarcane or sugar beets. This type of substrate has a sugar concentration that could lead up to 40% - 50% (Doelle and Doelle, 1990)

- Starch: a mixture of the polysaccharides amylopectine and amylose.

- Milk whey: a by-product of diary production that contains 3-8% of lactose (Petres, 2006).

- Grape marcs: the solid remains of grapes after pressing for juice. It contains the skins, pulp, seeds, and stems of the fruit (Favaro et al. 2013).

- Lignocellulose: one of the most abundant residues worldwide. It is composed of carbohydrate polymers (cellulose, hemicellulose), and an aromatic polymer (lignin). This substrate needs to be pretreated by enzymes or acids to get a fermentable feedstock, but this type of process can create some inhibitors that negatively affect the cell growth (Heer et al. 2008).

Unfortunately, media arranged with agri-food wastes contain compounds, derived from pre-treatment steps, like acetic acid and furfurals that inhibit yeast growth. Literature data show that some NCYs seem to be more resistant to high concentration of these compounds than S. *cerevisiae,* and for all these reasons application of NCYs could be promising in order to optimize the entire bioprocess. (Heer et al. 2008).

Fiugure 1. Agri-food wastes. Up on the left clockwise molasse, starch, milk whey, lignocellulose and grape marcs

In the last few decades NCYs has been investigated as promising candidates for the production of heterologous proteins. The application of these new yeasts brings much advantages. One of the majors is biomass-related: in comparison with *S. cerevisiae* the majority of NCYs has a respiratory metabolism that leads to higher biomass production and subsequently higher proteins productivity (Zaky et al. 2014; Rebello et al. 2018).

S. cerevisiae is used to introduce on protein post-tradutional modifications like hyperglycosilation and mannose addiction; this phenomenon not only have a negative effect on final protein activity, but also lead to adverse allergy response. Avoid allergy outbreak is important when the final product is

destined for pharmaceutical and food industries. This situation can be overcome using non-conventional yeasts (Stöckmann et al. 2009).
The real disadvantage of employ NCYs is lack of specific molecular tool to manipulate them (Minhas et al. 2009). In this respect, industry and academia are working to optimize NCYs cloning procedure. Currently are under investigation/developing specific vectors, efficient host recipient strains, proper selective markers and simpler transformation protocols. To date the protocols for NCYs transformation are based on protoplasting and electroporation (Sibirny and Voronovsky, 2009). Nowadays to perform industrial bioprocess with good results is fundamental to increase the knowhow on NCYs.
The search of new microorganisms with improved properties has led to an increased interest of scientific community on new habitats as location for enzyme bioprospecting activity. One of the most promising is marine habitat.

2.1.2 – Marine yeasts in modern biotechnology

A peculiar sub-class of NCYs are marine yeasts which assembled all strains isolated from marine environment. Marine yeasts are widely distributed in marine environments like seawater, marine sediment, weeds and algae, marine invertebrates and animals (Kutty and Philip, 2008; Chi et al. 2012). They can also be found in some extremely adverse sources such as deep sea, (Nagano and Nagahama, 2013), benthic animals and seafloor sediment at the depths ranging from 6400 to 11000 m (Gadanho and Sampaio, 2005). The genera of marine yeasts identified include *Rhodotorula* (Figure 2), *Rhodosporidium*, *Candida*, *Debaryomyces* (Figure 2), *Meyerozyma*, *Cryptococcus*, *Yarrowia*, *Aureobasidium*, *Metschnikowia*, *Torulopsis*, *Pichia*, *Kluyveromyces*, *Saccharomyces*, *Pseudozyma*, *Hansenula*, *Trichosporon*, *Filobasidium*, *Leucosporidium*.

Figure 2. Examples from our marine yeast collection. On the left *Debaryomyves hasenii* strain Bio2 (white yeast), o the right *Rhodotolura mucillaginosa* strain LM 18 (red yeast).

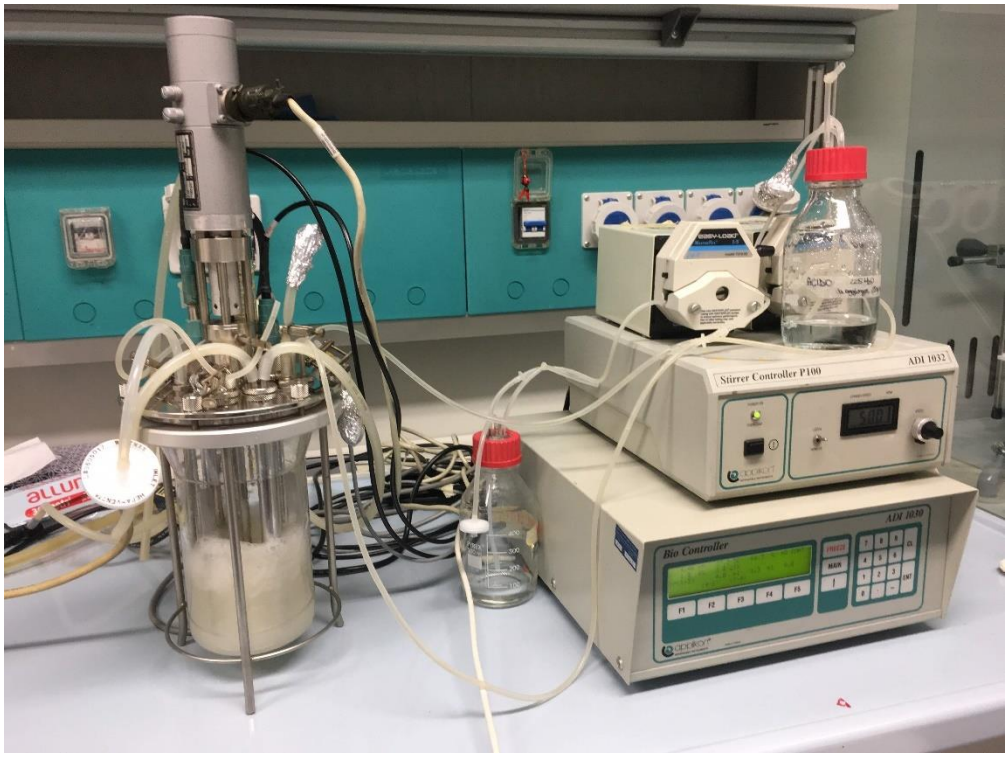
Since terrestrial yeasts are common in bioprocess, only little attention has been given to marine yeasts (Zaky et al. 2014), although the ability of these yeasts to live in the marine environments made them more resistant to the conditions that can be commonly found during industrial processes (Chi et al. 2016).

Marine yeasts show to be more resistant to high pressure and in presence of high salts concentration, than terrestrial yeasts. Literature data show that enzymes isolated from marine environment are halotolerant and osmotolerant and furthermore it has been observed a strict relation among salt and organic solvent tolerances (Trincone, 2017).

Moreover, the use of marine microorganisms opens the way to exploit seawater as alternative to potable water, both as fermentation and biotransformation medium, thus improving the greenness of bioprocesses. Exploitation of water as solvent in bioprocesses is highly desirable from the sustainable chemistry and, in this frame, seawater, accounting for 97% of the world's water, can be a promising alternative water resource (Anderson, 2012; Domínguez de María, 2013; Zambelli et al. 2015 – Figure 3). Further,

seawater contains a wide spectrum of minerals that can enrich the fermentation medium (Zaky et al. 2014).

Figure 3: Bioreactor fermentation of *D. hansenii* Mo40 performed in media arranged in seawater.



Analogously to NCYs, the little know of marine yeasts is a barrier for their industrial application. In my PhD work I characterized industrial stress response in marine yeasts, focusing my attention on osmotic stress response.

2.2 – Industrial relevant stress: osmotic stress

To date, researchers that works on development of new industrial processes are concentring forces to make a transition to green chemistry. (Sheldon and Pereira, 2017). An alternative to reduce the impact of conventional chemical synthesis is the use of microbial cells. Usually bioprocess often requires harsh conditions like high osmotic pressure, high temperature, unfavorable pHs, and presence of organic cosolvents, making necessary the identification of suitable microorganisms to work with. In my PhD research, I focused my attention on osmotic stress response in NCYs and in particular in marine yeasts. Currently osmoadaptation mechanisms have been mainly studied in the salt sensitive *S. cerevisiae*. Indeed, less data regarding osmotic stress response on NCYs are available. Due on this lack of information, it is interesting and intriguing to work in this field (Blomberg, 2000).

The principal constituents of cultural media are salts, sugars and polyols. These compounds are the principal osmotic stressors when are present at high concentration. For example, during the production of second-generation bioethanol the pre-treatments used to detoxify the hydrolysed materials from lignocellulose lead to an high final salt concentration (Sanchez and Cardona, 2008). Salt stress can compromise cellular performances causing a decreased production efficiency. For this reason, identification of a “good industrial yeast” is turned to be a major challenge for obtain sustainable process (Deparis et al. 2017). NCYs and in particular marine yeasts have been selected to solve this scope.

Understanding the mechanisms behind the adaptation to osmotic stress is fundamental, in order to increase bioprocesses productivity. (Bubnová et al, 2014; Dakal et al. 2014). Adaptation to altered osmolarity is an active process,

based on sensing of osmotic changes and on appropriate cellular responses aimed to maintain cellular activity. The mechanisms of osmoadaptation are generally conserved, but the response can be strains/species dependent (Gori et al. 2005).

Osmotic stress response in *S. cervisiae* is well known and documented and several approaches could help to analyse it. When a microorganism is exposed to hypertonic medium a rapid diffusion of water occurs from the cells into the surrounding medium. To avoid extreme loss of water, yeast accumulate compatibles solutes (Nevoigt and Stahl, 1997). The most common molecule is glycerol. Its production is activated by HOG-MAPK signalling cascade leading intracellular glycerol accumulation (Hohmann, 2015). Osmotic stress is able to drive change in fluidity and in membrane composition. In *S. cerevisiae* it has been observed a reduction of fatty acids chain length and an increase of degree of saturation that leads to a better membrane fluidity (Levin, 2011; Turk et al. 2007). In addition, osmotic stress due on ionic compounds like NaCl induce membrane depolarization. In homeostatic condition membrane potential is maintained by ions gradient across the membrane and for this reason the presence of this salt can modify this equilibrium (Ke et al. 2013).

A shift from higher to lower salt concentration could create stress condition (hyposmotic stress), stimulating HOG-MAPK cascade. In *S. cerevisiae* intracellular glycerol concentration is maintained by Fps1p, a plasma membrane channel. Increased external osmolarity induces its closure leading intracellular glycerol accumulation, whereas decreased osmolarity causes channel opening and glycerol release (Tamás et al. 1999).

In my PhD project I characterized osmotic stress response on two different strains of *Debaryomyces hansenii* isolated from marine environment. This yeast species is usually described for its halotolerant behaviour. In particular, I choose marine yeast strains, because marine environment is characterized by high salt concentration. It is possible to suppose, that

organisms isolated from this habitat, had evolved efficient mechanisms in order to live in that condition.

2.2.1 – *D. hansenii*

D. hansenii is an hemiascomycetes yeast and belong to NCYs groups. It is a member of *Saccharomycetaceae* family characterized by ovoid cells, and forms white and wrinkled colonies. This yeast is interesting from a biotechnological point of view because it possesses metabolic traits that look appealing for developing industrial processes.

Some strains of *D. hansenii* can tolerate up to 4 M NaCl that is a high concentration in comparison with *S. cerevisiae* tolerance that is less than 1 M (Lépingle et al. 2000; Onishi, 1963). Due to its halotolerance, it was able to acquire a particular ecological niche. This yeast is usually isolated from habitat characterized by high osmotic pressure like sea water and food matrix. *D. hansenii* strains isolated from food usually come from brines, cheese, salami and aged meat. In food matrix this yeast is involved in cheeses and salami maturation, contributing to the final aroma. For example, it produces S-methylthioacetate that confers their peculiar characteristic to Cheddar and Camembert (Gori et al. 2012).

D. hansenii is able to grow exploiting several carbon sources like hexososes and pentoses, but also starch, lactic acid, raffinose and melibose. This capability makes it able to grow on agri-food by-product such as hemicellulose hydrolysates. These compounds contain a sugar mixture in variable composition depending on the source of the raw material. Growth on pentoses is slower than growth on hexoses, but the values of biomass yields are very similar with the two types of sugars (Nombre et al. 1999).

D. hansenii is a non-pathogenic organism but the huge amount of literature data and, in particular, classification problems that occurred in the

last decades leads to confusion. The first classifications were based on phenotypic characterization and *D. hansenii* has been assimilated with *Candida* species, and with their photogenic status. Following the molecular classification era, *D. hansenii* species has been defined. To date, infections *D. hansenii*-dependent are found only in immunocompromised patients (Liu, 2011). Hence this yeast is included in QPS EFSA (Qualified Presumption of Safety- European Food Safety Authority - Koutsoumanis et al. 2019).

Due on its QPS status and its capability to growth in industrial conditions, *D. hansenii* is studied for several applications. Strains isolated from fish gut and cheese have been recently characterized as potential probiotic (Ochangco et al., 2016). Other *D. hansenii* strains could be “cell factory” to produce for xylitol, arabinol and riboflavin (vitamin B2). Furthermore *D. hansenii* is able to produce toxins with antibiotic activity versus *Candida spp* and ochratoxigenic molds. These toxins that usually are exopeptidases and thermophilic β-glucosidases, are interesting molecules that could be introduced as therapeutic agents (Prista et al. 2016, Iacumin et al. 2017). *D. hansenii* is been also described as ‘oleaginous’ yeasts (Breuer and Harms, 2006). The few data regarding this aspect make this research field interesting to be investigated. Thanks to its QPS status, lipids obtained from *D. hansenii* could be suitable also in food applications. One disadvantage is represented by the few specific molecular tools available to manipulate this yeast. Furthermore *D. hansenii* do not follow universal genetic code and it belongs to the CTG-clade group. In this group of yeasts CUG codon is translate a serine instead of leucine. This different language can lead problems for *D. hansenii* application as cells factory, because an incorrect translation could generate not active proteins. This ambiguous CUG decoding creates an evolutionary advantage. In particular this feature leads to a new protein variants and it is usually associated with development of microbial resistance and fitness improvement (Javid et al. 2014).

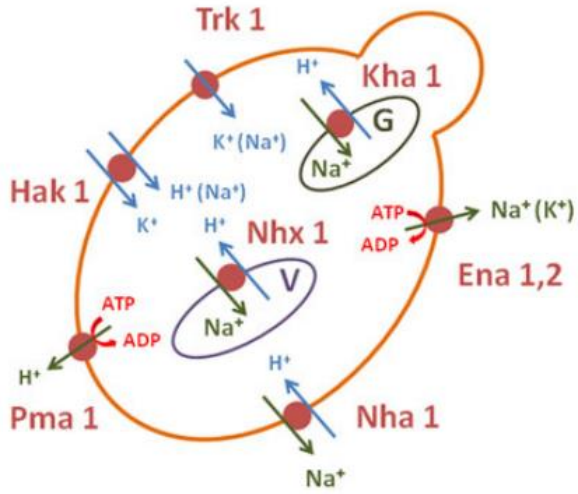
Osmotic stress response

Due on its industrial relevance science community has start to characterize *D. hansenii* with a physiological point of view. Researchers focused their forces to analyse mechanisms behind its halotolerant behaviour (Prista et al. 2016). In natural environments, especially in the marine environment, sodium chloride (NaCl) is present at variable concentration and is toxic to cell. Like in *S. cerevisiae,* when *D. hansenii* cells are exposed to hypertonic medium released water in the environment. To prevent extreme loss, glycerol accumulation occurs (Gancedo et al. 1968). In *D. hansenii* the stress response is mediated by HOG pathway (Prista et al. 2005). HOG 1 plays like a universal regulator for stress condition. In presence of high salt concentration, it activates several genes that mediate the adaptation to hyperosmotic stress. *DhGPD1* and *DhGPP* are genes directly connected with glycerol biosynthesis (Gori et al. 2005). Glycerol plays an important role also in adaptation of hyposmotic stress: in this shock condition cells reply with glycerol export. In *S. cerevisiae* efflux of glycerol is mediated by Fps1p channel. Curiously a gene encoding for Fps1p was not identified in *D. hansenii* (Prista et al. 2016). Since Fps1p role is essential for cell survival, in *D. hansenii* alternative mechanisms probably mediated from other membrane channel has been evolved.

In *D. hansenii* has been observed that salts stress has effect on membrane composition; in particular a modification on ratio between sterol and phospholipids. This phenomenon causes a decrease in the relative content of sterols, phosphatidylglycerol, phosphatidylinositol and phosphatidylethanolamine, whereas the relative content of phosphatidylserine increases. This change in ratio causes a reduction in membrane fluidity (Turk et al. 2007). In this regard, *D. hansenii* behaviour seems more similar to halophilic black yeasts (generally belonging to *Basidiomycota* clade) than the phylogenetically closer *S. cerevisiae,* suggesting that this response could be relate with its salt resistance.

The first studies on osmotic stress response in *D. hansenii* were performed by B. Norkans in Goteborg. He studied ions balance and he proposed that the key factor that confers salt resistance is the capability to maintain potassium and sodium homeostasis (Norkrans and Kylin, 1969). In *D. hansenii* transporters play an important role in maintain ions equilibrium. In this halotolerant yeast several genes encoding for this kind of proteins are identified. *DhTRK1 and DhHAK1* are involved in potassium influx. The first one is a constitutive gene, while expression *DhHAK1* requires ions starvation (Martinez et al. 2011 – Figure 4).

Figure 4. The major plasma membrane and intracellular cation transporters identified in the yeast *D. hansenii*. V, vacuole; G, Golgi complex (Prista et al. 2016)



DhEna1p/DhEna2p complex and DhNha1p Na^+/H^+ antiporter seem to be involved in salt resistance, but once expressed in *S. cerevisiae* are not able to confer this capability (Almagro et al. 2001 Figure 4)

DhKha1p and Nha1p are protein channels localized on intracellular compartment. DhKha1p has homologous in *S. cerevisiae,* its protein localized on Golgi apparatus is strictly correlated with Na^+ resistance, but it is not involved other ions tolerance. DhNhx1p is an Na^+/H^+ transporter that

contributes to ions compartmentalization in vacuoles (Montiel and Ramos, 2007 - Figure 4).

The interconnection of these proteins is still unknown. So far has been described that *D. hansenii* is a sodium-includer yeast. It means that it can reach high level of intracellular Na^+. This capability and the marginal role that transporters seem to play in salt stress response suggests that in *D. hansenii* others mechanisms are able to confers halotolerance (Prista et al. 2016). Understanding these mechanisms is interesting in order to optimize industrial bioprocess where the harsh condition can damage the final yield.

During my PhD I investigated osmotic stress response on two *D. hansenii* strains collected from deep sea hydrothermal vent. In particular, one isolated from gasteropod gills (*Ifremeria nautilei*) and one isolated from coral. We choose these yeasts due on their salt tolerance showed in primary screening and for their inclusion in QPS lists making them candidates for industrial application.

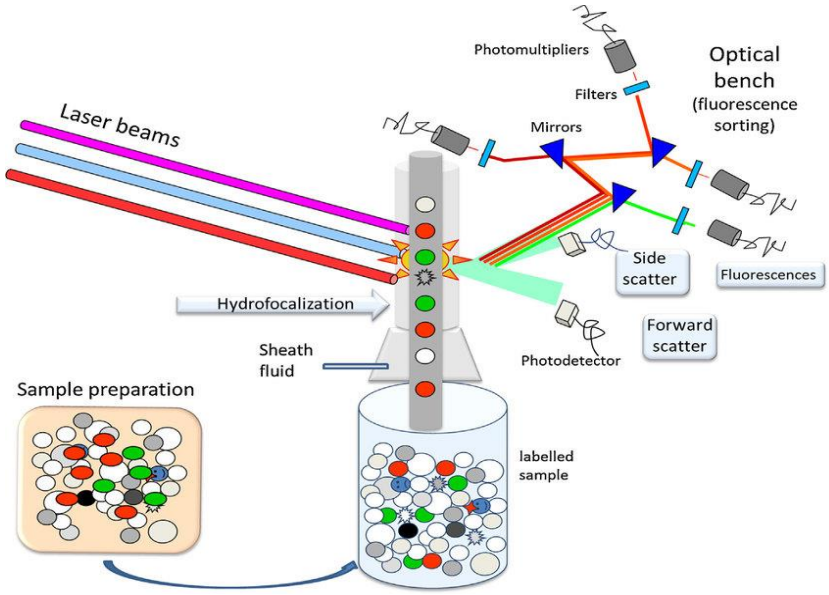
I set up a new approach: flow cytometry. This methodology permit to rapidly obtain accurate information regarding important cellular parameters at single cell level (ComasRiu and Rius, 2009).

2.2.2 – Flow cytometry

Flow cytometry (FC- Figure 5 pag 21) is a technique developed over the last 30 years. Initially it has been exploited for microrganisms analysis but to date playing an important role during the analysis of clinical samples (Cossarizza, 2010). It is an innovative methodology that permits to perform population study analyzing several physiological parameters at single cell levels in order to determine sample heterogeneity. It also permits to distinguish and analyze small subpopulations present in the sample obtaining segregated data (Davey and Kell, 1996). Application of FC in industrial field

could lead to intriguingly results and I will extensively elucidate it in chapter 4.1.

Figure 5. Schematic representation of flow cytometry principle (Depinige-Berger et al. 2016)



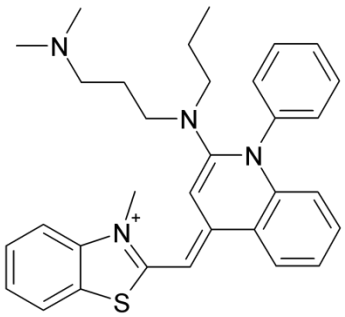
This technique permits to quickly analyse tens of thousand cells, and processes data gathered by a computer. The sample ideally flows as single cell through a laser beam; when the cell crosses the beam, light is scattered based on its components and characteristics. After the cell transitions instrument measured three different parameters: forward angle light scatter (FSC), side angle light scatter (SSC) and fluorescence (FL) at selected wavelengths. FCS and SSC parameter are strictly connected with light scatter. FSC is correlated with the size, shape, and state of cells aggregation, whereas SSC depends on the density of the particles/cells (i.e., the cytoplasmic granule number and membrane size). The third parameter (FL) gives information about cell fluorescence. It can be detected directly monitoring cells autoflorescence or indirectly staining cells with fluorescent dye labels. Autoflorescence is peculiar of cell fitness: an increase of this parameter is usually correlate with a presence of a kind of stress (Capusoni et al. 2016).

Several parameters could be observed by staining cells with fluorescent dyes and for this reason during my PhD project I worked with the following ones:

SYBER ® Green I (Figure 6) is an asymmetrical permanent cyanine dye used to stain nucleic acid. It is commonly used to observe DNA content in cells.

It was initially developed as a nontoxic and more quantifiable dye in replacement of ethidium bromide on agarose gels. When SYBER ® Green I bind DNA the resulting DNA-dye complex absorbs blue light (λmax = 497 nm) and emits green light (λmax = 520 nm). The dye preferentially binds dsDNA, but stains also ssDNA with lower performance (Zipper et al. 2004)

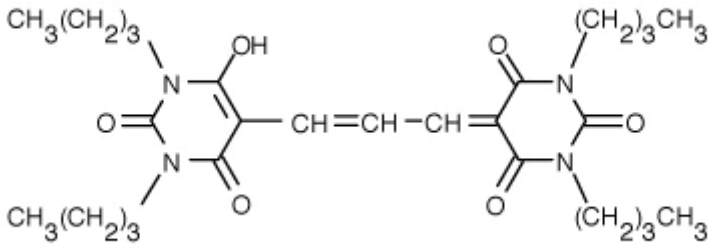
Figure 6: Chemical structure of Syber Green I



SYTO™24 is another permanent nucleic acid stain. It has a very similar mechanism of action of SYBER ® Green I (Morris et al. 2006)

$DiBAC_4$(3) (Figure 7 page 23) is a voltage-sensitive fluorescent dye which measure membrane potential. This dye enters in depolarized cell and binds proteins and membranes. Enhanced fluorescence causes a red spectral shift. Increase in fluorescence is connected with an increased depolarization since in this condition is detected an additional influx of the anionic dye.

Figure 7. Chemical structure of $DiBAC_4(3)$



Conversely, decrease in polarization level is indicated by a decrease in fluorescence. This dye has an “excitation maxima” in blue light (λmax=497 nm) and emission maxima in green light (λmax = 526 nm) (Gamalei et al. 1991).

Propidium iodide (PI): is a fluorescent intercalating agent that can stain nucleic acids. PI has a fluorescent excitation maximum in blue-green (λmax = 493 nm) and an “emission maxima” in red (λmax = 636 nm). PI binds DNA by intercalating between the bases with no sequence preference. After binding DNA, the quantum yield of PI is enhanced 20-30 folds. PI is not membrane-permeable, making it useful to differentiate necrotic, apoptotic and healthy cells based on membrane integrity (Davey and Hexley, 2011).

FC is a promising technique to study stress response, because when a population of cells is exposed to stress a heterogeneous response occurred. In this reaction some cells are killed, others are damaged, and yet others may show no observable phenotypic changes. In this case aggregated data are not significant but using FC analysis is possible to investigate the heterogeneity of population fitness (Heins et al. 2019).

In some experiment belonging to my PhD project I performed different experiments with FC in order to characterized osmotic stress response.

2.3 – Biocatalysis

Yeasts playing a pivotal role during the process of biocatalysis, which is a mainstream technology not only for the sustainable production of bulk chemicals and pharmaceutical ingredients but also for food production in food-industry. Term biocatalysis is generally referred to the utilization of isolated enzymes or whole cells for converting a series of natural and non-natural compounds (Schmid et al. 2001)

The wide application of biocatalysis can be due on its numerous environmental and economic benefits that shows in comparison with chemical catalysis. Enzymes are “natural” and “sustainable” catalysts. Natural, because they are isolated from biocompatible and renewable resources; in addition, they are biodegradable and non toxic. Sustainable, because derived from renewable resources. Biocatalysis permit to avoid chemical catalysts that usually are precious metals; these compounds are not only expensive, but also their removal from final products has prohibitive cost (Sheldon and Pereira, 2017) Furthermore enzymatic reactions are performed in water under mild condition like physiological pH and ambient temperature and pressure, avoiding harsh condition usually required in chemical processes. Often conventional organic syntheses require protection and deprotection steps, but in biocatalysis, thanks to enzymatic higher selectivity these steps can be avoided. This results in a shorter syntheses capability, purer products and less waste. Due on higher enzymatic specificities the catalyzed reactions are regio-, chemo- and stereoselective. These features make biocatalysis one of the most important tools to create new molecules in a “green techniques” manner (Atalah et al. 2019).

Biocatalysis can be performed in two different way: using isolated enzymes or whole cells (that can be bacteria, fungi, microalgae and plants). In particular during my PhD project I performed biotransformation reaction

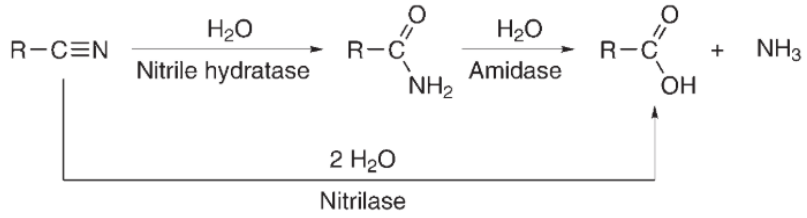
using yeast whole cells. In industry this technique confers important advantages compared to one based on isolate enzymes (Garzón-Posse et al. 2018) The first advantages is represented by low costs: processes performed with isolated enzymes, to work efficiently, requires presences of cofactor (like NAD^+(P), NAD(P)H, FAD) or auxiliary (Sheldon and Pereira, 2017) enzymes that are able to recycling them. Secondly preparation of enzymatic pure solution is complex and requires specific techniques and resources. Isolated enzymes are obtained by genetically engineered microorganisms, requiring a good expertise in molecular engeneering. Several publications (Garzón-Posse et al. 2018) on biocatalysis underline that biotransformation performed with whole cells instead of isolated enzyme are a simpler and equally effective method to obtain high product yields with a good enantiomeric excess. Usually experiment performed with whole cells are generally the first step in search new enzymatic activities.
Despite the substantial advances, whole cells present also significant disadvantages as substrates permeability, substrates/products toxicity and difficulties in process scale up.

Literature data show that strains of *Meyerozyma guilliermondi* and *Rhodotolura mucillaginosa* are able reduce stereoselectivelly ketones in alcohol in seawaters. This capability has been employed to produce, in seawaters, intermediates for the synthesis of molecule of pharmaceuticals interest as desogestrel, norgestrel, gestodene and pramipexole (Serra et al. 2016).
In my PhD thesis I characterized a collection on NCYs in order to identify two different classes of enzyme: nitrilases and phytases. Both are class of enzymes that are common in industrial bioprocess including the pharmaceutical (nitrilase – Chen et al. 2019) or the food-related (phytase – Lei et al. 2013).

2.3.2 – Nitrilase

Nitrilases (EC 3.5.5.1) are tyolitic enzyme that catalyze the direct conversion of nitrile to the corresponding carboxylic acids, yielding ammonia as side product. Nitriles may also be converted to acids via bienzymatic pathway that involve nitrile hydratases (EC 4.2.1.84) and amidases (EC 3.5.1.4). In particular hydratases catalyse a two-step reaction: the hydration of nitriles to the corresponding amides and then their conversion to acids (Singh et al. 2006 - Figure 8).

Figure 8. Types of Reactions carried out by nitrilase-superfamily members



Nitrilase are ubiquitous and can be found in bacteria, filamentous fungi and plants. Yeast nitrilases are less frequently described and exploited, indeed only 60 nitrile-metabolizing yeasts have been described (Gong et al. 2012). In addition, the two-step hydrolysis catalysed by nitrile hydratase and amidase is more frequently described in yeast than other organisms (Singh et al. 2006).

Nitriles are compounds of natural or synthetic origin that contain a cyano group in their structure. In nature, they are produced by plants in various forms such as cyanoglycosides, cyanolipids, ricinine, phenylacetonitrile. Due to their versatility nature, in industries these enzymes are exploited non only during polymers synthesis but also as biding blocks for synthesis of drugs, cosmetics and pesticides (Gong et al. 2012). The majority of these compounds are toxic, mutagenic and suspected to be carcinogenesis (Banerjee et al. 2002; Zheng et al 2018). Given their presence in the environment, it no surprises that

specific microorganisms such *Rhodococcus* spp. Have been found able to metabolize them (Atalah et al. 2019). Usually this detoxification procedure involves enzymes with nitrilase activities.

Nitrilase has attracted researchers' attention in the last six decades, since these enzymes are able to work on a wide spectrum of substrates (Gong et al 2012; Chen et al. 2019). Nitrilase are usually found as homooligomers and their catalytic activity do not require the presence of metal ions or other cofactors. These enzymes are attractive because conventional chemical methods for nitrile hydrolysis entail the use of severe conditions like concentrated acid or basic solution and high temperatures (over 100°C-Debabov and Yanenko, 2011). Such harsh conditions are not sustainable for a "green process". Chemical synthesis is not stereoselective, like enzymatic synthesis, and lead to side products production and subsequently to yield losses (Singh et al. 2006).

During PhD experiments I focused my attention on NCYs' nitrilase activity. In particular I analyzed yeasts isolated from deep-sub-seafloor sediment (Rédou et al. 2015). Indeed, the yeast marine habitat could be an interesting location of enzyme bioprospecting activity. Marine enzymes show great potential compared to the terrestrial counterparts (Serra et al. 2016). Hydrothermal vents and oceanic caves, which constitute the ecological niches for marine yeast, are characterized by high pressure and salinity. For reasons described above the enzymes of marine yeasts are expected to be resistant to "extreme" conditions (Trincone, 2017). Finally, it is important to remark that few literature data are available on marine yeasts nitrilase.

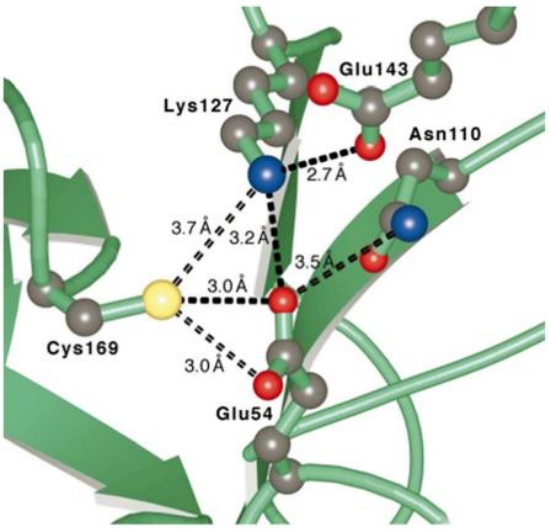
Biochemycals characterization

This class of enzymes can be classified on their substrate preference "arylaceto-", "aromatic-" and "aliphatic-" nitrilases. This is very important to take into account in the initial phase of a screening to detect enzymatic activities. The choose of the right substrate is pivotal because some enzymes

show no activity on substrate where nitrile group is attached to an aromatic nucleus, but prefers molecules where nitrile is on an aliphatic backbone and *vice versa* (Kobayashi and Shimizu, 1994).

To date structure of yeast nitrilases are not available, but several works on bacteria nitrilase confirms that protein structure is phylogenetically conserved (Brenner et al. 2002). The structure of bacterial nitrilase is a novel α-β-β-α sandwich fold, with a triad of residues, Glu-Lys-Cys (Figure 9).

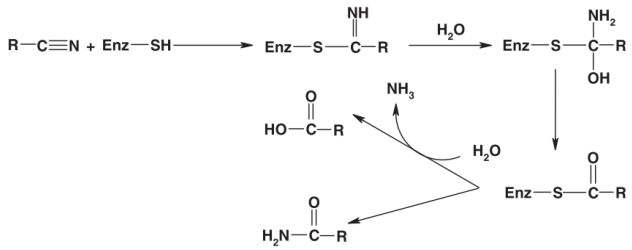
Figure 9. Putative nitrilase active site proposed by Pace et al. 2010. A putative Nit active site. The region around Cys169, a residue conserved in nitrilases, is conserved in Nit proteins. Residues aligning with Cys169, Glu54 and Lys127 are predicted to form a catalytic triad in the nitrilase superfamily.



This catalytic domain is essentials in function and enhances nitrilase's performance (Chen et al. 2019). Catalytic mechanism was characterized by Mahadevan's groups. They postulated that nitrile carbon bearing a fractional positive charge, was subjected to a nucleophilic attack, probably by one of the two SH groups on the nitrilase. The resulting imine was then hydrolyzed to corresponding ketone while having NH_3 as a by-product. Acyl-enzyme was

then hydrolyzed by the addition of H_2O, and finally liberated the carboxylic acid along with the regenerated enzyme (Thimann and Mahadevan, 1964). The proposed mechanism is still valid and is widely accepted by researchers (Figure 10).

Figure 10. Proposed mechanism for nitrilase-mediated biocatalysis by Gong et al. 2012



Some nitrilases possessed a subunit size between 30 kDa and 45 kDa, and must form heterocomplexes to gain catalytic activity (Gong et al. 2012). In *Rhodococcus* the aromatic ring in the substrate can lead nitrilase aggregation. Through HPLC gel filtration it possible detect that some substratse are able to activated the enzyme leading to form 560-kDa complex through the association of the 47-kDa monomer. This association is often observed in presence of salts and organic solvents and confers major stability to the enzyme (Nagasawa et al. 2000).

Nitrilases are not constitutive proteins and in the majority of organism nitrilase activity is inducible (Nageshwar et al. 2011). A study on *Cryptococcus* reports that this yeast is able to degrade benzonitrile only when it is present as sole nitrogen source (Rezende et al. 2000). Interestingly in *Fusarium solani* the presence of nitrile is able to increase 100-fold enzymatic activity in comparison with other inducers (Gong et al. 2012).

For industrial application nitrilase need to be resistant to organic solvent. Some bioconversion could require the presence of cosolvent to increase substrate solubility. Hence the degree of resistance to organic solvent was investigated due poor solubility of nitrile compounds. It is been discovered that these enzymes are fairly stable in organo-acqueous media (Gong et al. 2012).

The possibility to employed nitrilase isolated from marine environment could be promising in order to increase solvent tolerance. This last feature is usually associated with enzymes halotolerance (Trincone, 2017).

Industrial application

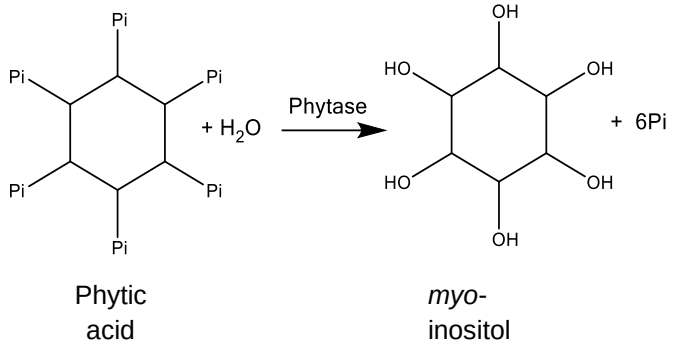
Bacterial nitrilase are successfully exploited on industrial scale. For example, nitrilase from *Acidovorax facilis* plays an important role during the chemoenzymatic production of 1,5-dimethl-piperdone (Chen et al. 2019). This compound is commercialized by DuPont with the name of XolvoneTM: a new biodegradable cleansing solvent. An engineered version of this enzyme with enhanced substrate tolerance is used for the production of iminodiacetic acid a precursor for the manufacture of chelating agent and surfactants. Nitrilase are exploited in food industries for production of food supplements like α- and β- amino acids and nicotinic acid (vitamin B3) (Fan et al. 2017).

Nitrilases plays an important role in bioremediation. Nitrile compounds like acetonitrile and benzonitrile, but also plastic materials and pesticides are often charged into the environment as a result of human activities. Nitrilases are able to degrade these compounds into harmless intermediates or, ultimately, carbon dioxide (Atalah et al. 2019).

2.3.2 – Phytase

With phytase word is identified a class of enzyme that hydrolyzes phosphomonoester bond from phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate), liberating organic phosphate (Irving and Cosgrove, 1974 – Figure 11). Phytases are produced by a wide range of organisms: plants, animals, bacteria, fungi and yeasts (Vats and Banerjee, 2004).

Figure 11. Phytase reaction scheme



Environmental phytic acid accumulation

Phytic acid is present in nature because is the main source of stored phosphorus in grains, pulse, oil seeds and nuts (Mullaney and Ullah, 2000). It represents up to 5% of seed dry weight and up to 80% of total phosporous in seed. Phytic acid is synthesized by plants starting from glucose 6-P, that is first converted to 1D-myo-inositol-3-phospate and subsequentially completely phosphorylated (Shi et al. 2005). This compound plays an important role during seed germination and seedling growth, because it serves as storage form of *myo*-inositol and phosphorus. Due to its nature of polyanionic chelating agent, phytic acid is characterized as antioxidant specie. It is able to form stable complexes with Cu^{3+} and Fe^{3+}, preventing formation of reactive oxygen species responsible of cellular damages and carcinogenesis (Graf et al. 1987).

Presence of phytic acid, despite its antioxidant effect, creates various problems environment-related (Vats and Banerjee, 2004). In the last decades scientists who are working in the field of environmental protection, animal nutrition and human health have focused their attention on phytase. This class of enzymes has been accepted worldwide as feed supplement for breeding and aquaculture (Lei et al. 2013). Problems in breeding occurs because feed is mainly composed by vegetal materials that are rich in phytic acid. Differently from monogastric animals the polygastric ones are able to degrade phytate,

thanks to their particular gut microbiota (Lei et al 2013). Accumulation of phytic acid has a negative effect on animal and human health because this acid reduces bioavailability of proteins and ions like Fe^{3+}, Ca^{2+}, Zn^{2+} and Mg^{2+} forming insoluble complex (Reddy et al. 1982). Moreover, undigested phytate accumulates in manure and liquid effluent, leading to phosphorous pollution that causes waters eutrophication. When phytic acid is released in aquatic environment causes problem like cyanobacteria blooms, hypoxia and death of marine/freshwater animals (Puppala et al. 2018). Since phytic acid cannot be employed as phosphorous sources, feeds for monogastric animals are commonly fortified with inorganic phosphorous, increasing final costs of the product. The combined effect of phytate as antinutritional factor, unviable sources of phosphorous and as causes of environmental pollution makes phytase a lucrative target for investigation (Lei et al. 2013).

In order to avoid/contain phytic acid released in the environment a strict regulatory measure has been taken and for these reasons phytase value on market is becoming important. At the end of the twenty centuries the annual sales of phytase as an animal feed additive were estimated to be US$ 500 million (Puppala et al. 2019).

Biochemycals characterization

Among phytases is possible to observe several different mechanisms of phosphate cleavage as well as structural differences (Vats and Banerjee, 2004).

One of the mainclassification is based on three different subclasses

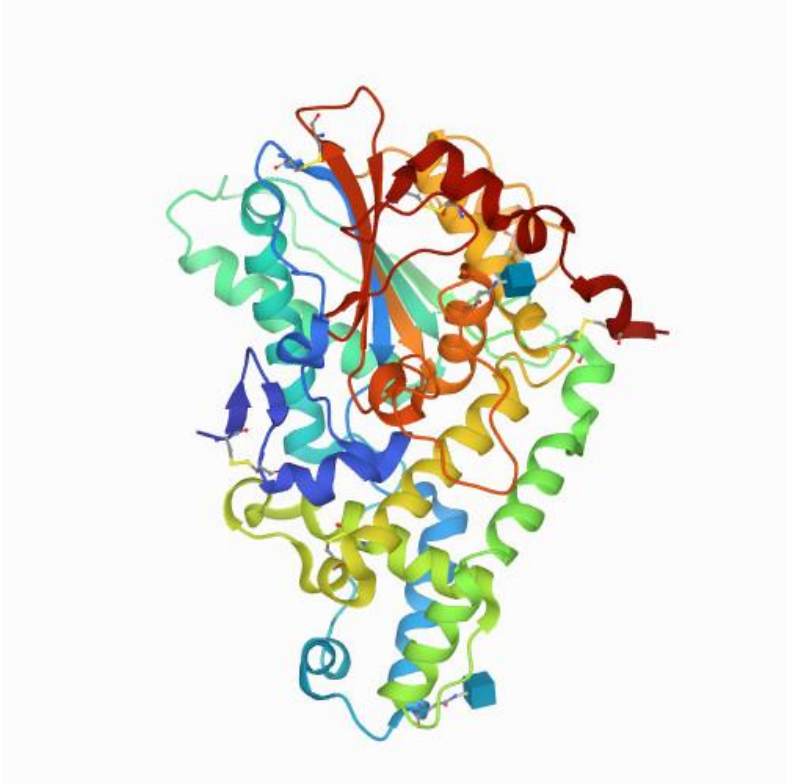
1. histidine acid phosphatases (HAP),
2. β – propeller phytase (BPP),
3. purple acid phosphatases (PAP)

Since during my PhD project I've investigated only phytases belonging to HAP class I will describe meticulously their mechanism of actions. Other class will be described briefly.

The first and most common class of phytase is represented by HAPs (EC 3.1.3.8 – Greppi et al. 2015) and are spread in yeast, bacteria, filamentous fungi, but also in upper eukaryotes (Lei et al. 2013). HAPs are able to start phytic acid hydrolysis starting from at either the C^3 or C^6 position of the inositol ring and produced myo-inositol 2-phospate.

All members of this class hydrolyzed phosphate from phytate in the acid pH range. They maintain two different common domains. N-terminal heptapeptide active site RHGXRXP (38-44 aa) and a C-terminal catalytically active dipeptide HD (325-326 aa). Enzyme is composed by two different domains a large α/β domain and a small α domain. The catalytic site is in the interface of the two domains (Kostrewa et al. 1997 - Figure 12).

Figure 12. Structure of PhyAp belonging to *A. niger deposited* RCSB PDB by Oakley A.J. 2010.



The hydrolyzation of phytic acid by HAP is carried out through a two-step mechanism. The first step is a nucleophilic attack on the phosphoester bond by a conserved histidine in the long active-site motif (RHGXRXP). The histidine side chain from the conserved HD motif protonates the leaving group. The second step comprises hydrolysis of the resulting covalent phosphohistidine intermediate. The final product of histidine acid phytases is myo-inositol monophosphate. Alkaline phosphatases like BBP are only able to hydrolyze three phosphate groups resulting in myo-inositol triphosphates as final product (Böhm et al. 2010). The enzyme that belong to this class are characterized by different substrate specificity. Some members show broad substrate specificity but low activity (namely PhyAp), whereas others have narrow substrate specificity and high specific activity for phytic acid (namely PhyBp – Mulaney and Ullah, 2003). Comparing PhyAp and PhyBp no significant variation in the catalytic sites of these fungal phytases has so far been discovered. Kostrewa and co-workers explain the high PhyA specificity thanks to a Substrate Specific Site (SSS) optimized to bind the negatively charged phytic acid. This enzyme is commercially available on market as Naturphos®. PhyBp shows different catalytic properties from phyA. Indeed, it appeared for the first time in literature as phosphatase that works with a pH optimum of 2.5 (Ullah and Cummins 1987; Ullah and Phillippy 1994). Phytase activity was not detected because initial screening was performed at pH 5. The reason of this phenomenon is due to the SSS of PhyBp, which is composed by two aminoacid (D75 and E272). This composition lead to a more electrostatically neutral SSS that can accommodate a broader substrate spectrum than PhyAp. At pH 2.5 the SSS is uncharged and can accept phytate as substrate, but at pH 5 it is negatively charged and would repel negative-charged substrate like phytic acid (Kostrewa et al. 1997).

A second class of phytases is represented by β-Propeller phytase (BPP) (EC 3.1.3.8) which has recently discovered and is characterized by a novel

hydrolysis mechanism (Ha et al. 2000). It is known that the hydrolysis of substrates belonging BPP occurs at the “cleavage site”. The BPP is the only phytase class with observed activity in neutral and alkaline pH conditions and exhibit high thermostability, compared to the HAPs and PAPs (Vats and Banerjee, 2004).

The third and last phytases’ class are Purple acid Pospahtase (PAP). This class of metalloenzyme has been recently isolated from soybean. Intriguingly an analysis on genomic database indicates that these sequences are present also in mammals, fungi, and bacteria (Lei et al. 2013). PAPs isolated from soybean are the only one that shows significant activity on phytate. This activity is lower in comparison to phyA, probably because seed germination required a steady breakdown of phytate over a period of several days. A lower activity is necessary to avoid phosphate accumulation during seedling process (Mulaney and Ullah, 2003).

Yeast phytase application in feed production

Microbial phytase are available on market in different formulations, like Allzyme®SSF, RoNozyme® and Natuphos®, isolated from *Aspergillus niger* (Lei et al. 2013). Yeasts are good candidate for phytases production as feed additive and some of them have been already characterized (Olstorpe et al 2009, Greppi et al. 2015). In different species these enzymes show different localizations: in *A.niger* (Neira-Vielmaa et al. 2017; Vats and Banerjee 2004), *Saccharomyces crevisiae* (In et al. 2009; Klosowski et al. 2018), *Candida tropicalis* (Puppala et al. 2018), *Debaryomyces castelli* (Rangoon et al. 2008) phytases are classified as extracellular enzymes and in some case, like in *Kodamea ohmeri* (Li et al. 2008), signal sequences for secretion have been identified. Curiously others yeast phytases are defined as intracellular enzymes like in *Cryptococcus laurenti* (Pavlova et al. 2008) or cell-bound, like in *Wiccheranomyces anomalus* (Vohra and Satyanarayana, 2001).

A good phytase suitable as feed additive display three major characteristics:

1. ability to hydrolyze phytate pH optimum 3.5 - 5.5
2. cheap production cost
3. resilience up to 65-90°C

These three features have been described in several publications (Lei et al., 20013; Vasudevana et al. 2019).

Yeast phytases, in particular HAPs, have a pH optimum in acid range, situation that is found in the upper digestive tract of animals. Based on this HAPs are a good candidate to be use as feed additive.

Recent literature data indicate yeast as good candidate to decrease the final costs of enzyme production since they are able not only to produce high level of phytases but also to grow using cheap substrates (Vohra and Satyanarayana, 2004)

The last features consist in resilience to high temperature. During feed production process, substrates are subjected to specific heat treatment (Lei et al. 2013). Some need to be performed in order to control *Salmonella* spoilage and others are employing during pelletting procedure. Heat treatment can negatively affect final enzymatic activity of phytase if the enzyme is not thermostable (Vasudevana et al. 2019). An alternative could be to add in liquid phytase after heat treatment. Sophisticated instruments are required to perform an homogeneous incorporation of enzyme. Hence this aspect results in higher final cost of the feed and suggests that the good solution is represented by a thermostable phytase.

In my PhD work I analyse several yeasts species in order to identify a novel phytase activity. The idea was to find the phytase with good characteristics suitable as feed additive.

2.4 - References

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3 - AIMS and OBJECTIVES

Academia and industries are focusing their studies in order to shift from traditional chemical synthesis to new eco-friendly production processes. A good alternative could be the use of microorganisms to set up bioprocesses. Companies need to find new organisms, different from the "old" *S. cerevisiae*, with suitable physiological characteristics. In particular a good industrial yeast should exhibit traits like osmotolerance, halotolerance, thermotolerance and barotolerance, to cope the harsh conditions often present in industrial processes. On the other hand few data compared to *S. cerevisiae* are available in literature. To fill this gap, researchers have started to investigate the world of non-conventional yeast (NCYs).

In my PhD work I analysed a collection of NCYs with a dual purpose: to identify good candidates in order to exploited them in bioprocesses and to characterize new promising enzymatic activities useful for industrial application.

In the first part of my work I examined physiological mechanisms involved in salt tolerance. In the second part I inspected our yeast collection (yeasts isolated from marine environment and food matrix) for enzyme bio-prospecting activities, focusing my attention on nitrilases and phytases.

4 - RESULTS CHAPTERS

4.1 - Hyper-osmotic stress elicits membrane depolarization and decreased permeability in halotolerant marine *Debaryomyces hansenii* strains and in *Saccharomyces cerevisiae*

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4.1.1 - Abstract

The use of seawater and marine microorganisms can represent a sustainable alternative to avoid large consumption of freshwater performing industrial bioprocesses. *Debaryomyces hansenii*, which is a known halotolerant yeast, possess metabolic traits appealing for developing such processes. For this purpose, we studied salt stress exposure of two *D. hansenii* strains isolated from marine fauna. We found that the presence of sea salts during the cultivation results in a slight decrease of biomass yields. Nevertheless, higher concentration of NaCl (2 M) negatively affects other growth parameters, like growth rate and glucose consumption rate. To maintain an isosmotic condition, the cells accumulate glycerol as compatible solute. Flow cytometry analysis revealed that the osmotic adaptation causes a reduced cellular permeability to cell-permeant dye SYBR Green I. We demonstrate that this fast and reversible phenomenon is correlated to the induction of membrane depolarization, and occurred even in presence of high concentration of sorbitol. The decrease of membrane permeability induced by osmotic stress confers to *D. hansenii* resistance to cationic drugs like Hygromycin B. In addition, we describe that also *in Saccharomyces cerevisiae* the exposure to hyper-osmotic conditions induced membrane depolarization and reduced the membrane permeability. These aspects are very relevant for the optimization of industrial bioprocesses, as in the case of fermentations and bioconversions carried out by using media/buffers containing high nutrients/salts concentrations. Indeed, an efficient transport of molecules (nutrients, substrates, and products) is the prerequisite for an efficient cellular performance, and ultimately for the efficiency of the industrial process.

4.1.2 - Introduction

The use of microbial cells for industrial bio-productions has been promoting the transition to green chemistry for the synthesis of molecules by

means of more eco-compatible processes. The isolation and study ofmicroorganisms with useful properties is therefore a fundamental step in the development of such processes. At industrial level, microbial cells are often cultivated under conditions that can exert stress. Salts (ionic) and sugars/polyols (non-ionic) are some of the principal constituents of cultural media and represent the principal osmotic stressors when used at high concentration as in several fermentation processes. High salt levels are present in the second-generation bioethanol production process, due to the pretreatments used to detoxify the hydrolysate from lignocellulosic materials (Sanchez and Cardona, 2008). These stressful conditions compromise the cellular performance resulting in decreased production efficiency. In this regard, adequate stress tolerance of the industrial strains has turned out to be a major challenge for obtaining economically sustainable production (Deparis et al., 2017). Recently, marine yeasts have been explored with the aim to develop industrial bioprocesses employing seaweeds as carbon source as well as seawater, which can represent a sustainable alternative to the large consumption of freshwater (Domínguez de María, 2013; Zaky et al., 2014; Zambelli et al., 2015; Serra et al., 2016). Their industrial potential as biocatalysts is increasing also, due to the strict relation among salt and organic solvent tolerances often observed for enzymes obtained from halotolerant species (Trincone, 2011). Therefore, the understanding of adaptive responses triggered to maintain proper cell homeostasis under hyperosmotic conditions is a priority for the improvement of such biotechnological processes.

Yeast species exhibit different levels of osmotic tolerance, and the studies on the cellular mechanisms behind highlight how yeasts employ different strategies to survive (Ramos et al., 2011; Bubnová et al., 2014; Dakal et al., 2014; Mukherjee et al., 2017). In *Saccharomyces cerevisiae*, the osmotic response has been widely explored due to its high biotechnological importance and the broad availability of analytical methods. High osmolality activates the HOG-MAPK signaling cascade (Hohmann, 2015), resulting in

adaptive responses such as accumulation of glycerol. Structural properties of the cell wall and plasma membrane are important factors influencing the yeast osmotolerance (Levin, 2011). *S. cerevisiae* has been reported to shorten the fatty-acid chain length, and to increase their saturation level upon the rise of salinity (Turk et al., 2007). Membrane potential is generated by ions gradients across the membranes of living cells, and it is obviously affected by salt-induced osmotic stress. Plasma membrane depolarization caused by NaCl has been observed in *S. cerevisiae*, and predicted by mathematical models of ion fluxes regulation (Ke et al., 2013; Plášek et al., 2013).

Osmotic response has been studied in few halotolerantspecies, like *Debaryomyces hansenii* (Prista et al., 2005). Detailed researches on Na^+ and K^+ movements during osmotic stress and on expression of genes encoding the ion transporters/exchangers demonstrated the important role played by these mechanisms (Almagro et al., 2001; González-Hernández et al., 2004; Velkova and Sychrova, 2006; Michan et al., 2013). Recently, a mechanism that entails the sequestration of surplus Na^+ cations in intracellular compartments has been reported (Herrera et al., 2017), confirming its previously reported attitude as “Na-includer” yeast.

In the present work, we have investigated aspects related tothe osmotic adaptation in marine yeasts collected at a deep-sea hydrothermal vent (South Pacific West, Lau Basin, 2620 m below sea surface level) (Burgaud et al., 2010). In particular, two *D. hansenii* strains, isolated from gasteropod gills (Ifremeria nautilei) and from coral, showed halotolerance and were studied in detail. *D. hansenii* possesses metabolic traits that look appealing for developing industrial processes (Prista et al., 2016). Strains associated with cheese and meat processing, have been reported to contribute to their final aroma and, by the activity of particular proteolytic enzymes, to alter food composition (Gori et al., 2012). Strains isolated from cheese and fish gut have been recently investigated for potential probiotic properties (Ochangco et al., 2016). Other examples ofits biotechnological interest are its usage for

biocontrol of ochratoxigenic molds (Iacumin et al., 2017), production of enzymes like exopeptidases and thermophilic β-glucosidases, production of fine chemicals, such as xylitol and riboflavin as well as for its ability to use a broad spectrum of carbon substrates (Breuer and Harms, 2006). Considering the potential for applications of this species, there is an interest for the development of sustainable industrial bioprocesses.

By using flow cytometry, a methodology that rapidly allows obtaining accurate information regarding important cellular parameters at single cell level, monitoring in this way the heterogeneity of the cellular population (Comas-Riu and Rius, 2009; Müller and Nebe-von-Caron, 2010), we describe that membrane depolarization and decrease of membrane permeability reversibly occur upon hyper-osmotic stress in *D. hansenii* strains. The decrease of membrane permeability confers to *D. hansenii* resistance to cationic drugs like Hygromycin B. In addition, we show that these osmotic responses are induced also in less osmotolerant species like *S. cerevisiae*. These effects are relevant for the development of industrial bioprocesses, particularly in the case of seawater-based processes. High concentrations of nutrients and salts in cultivation media as well as in bioconversion buffers exert in fact a strong osmotic pressure, in this way influencing the efficiency of solute transport (nutrients, substrates, and products) and, in turn, the efficiency of the whole process.

4.1.3 - Materials and methods

Strains and Growth Conditions

The strains used in this work belong to a collection of marine yeasts created previously from deep sub-seafloor sediments and deep-sea hydrothermal vents (Burgaud et al., 2010). All isolates are available at the UBO Culture Collection. For long-term storage, yeast strains, including *S. cerevisiae* strain CENPK113-7D, were maintained at −80°C on 15% (v/v)

glycerol and 85% (v/v) YPD (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose).

The screening in presence of NaCl was performed on plates containing defined minimal medium Yeast Nitrogen Base (YNB, Difco, Italy) containing glucose 2% (w/v, Sigma-Aldrich, Italy), 2% agar (w/v, Conda, Spain), supplemented with 2-(N- Morpholino) Ethane Sulfonic acid (MES, Sigma-Aldrich, Italy) 0.1 M at pH 6, and containing different concentrations of NaCl, ranging from 0.5 to 2 M.

Broth cultures were performed at 28°C in flasks under shaking conditions at 150 rpm (INFORS HT, Multitron Standard). Cells from pre-cultures grown in YNB-glucose-MES were harvested during the exponential growth phase by centrifugation and inoculated at $OD600_{nm}$ 0.1 into the same medium supplemented with 4% (w/v) sea salts (SS, Sigma-Aldrich, Italy), or 2 M NaCl, or 2 M sorbitol (Sigma-Aldrich, Italy), or not supplemented (control cultures). The growth was monitored through the increase in OD at 600_{nm} using a spectrophotometer (Jenway, 7315^{TM} Bibby Scientific Limited, Stone, United Kingdom). Broth cultures were performed in triplicate.

In *D. hansenii* to apply hyper-osmotic shock, cells growingin YNB-glucose-MES (control condition) were harvested during the exponential growth phase, centrifuged at 2300 g for 5 min and shifted to flasks containing YNB-glucose-MES supplemented with 4% (w/v) SS (naturally containing 0.55 M NaCl), 2 M NaCl, for 30 min up to 2 h, or 2 M sorbitol and incubated for 2 h. In *S. cerevisiae* to apply hyper-osmotic shock, cells growing in YNB-glucose-MES (control condition) were harvested during the exponential growth phase, centrifuged 2300 g for 5 min (Eppendorf 5415D) and shifted to flasks containing YNB-glucose-MES supplemented with 0.55 M NaCl for 2 h.

For hypo-osmotic shock, cells growing in YNB-glucose-MES in presence of 4% (w/v) SS or 2 M NaCl were incubated in the same medium without salts for 2 h.

The effect of hyper- and hypo-osmotic stress was assessed by flow cytometry (FCM) by evaluating cell membrane permeability and cell membrane potential (see below).

For testing cationic drug sensitivity after exposition toward salts, aliquots of tenfold serial dilutions were spotted on YNB-glusose-MES plates supplemented or not with 2 M NaCl and in presence of increasing concentrations of Hygromycin B (10, 25, and 75 µg/ml) (Sigma-Aldrich, Italy). Then the plates were incubated at 28°C for 3 days.

Dry weight and metabolite assays

For dry weight measurements (DW), samples from different culture conditions were collected at different times (in triplicate at each point). Cells were filtered through a glass microfiber GF/A filter (Whatman), washed with three volumes of deionized water and dried at 100◦C for 24 h. Glucose, ethanol, and glycerol concentrations in the supernatants of the cell cultures were assayed by using commercial enzymatic kits (Hoffmann La Roche, Basel, Switzerland). For intracellular glycerol determination, cells (30 OD) were collected by centrifugation and suspended in 500 µl of water, boiled boiled at 100°C for 15 min, then centrifuged at 16,100 g for 5 min, and the supernatants were assayed by using the commercial enzymatic kit (see above). For all the assays, the standard deviation was lower than 5%. Biomass yields and specific glucose consumption rates have been calculated according to van Hoek et al. (2000).

FCM analysis

SYBR Green I, propidium iodide (PI), 5(6-)-carboxyfluorescein diacetate N-succinimidyl ester (cFSE), and $DiBAC_4(3)$ were obtained from Sigma-Aldrich (Italy). SYTOTM 24 was purchased from Thermo Fisher Scientific (Milan, Italy).

Cells were stained with SYBR Green I 1X or with SYTO™ 24 5 µM at 25°C for 15 min in the dark. For the evaluation of membrane integrity or cell viability the cells were stained with PI 7.5 µM or with cFSE 5 µM and incubated in the dark for 15 min before measurement. For the evaluation of membrane depolarization, the cells were stained with $DiBAC_4(3)$ 5.7 µM and incubated in dark for 15 min before measurement. Briefly, cells were harvested by centrifugation (2,300 g 5 min) during early exponential growth phase and suspended at 10^6 cells/ml in YNB- glucose-MES pH 6, or in the same medium supplemented with SS or 2 M NaCl. The dyes were then added at the appropriate final concentrations, and incubated as required. To cause membrane depolarization, cells growing in YNB medium were exposed for 15 min to 1 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma-Aldrich, Italy) and then stained as described above. Cell count and fluorescence detection were performed using an Accuri C6 flow cytometer (BD Biosciences, Milan, Italy). Cell suspensions were analyzed using the FCM with the following threshold settings: FSC 5000, SSC 4000, and 20000 total events collected. All the parameters were collected as logarithmic signals and 488 nm laser was used to measure the FSC values. When necessary, samples were diluted in filtered fresh media just before measurement, so that the rate of events in the flow was generally lower than 2,000 events/s. The data were analyzed using BD Accuri™ C6 software version 1.0 (BD Biosciences, Milan, Italy). The SYBR Green I, SYTO™ 24, cFSE and $DiBAC_4(3)$ fluorescence intensities of stained cells were recovered in the FL1 channel (excitation 488 nm, emission filter 530/30 nm, BD Biosciences, Milan, Italy). The PI fluorescence was recovered in the FL3 (excitation 488 nm, emission filter 610/20 nm, BD Bioscience, Milan, Italy).

4.1.4 - Results

Impact of sea salts and 2 M NaCl on *D. hansenii* growth parameters

A screening performed on solid medium containing different NaCl concentrations (range 0.5–2 M) was performed in order to test the salt tolerance of yeasts isolated from endemic animal fauna living at deep-sea hydrothermal vents (Burgaud et al., 2010). This analysis revealed that *D. hansenii* was among the most tolerant species (Supplementary Table S1).

In order to study the effects of SS or higher NaCl concentration on the growth parameters, we performed broth cultures of *D. hansenii* Bio2 and Mo40 strains. The cultures were carried out under aerobic conditions, on defined minimal medium YNB containing: (i) 4% (w/v) SS, the concentration in seawater (containing 0.55 M NaCl) or (ii) 2 M NaCl, which exerts a stronger osmotic pressure.

The presence of SS in the growth medium had a faint effect on the growth of both strains: their specific growth rates and specific glucose consumption rates were similar to those of respective control cultures (without salts) (Table 1 and Figure 1). On the contrary, higher salt concentration (NaCl 2 M) slowed down the growth, with reductions of their respective growth rates of 42 and 43% for Bio2 and Mo40, respectively (Table 1). Slower specific glucose consumption rates were also detected, with decreases of 23 and 24% for Bio2 and Mo40, respectively. The growth kinetics showed an adaptation phase lasting at least 8 h for Bio2 and 13 h for Mo40 (Figures 1C, F).

Table 1. Growth parameters of *Debaryomyces hansenii* Bio2 and Mo40 cultivated in presence of sea salts (SS) and 2 M NaCl.

	Bio 2				Mo40			
	μ_{max} [h^{-1}]	Biomass yield (Y) [g_{dw}/g_{glc}]	q Glucose [$mmol_{glc}/g_{dw}$ /h]	Final biomass g/L	μ_{max} [h^{-1}]	Biomass yield (Y) [g_{dw}/g_{glc}]	q Glucose [$mmol_{glc}/g_{dw}$ /h]	Final biomass g/L
YNB	0.26 ± 0.01	0.42 ± 0.02	2.52 ± 0.03	8.88 ± 0.24	0.28 ± 0.01	0.39 ± 0.01	2.71 ± 0.13	7.71 ± 0.14
YNB SS	0.26 ± 0.01	0.36 ± 0.01	2.56 ± 0.04	7.45 ± 0.64	0.31 ± 0.01	0.37 ± 0.01	2.59 ± 0.13	7.90 ± 0.19
YNB 2M NaCl	0.15 ± 0.02	0.37 ± 0.01	1.95 ± 0.13	7.40 ± 0.14	0.16 ± 0.04	0.34 ±0.02	2.05 ± 0.11	6.61 ± 0.16

The cultivation in presence of either SS or 2 M NaCl resulted in 13–14% lower biomass yields (Table 1), although the cells showed a respiratory metabolism (no production of ethanol was found in all the cultures). The discrepancy between respiratory metabolism and low biomass yield can reflect a higher amount of ATP to be redirected from the biomass synthesis toward the mechanisms of cation extrusion of either SS or 2 M NaCl resulted in 13–14% lower biomass yields (Table 1), although the cells showed a respiratory metabolism (no production of ethanol was found in all the cultures). The discrepancy between respiratory metabolism and low biomass yield can reflect a higher amount of ATP to be redirected from the biomass synthesis toward the mechanisms of cation extrusion.

In parallel, we measured the intracellular glycerol accumulated during the growth in presence of SS and NaCl. In Bio2 and Mo40 glycerol was maintained at higher intracellular level in presence of 2 M NaCl than in sea salts, and this level decreased when the growth approached the stationary phase, concomitantly with its leakage into the medium (Figures 1C, F).

In conclusion, our results indicated that the presence of SS during the cultivation of *D. hansenii* marine strains produces only a limited decrease of the biomass yields. Nevertheless, a higher salt concentration (2 M NaCl) exerts greater negative impact on the other growth parameters (growth rate and glucose consumption rate).

Effects of growth conditions on cell volume, membrane permeability and membrane potential

Debaryomyces hansenii Bio2 and Mo40 cells cultivated on media containing salts were analyzed by FCM. Bio2 exhibited a reduced cell volume in terms of forward light scattering (FSC), namely a 18 and 23% of volume decreasing when the cells were cultivated in presence of SS or 2 M NaCl, respectively. This effect was not evident in Mo40 cells cultivated in presence of salts (Supplementary Figure S1).

Membrane-permeant dyes that specifically bind to nucleic acids, like SYBR Green I or SYTO™ 24, are usually employed to stain cells (Müller and Nebe-von-Caron, 2010). On the contrary, intact membranes of viable cells exclude PI, which is used to monitor membrane damages or cell death (Davey and Hexley, 2011).

Figure 1. Growth of *Debaryomyces hansenii* Bio2 and Mo40 strains under different conditions. (A, D) Growth on YNB, control condition; (B, E) growth in presence of sea salts (SS); (C, F) growth in presence of 2 M NaCl. ● OD 600 nm, ■ glucose (g/l), ▲ extracellular glycerol (g/l), ∆ intracellular glycerol (g/g of dry weight). Broth cultures were performed in triplicate and standard deviations were lower than 5%.

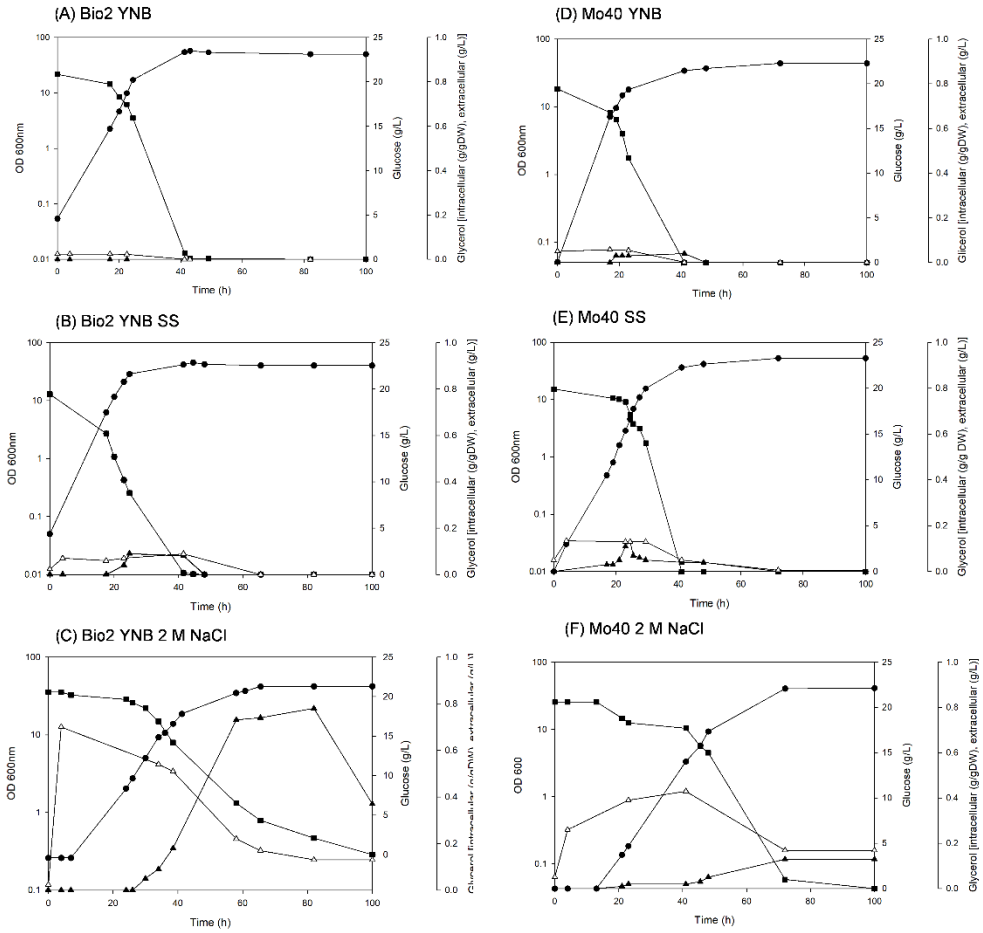
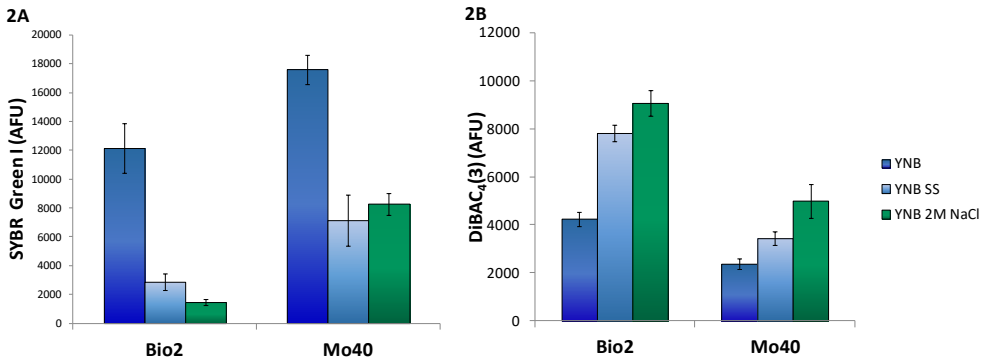


Figure 2. FCM analysis showing SYBR Green I fluorescences detected in FL1 channel. (A) Bio2 cells stained after growth on YNB (control cells); (B) after growth in presence of sea salts; (C) after growth in presence of 2 M NaCl.; (D) Mo40 cells stained after growth on YNB (control cells); (E) after growth in presence of sea salts; (F) after growth in presence of 2 M NaCl.



Samples of Bio2 and Mo40 exponentially growing cells cultivated on YNB without salts (control cultures) or on YNB in presence of SS or 2 M NaCl were collected and stained by maintaining the cells in the same conditions of the growth. In comparison with control cultures, cells grown on salts-containing media (therefore exposed to hyper-osmotic stress during their growth) exhibited lower levels of SYBR Green I (Figure 2A and Supplementary Figure S2). For Bio2 strain, the fluorescence decrease was of 76% on SS and 88% on 2 M NaCl. For Mo40 the decrease was of 60% on SS and 53% on 2 M NaCl (Figure 2A). On the other hand, PI stained only 1–2% of cells under all the growth conditions, indicating that most of the cells were viable and with intact membrane. To confirm the reduced membrane permeability toward permeant dyes due to the hyper-osmotic stress, Bio2 cells were stained with SYTO^{TM} 24, another cell permeant nucleic acid staining. As figured out for SYBR Green I, we measured a decreasing of fluorescence when cells were cultivated in presence of salts (Supplementary Figure S3). Moreover, when Bio2 cells growing under hyper- osmotic conditions were permeabilized by treatment with 70% ethanol their SYBR Green I fluorescence levels were equal to the control cells (Supplementary Figures S4A, C). Interestingly, if the staining was performed by diluting the cells in PBS, which is a common

isotonic solution, we observed that cells growing on salts-containing media were stained at levels equal to the control cells (Supplementary Figures S4D, F). We can hypothesize that this buffer can cause a hypo-osmotic shock during the staining procedure of cells growing on salts-containing media, suggesting that this condition rapidly (30 min) restores the membrane permeability to the dye.

These results indicated that the growth in presence of salts alters the cell membrane permeability. To test if the effects observed with SYBR Green I and SYTO™ 24 were exhibited also with dyes possessing different chemical characteristics, we stained Bio2 and Mo40 cells with cFSE, a lipophilic compound mainly used to assess cell viability and intracellular pH (Capusoni et al., 2016).

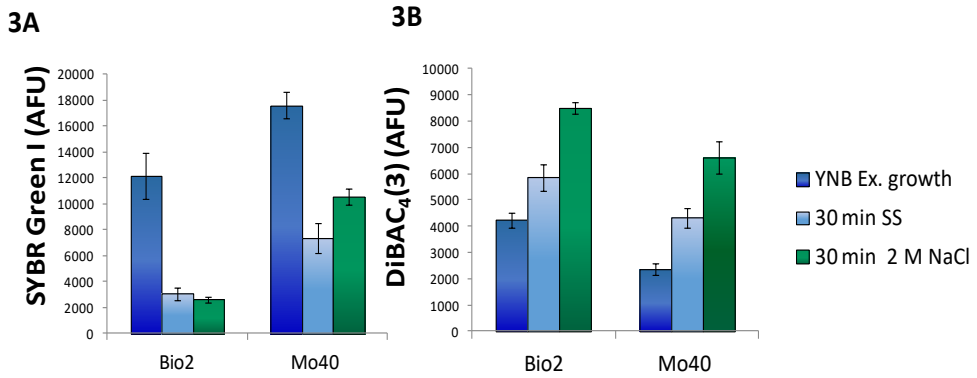
These results indicated that the growth in presence of salts alters the cell membrane permeability. To test if the effects observed with SYBR Green I and SYTO™ 24 were exhibited also with dyes possessing different chemical characteristics, we stained Bio2 and Mo40 cells with cFSE, a lipophilic compound mainly used to assess cell viability and intracellular pH (Capusoni et al., 2016). In this case, both cells growing on media containing SS or 2 M NaCl resulted stained like the control cells (Supplementary Figure S5).

Then, we estimated membrane potential on Bio2 and Mo40 cells growing in presence of salts by staining with the oxonol dye $DiBAC_4(3)$, a voltage-sensitive fluorescent dye (Comas-Riu and Rius, 2009). Cells with depolarized membranes result brighter than cells with normal membrane potential (Guyot et al., 2015). Cultivation on media with salts caused membrane depolarization in both strains (Figure 2B). Specifically, for Bio2 and Mo40 cells we measured an increase of $DiBAC_4(3)$ fluorescence when cells were cultivated in presence of SS and NaCl. Interestingly, higher fluorescence values were detectable when cells were exposed to NaCl rather than SS during their growth (Figure 2B).

Effect of hyper- and hypo-osmotic conditions on membrane permeability and membrane potential

To better understand the kinetic of the salt effect in changing membrane permeability and membrane potential, we analyzed the the short-time response (from 30 min up to 2 h) toward hyper- and hypo-osmotic stress. Bio2 and Mo40 cells exponentially growing on YNB (control condition) were exposed to SS or 2 M NaCl (hyper-osmotic). By FCM we observed that 30 min of exposure to SS or 2 M NaCl were enough to determine, respectively, 75 and 78% decrease of SYBR Green I staining in Bio2 strain (Figure 3A). Similarly, in Mo40 the fluorescence decrease caused by salt stress was of 60% for SS and of 40% for 2 M NaCl (Figure 3A). After 2 h, the loss of membrane permeability in terms of reduced fluorescence was comparable to that observed in cell growing in presence of salts (not shown).

Figure 3. Quantitative analysys of (A) SYBR Green I and (B) $DiBAC_4(3)$ fluorescences detected by FMC in FL1 channel in cells collected in exponential phase of growth on YNB (control condition), in presence of sea salta (SS) or 2 M NaCl. Arbitrary Fluorescence Units (AFU).

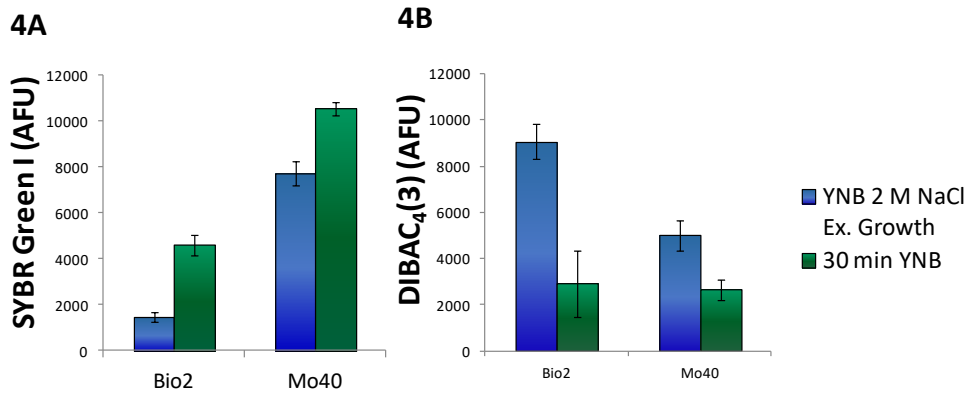


On the other hand, exposure to salts caused membrane depolarization: after 30 min an increase of $DiBAC_4(3)$ fluorescence of 38 and 100% was detected in Bio2 cells, and of 83 and 180% in Mo40 cells exposed to SS and 2 M NaCl, respectively (Figure 3B).

Then we investigated if hyper-osmotic conditions inducedby sugars, as high concentration of sorbitol, triggered the same kind of response. The exposition of Bio2 cells to 2 M sorbitol in the growth medium resulted in 85% decrease of SYBR Green I fluorescence and generated membrane depolarization in 2 h (Supplementary Table S2). We concluded that loss of permeability and membrane depolarization are a general hyper-osmotic stress response.

In order to test if the shift to hypo-osmotic conditions restored membrane permeability and membrane polarization, Bio2 and Mo40 cells cultivated in presence of 2 M NaCl were stained in YNB without salt. The FCM analysis showed that after 30 min an increase of SYBR Green I fluorescence was already visible in both strains (Figure 4A), concomitantly with the process of membrane repolarization, detected by the decrease of $DiBAC_4(3)$ fluorescence (Figure 4B).

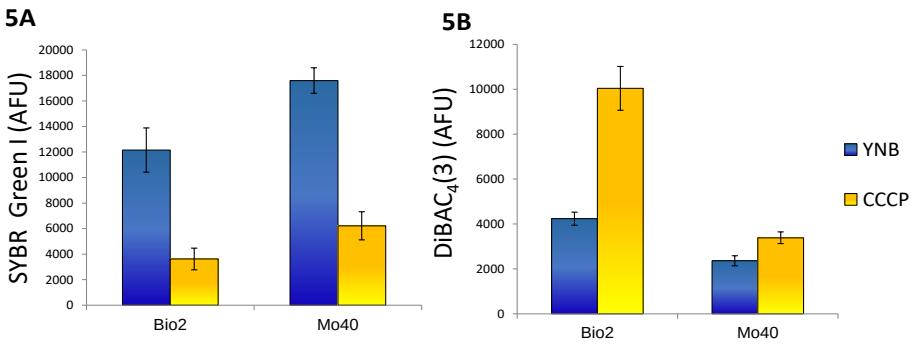
Figure 4 Quantitative analysis of (A) SYBR Green I and (B) $DiBAC_4(3)$ fluorescences detected by FMC after exposure to hypo-osmotic stress for 30 min. AFU, Arbitrary



Membrane depolarization and loss of permeability are linked in *D. hansenii*

The results obtained suggested that the cultivation under hyper-osmotic conditions induced in Bio2 and Mo40 a loss of permeability to cationic dyes like SYBR Green I, as well as a concomitant membrane depolarization. To assess if the two phenomena were correlated, cells growing under control condition (without salts) were exposed to CCCP, a protonophore that causes membrane depolarization (Zahumenský et al., 2017). The cells were then stained with $DiBAC_4(3)$, to detect membrane depolarization, and with SYBR Green I, to detect loss of permeability. In comparison to the control cells, at higher levels of $DiBAC_4(3)$ fluorescence (increase of 137% for Bio2 and 43% for Mo40) corresponded lower levels of SYBR Green I fluorescence (decrease of 80% for Bio2 and 65% for Mo40) (Figure 5). In conclusion, these effects (depolarization and impermeability) were similar to those observed when cells growing on control media (without salts) were exposed to salts (Figure 2).

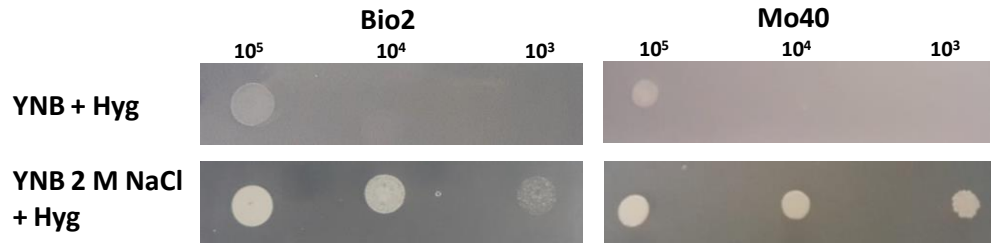
Figure 5. Quantitative analysis of (A) SYBR Green I and (B) $DiBAC_4(3)$ fluorescences detected by FMC after exposure to carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a protonophore inducing membrane depolarization. Arbitrary Fluorescence Units (AFU).



Membrane potential is known to play a role in the mechanism of cationic drugs toxicity in *S. cerevisiae* (Maresova et al., 2009). Mutants exhibiting hyper-polarization of plasma membranes showed hyper-sensitivity to Hygromycin B (Kodedová and Sychrová, 2015). When *D. hansenii* Bio2 and

Mo40 strains were cultivated on solid medium containing 2 M NaCl, they displayed a decreased sensitivity toward this drug (Figure 6), reinforcing the conclusion that the growth under hyper-osmotic conditions alters the membrane permeability to cationic compounds, due to an acquired level of depolarization.

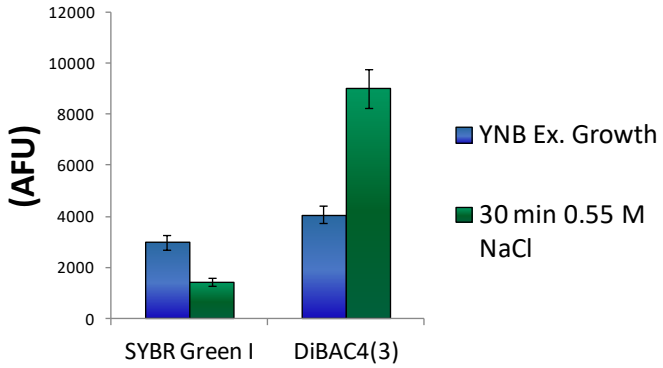
Figure 6. Test for cationic drug resistance. *D. hansenii* cells were spotted on YNB plates supplemented with 75 µg/ml hygromycin B and containing or not 2 M NaCl. The plates were recovered after three days of growth at 30°C.



Membrane depolarization and loss of permeability occur also in *S. cerevisiae*

To verify if this correlation occurred also in other less salt tolerant yeast species, we exposed *S. cerevisiae* to hyper-osmotic stress caused by the presence of 0.55 M NaCl. *S. cerevisiae* is known to exhibit maximum tolerance to NaCl 2 M (Lages et al., 1999). The exposition to salts for 30 min triggered membrane depolarization, detected by 122% increase of DiBAC4(3) fluorescence in comparison to control cells (Figure 7). Concomitantly the level of SYBR Green I staining decreased of 52% (Figure 7 and Supplementary Table S3). These results demonstrate that the membrane depolarization induced by hyper-osmotic stress causes a loss of membrane permeability also in *S. cerevisiae.*

Figure 7. Quantitative analysys of (A) SYBR Green I and (B) DiBAC$_4$(3) fluorescences detected by FMC in *S. cerevisiae cells* after exposure to hyper-osmotic stress (0.55 M NaCl). Arbitrary Fluorescence Units (AFU).



4.1.5 - Discussion

In order to develop bioprocesses using seawater or high salt-containing feedstocks, an important point is to analyze their impact on the growth parameters. A screening performed on solid medium containing different concentration of NaCl (range 0.5–2 M) revealed that among yeasts species isolated from endemic animal fauna living at deep-sea hydrothermal vents (Burgaud et al., 2010). *D. hansenii* was the most halotolerant. In this study, we further investigated the halotolerance of two *D. hansenii* strains, Bio2 and Mo40, by carrying out broth cultures in presence or absence of SS 4% (w/v) (containing 0.55 M NaCl) or 2 M NaCl. The presence of SS did not affect the growth in terms of rate and exerted a faint negative effect only on the biomass yield. The presence of higher NaCl concentration (2 M) produces the same effect on the biomass yield. This can reflect a greater energy expenditure to maintain balanced the internal osmotic pressure in presence of hypertonic conditions. The other growth parameters resulted more influenced in presence of 2 M NaCl, with reduction of 42–46% for the growth rate and of 22–24% for the glucose consumption rate (Table 1). Nevertheless, it is noteworthy that for

the black yeast *Hortaea werneckii*, which is considered a halotolerant/halophilic species, a growth rate decreases of 60% at salinities between 10 and 17% NaCl on defined minimal medium has been reported (Petrovic et al., 2002). Glycerol was accumulated as a compatible solute to maintain osmotic equilibrium (Figure 1). This response, which can be expected at high salt concentration, as already reported (Gori et al., 2005), was a bit surprising in presence of sea salts, due to the ecological origin of the strains. By using FCM, we analyzed at single cell level effects related to the hyper-osmotic stress response. The presence of salts in the growth medium causes a reduced cell volume in Bio2 strain, detected in terms of FSC (Supplementary Figure S1). Conversely no significative variations in terms of cell dimension were detectable in Mo40, suggesting that this response to osmotic stress is strain dependent. This effect has been previously described in *S. cerevisiae* and in *D. hansenii*, but using different analytical methods (Prista et al., 1997; Petelenz-Kurdziel et al., 2011). By staining the cells with permeant dyes like SYBR Green I (Figure 2A) and SYTO^{TM} 24 (Supplementary Figure S3), we observed that the cultivation under hyper-osmotic conditions causes a decreased membrane permeability. In addition, we found that the adaptation to hyper-osmotic conditions is strictly associated to the acquisition of plasma membrane depolarization, which is, however, compatible with the ability to grow under these conditions (Figures 1, 2B). These responses are fast, being detectable after 30 min of salt exposure (hyper-osmotic stress) (Figure 3).

By using CCCP as selective proton gradient uncoupling, we demonstrate that membrane depolarization and loss of membrane permeability are strictly associated (Figure 5). Moreover, these responses are not only specifically triggered by salt-generated stress, but seem to be induced also by high concentration of sorbitol (Supplementary Table S2). Our results indicate that the membrane depolarization and the consequent reduced membrane permeabilization can be considered as general osmotic stress

responses. Interestingly, we observed that hyper-osmotic induced membrane depolarization is reversible. Indeed, the elimination of high osmotic pressure causes a rapid restoration of membrane polarization and in turn restores the cellular permeability (Figure 4), underlining how the coordination of these mechanisms is fundamental in the control of cellular homeostasis. Plasma membrane depolarization can then represent another important mechanism contributing to the fast restoration of ions balance by reducing transporter activities. Upon addition of 1 M NaCl, a significant decrease in the abundance of Pma1 H^+-ATPase, which is the main system pumping protons out of cells, in the *S. cerevisiae* plasma membrane has been reported (Szopinska et al., 2011). Its decreased activity can result in a relative depolarization, and then in a concomitant decrease in the potential-driven influx of toxic sodium cation. On the other hand, a rapid and sharp rise in cytoplasmic calcium level occurs upon osmotic stress, due to a combination of uptake of extracellular Ca^{2+}, release of Ca^{2+} from intracellular stores and its limited vacuolar sequestration (Matsumoto et al., 2002), contributing to the membrane depolarization. Depolarization can change in turn the general properties of the membrane, by a different distribution of the plasma membrane components (Grossmann et al., 2007; Herman et al., 2015). Changes in membrane packing related to more disordered structure and sterol relocalization can make depolarized membrane less accessible, and this could play a significant role in fast cellular responses to acute stress conditions. In accordance with these observations, the plasma membrane of depolarized *S. cerevisiae* cells is less sensitive to detergents and cationic drugs (Grossmann et al., 2007; Maresova et al., 2009). By using this approach, we show that the presence of 2 M NaCl increases the resistance of *D. hansenii* to cationic drugs like Hygromycin B (Figure 6).

We describe that these osmotic stress responses are not species-specific but occur also in the less osmotolerant species *S. cerevisiae* (Figure 7). We can then speculate that similar mechanisms can be operative in *D.*

hansenii as well as in *S. cerevisiae*, resulting in membrane depolarization and in strongly reduced permeability to cationic compounds like SYBR Green I and Hygromycin B.

In addition, we show that permeant dyes like SYBR Green and SYTO™ 24 can be used to evaluate membrane permeability by FCM. In this respect, it is noteworthy that depending on the physiological conditions, the usage of permeant dyes can be problematic. We reasoned in fact that being well known that response to osmotic stress is very fast (Hohmann, 2015), the conditions employed during the staining (taking approximately 30 min) could affect the cells status. In order to maintain the cells during the staining procedure under the same osmotic pressure present during their growth, avoiding hypo-osmotic shock, cells were stained in their respective growth media. When cells growing under hyper-osmotic condition were stained using hypo-osmotic conditions, like PBS or medium without salt (Supplementary Figure S4 and Figure 4), we obtained different results. Due to the reversibility of some cellular responses, like membrane depolarization and altered permeability, the staining conditions are in fact very relevant, and this point to the importance of optimizing the staining procedures in order to obtain correct results and to avoid misinterpretations (Davey, 2011).

Yeasts are exposed to variations of osmotic pressure during biotechnological processes, and such changes are known to play important roles on cellular viability and metabolic activities, by challenging the integrity of cells. The deep knowledge of these aspects is very relevant for the optimization of industrial bioprocesses, particularly in the case of fermentations/bioconversions performed on high nutrients and/or seawater-based media. An efficient transport of nutrients/substrates and products is in fact at the basis of an efficient cellular performance, and this is the essential prerequisite for the efficiency of the whole industrial process. Moreover, these aspects can be also important for the development of new probiotic strains able to survive to bile salts in the gastrointestinal tract.

4.1.6 - References

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4.1.7 - Supplemental materials

Supplementary Table S1. Screening of yeast growth on YNB solid medium containing different concentration of NaCl.

Time (h)	NaCl (%)	Yeast Strains													
		Mo40	Mo 29	Mo35	Mo38	Mo39	Ex7	Ex15	Bio1	Bio2	Mo36	Mo34	Mo31	Mo30	Mo22
24	0	**	**	**	**	**	**	***	***	**	*		*		
	3	*	*	*	*	*	*	**	**	**			*		
	6	*				*		*		**					
	9	*								*					
	12														
48	0	****	****	****	****	****	****	****	****	****	***	*	****		**
	3	****	****	****	****	****	****	****	****	****	***	*	****		**
	6	****	***	****	***	***	***	****	****	****	*	*	****		
	9	***		*		***	*	**	**	**			**		
	12					*				*					
120	0	****	****	****	****	****	****	****	****	****	****	***	****		****
	3	****	****	****	****	****	****	****	****	****	****	****	****	*	****
	6	****	****	****	****	****	****	****	****	****	****	****	****	**	***
	9	****	***	****	***	****	****	****	****	****	***	***	****	**	
	12	****			*	****		****	**	****		**	****		

Abbreviation

Name	Genus	Species	ID number
Bio1	*Candida*	*viswanathii*	UBOCC-A-208001
Bio2	*Debaryomyces*	*hansenii*	UBOCC-A-208002
Ex15	*Pichia*	*guilliermondii*	UBOCC-A-208004
Ex7	*Rhodotorula*	*mucilaginosa*	UBOCC-A-208010
Mo22	*Sporobolomyces*	*Roseus*	UBOCC-A-208018
Mo29	*Cryptococcus*	Sp	UBOCC-A-208024
Mo30	*Phaeotheca*	*triangularis*	UBOCC-A-208025
Mo31	*Candida*	*Atlantica*	UBOCC-A-208026
Mo34	*Hortaea*	*werneckii*	UBOCC-A-208029
Mo35	*Rhodotorula*	*mucilaginosa*	UBOCC-A-208030
Mo36	*Leucosporidium*	*Scottii*	UBOCC-A-208031
Mo38	*Rhodosporidium*	*diobovatum*	UBOCC-A-208033
Mo39	*Candida*	*Marinus*	UBOCC-A-208034
Mo40	*Debaryomyces*	*hansenii*	UBOCC-A-208035

Table S2. SYBR Green I and DiBAC$_4$(3) fluorescences (Arbitrary fluorescence units – AFU) detected by FMC in FL1 channel in *D. hansenii* cells collected under different conditions.

	Bio2		Mo40	
	SYBR Green I	DiBAC$_4$(3)	SYBR Green I	DiBAC$_4$(3)
Exponential growth				
YNB	12152 ± 1725	4236 ± 294	17595 ± 996	2362 ± 225
YNB SS	2866 ± 556	7834 ± 343	7134 ± 1766	3426 ± 285
YNB 2 M NaCl	1452 ± 203	9071 ± 526	7726 ± 760	5583 ± 703
Hyper-osmotic stress	SYBR Green I	DiBAC$_4$(3)	SYBR Green I	DiBAC$_4$(3)
YNB exp growth	12152 ± 1752	4236 ± 294	17595 ± 996	2362 ± 225
30 min YNB SS	3081 ± 508	5853 ± 500	7355 ± 1134	4328 ± 373
30 min YNB 2 M NaCl	2647 ± 225	8495 ± 256	10535 ± 619	6621 ± 392
CCCP	3619 ± 855	10043 ± 979	6217 ± 1107	3383 ± 259
2h YNB 2 M sorbitol	1895 ± 236	10835 ± 563	Nd	Nd
Hypo-osmotic stress	SYBR Green I	DiBAC$_4$(3)	SYBR Green I	DiBAC$_4$(3)
YNB 2 M NaCl exp. growth	1452 ± 203	9071 ± 526	7726 ± 760	4999 ± 703
30 min on YNB	4618 ± 448	2915 ± 288	10568 ± 1442	2658 ± 438

Table S3. SYBR Green I and DiBAC4(3) fluorescences (Arbitrary fluorescence units – AFU) detected by FMC in FL1 channel in *S.cerevisiae* cells collected under hyper-osmotic stress.

	SYBR Green I	DiBAC$_4$(3)
YNB exp. growth	2985 ± 284	4064 ± 332
30 min YNB 0.55 M NaCl	1430 ± 146	9028 ± 772

Figure S1. FCM analysis showing Bio2 and Mo40 cell size (as FSC) under different growth conditions. Blue line: cells exponentially growing on YNB (control cells); green line: cells growing in presence of SS; red line: cells growing in presence of 2 M NaCl.

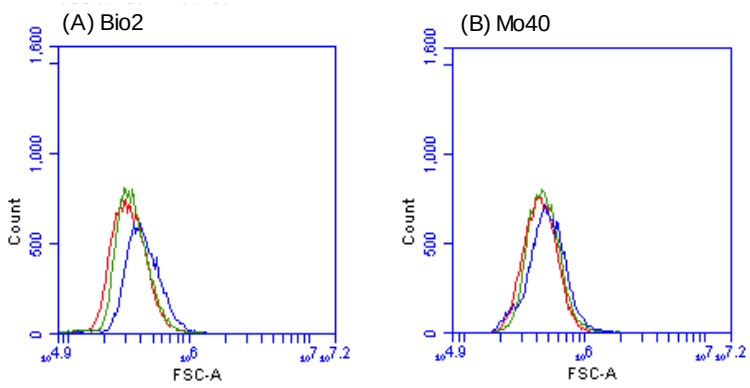


Figure S2. FCM analysis showing SYBR Green I fluorescences detected in FL1 channel. (A) Bio2 cells stained after growth on YNB (control cells); (B) in presence of SS; (C) in presence of 2 M NaCl; (D) Mo40 cells stained after growth on YNB (control cells); (E) in presence of SS; (F) in presence of 2 M NaCl.

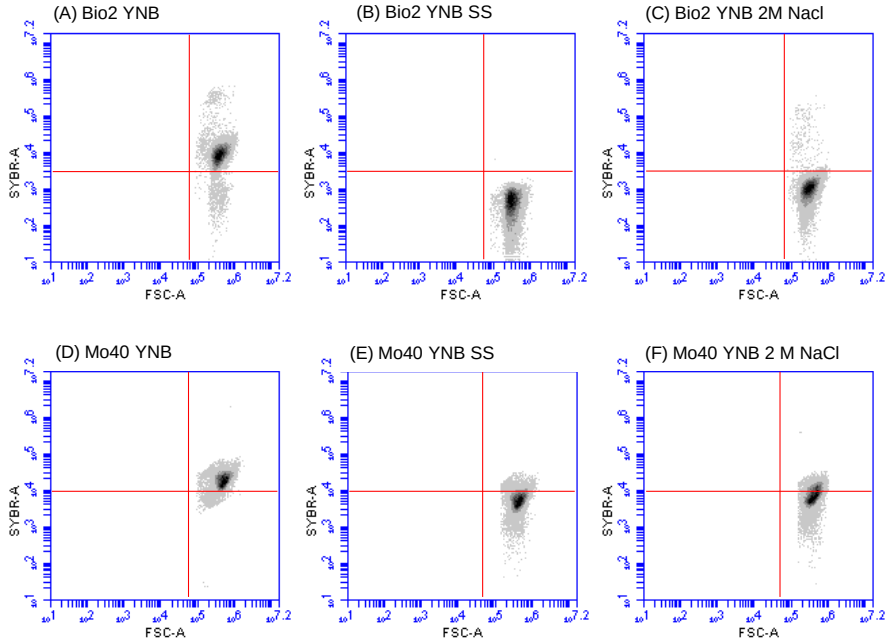


Figure S3. FMC analysis showing SYTO™ 24 fluorescence detected in FL1 channel in Bio2 cells stained after growth (A) on YNB (control cells); (B) on YNB plus SS; (C) on YNB plus 2 M NaCl.

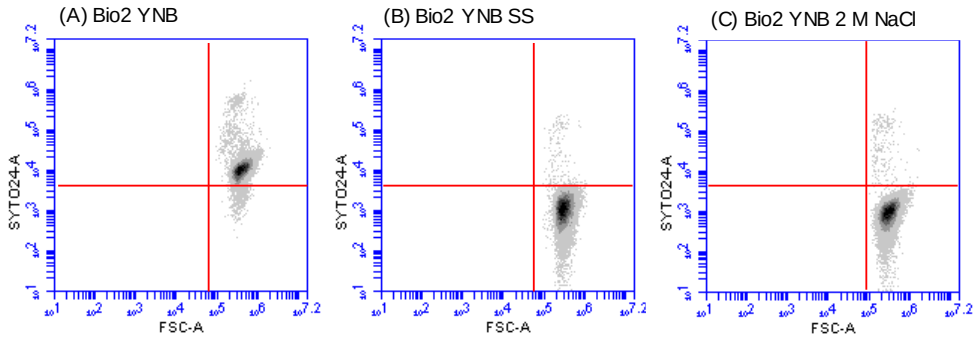


Figure S4. FMC analysis showing SYBR Green I fluorescence detected in FL1 channel in Bio2 cells after permeabilization with ethanol 70 % (v/v) or in cells stained in PBS after growth on YNB (control cells) (A, D), on YNB plus SS (B, E), on YNB plus 2 M NaCl (C, F).

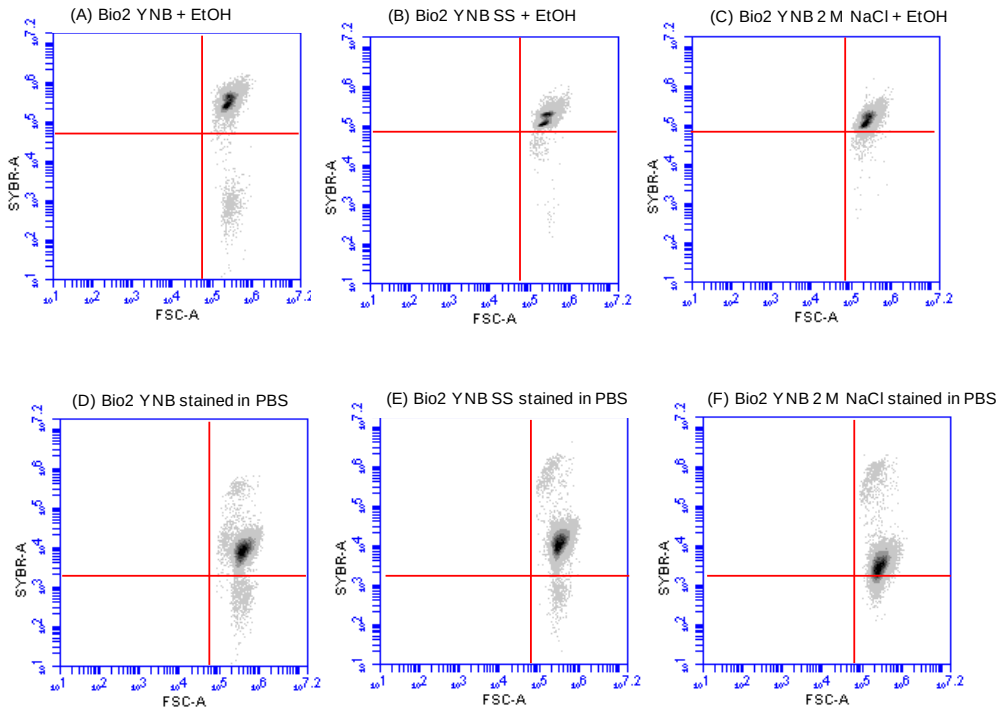
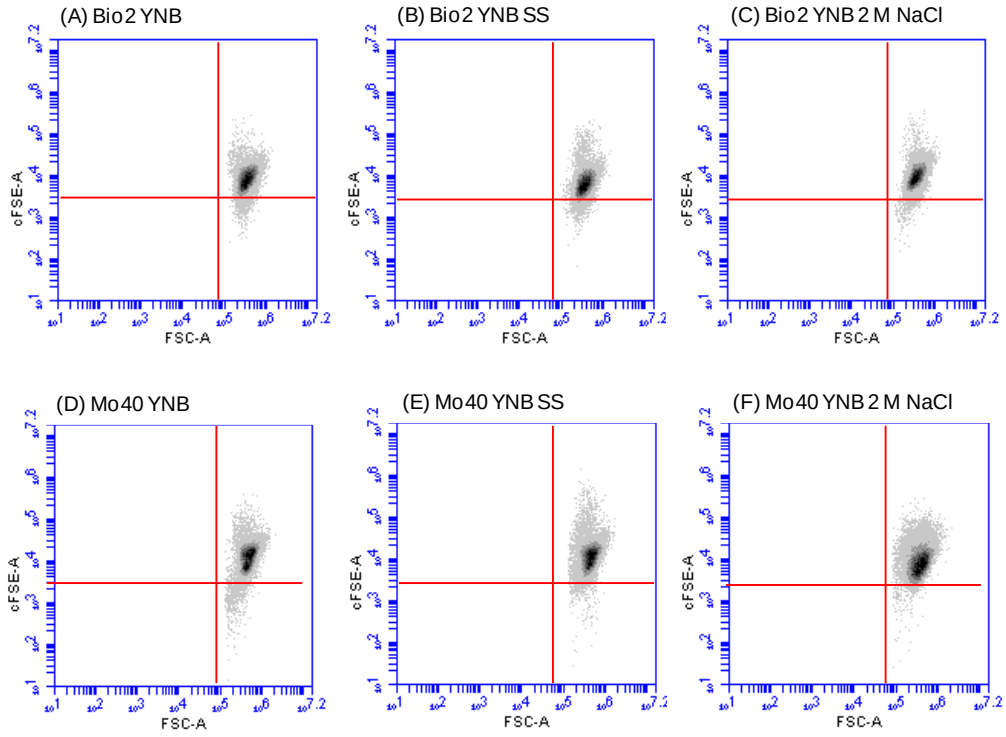


Figure S5. cFSE fluorescence detected in FL1 channel in Bio2 cells collected (A) after growth on YNB (control cells); (B) on YNB plus SS; (C) on YNB plus 2 M NaCl, and in Mo40 cells (D) grown on YNB (control cells); (E) grown on YNB plus SS; (F) grown on YNB plus 2 M NaCl.



4.2 - Optimization of growth conditions for developing bioprocesses with reduced ecological footprint by marine *Debaryomyces hansenii* strain and its potential application in food/feed industries.

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Manuscript in preparation

4.2.1 - Abstract

Science community has focused his attention on the development of new processes with a reduced ecological footprint. In this scenario, the possibility to perform a seawater-based process permits to save huge amount of fresh water. Not all microrganisms are able to grow on media arranged with this water source, due on its salt concentration. In this work we focus our attention on a marine strain of *Debaryomyces hansenii.* We show that this strain is able to grow on industrial media arranged with seawater, exhibiting good growth rate and high biomass yield ($0.627 g_{d.w.}/g_{c.s.}$). This strain has been also characterized for its potential application in food industry, due to its inclusion in QPS EFSA list (Quality Presumption as Safe - European Food Seafty Autority). Capability to produce lipids and to degrade phytic acids could represent an interesting starting point for its potential employment as probiotic as well as yeast biomass additive in food/feed industries.

4.2.2 - Introduction

The importance to develop sustainable processes has been increasing in parallel with the concerns regarding global climate changes. Microbial fermentations are widely used for production of food, fuels and chemicals. This system offers several advantages compared with traditional techniques, by converting waste into high value-added products in a “green manner”. Hence, this technology can now offer a big opportunity to support human reducing fossil fuel dependence and promoting a sustainable society. In this context, optimization of industrial bioprocesses is very relevant (D'Amato et al. 2017). An efficient cellular performance requires capability of cultivating selected microorganisms under conditions suitable for their growth and metabolism. On the other hands, in term of economic and environmental sustainability, biotechnological processes are often performed under limiting

conditions. These challenging conditions could be caused by the presence of high osmotic pressure and/or inhibitors (Trincone, 2017). Inhibitors are common after pre-treatments of feedstocks (Heer et al. 2008).

Among yeast species, *D. hansenii* is considered of great interest due to its salt tolerant features (Gadanho and Sapaio, 2005; Butinar et al. 2005; Prista et al. 2005) which permits its cultivation on media at high concentration of nutrients, and/or on seawater-based media. Cultivation on seawater-based media makes possible the use of more precious fresh water, which is largely consumed during submerged fermentations. In addition, *D. hansenii* is known for its ability of metabolising a broad range of carbon sources by respiratory metabolism. Its biotechnological relevance is linked to the food final aroma and to an altered food composition (Gori et al. 2012). Strains isolated from cheese and fish gut have been recently investigated for potential probiotic properties (Ochangco et al. 2016). Other examples of its biotechnological interest are the use in biocontrol of ochratoxigenic moulds (Iacumin et al. 2017), production of enzymes like exopeptidases and thermophilic β-glucosidases, production of fine chemicals, such as xylitol and riboflavin (Vitamin B2, Breuer and Harms, 2006).

Until recent times *D. hansenii*'s biotechnological potential has been suffering because of a limited availability of efficient molecular tools (Prista et al. 2016), as well as lack of information about cultivation under controlled conditions in bioreactors. Tavares et al. reported about growth in continuous culture on a chemically defined medium at controlled pH 5.5 for production of xylitol (Tavares et al. 2016). Studies on genes involved in xylose transport and industrial use have been carried out due to their biotechnological importance (Pal et al. 2013).

Recently, we have investigated the osmotic adaptation in *D. hansenii* strains, isolated from gasteropod gills (*Ifremeria nautilei*) and from coral (Burgaud et al. 2009). In particular, we demonstrated that membrane

depolarization and decrease of membrane permeability is associated with osmotic stress response (Capusoni et al. 2019). These effects are relevant for the optimization of industrial bioprocesses, particularly in the case of seawater-based processes. High concentrations of nutrients and salts in cultivation media as well as in bioconversion buffers exert a strong osmotic pressure. This might influence the efficiency of solute transport (nutrients, substrates, products) and, in turn, the efficiency of the whole process.

In the present work, we investigated the ability of marine *D. hansenii* strain Mo40 to grow under controlled conditions of bioreactor simulating industrial bioprocesses: a low pH in seawater-based media. In addition, we evaluated the use of glucose/xylose mixtures similar to lignocellulosic hydrolysates, and the use of urea as nitrogen source. *D. hansenii* is a strain included in QPS EFSA list and for this reason it looks like a good candidate for food industrial application (Koutsoumanis et al. 2019).

Literature data show that *D. hansenii* had been characterized as lipids producing strains (Prista et al. 2005). Microbial oils could be employed for biodiesel production or as food/feed supplement.

Furthermore, in the last few decades, marine environment has been identified as good habitat for bioprospectig of enzymatic activity. Marine enzymes are good candidates to be employed in bioprocess thanks to their halotolerance, osmotolerance and organic solvent tolerance (Trincone, 2017). We have also characterized the phytase activity of *D. hansenii,* which is able to grow employing phytic acid as sole phosphorous sources (Olstrope et al. 2009). Phytases are enzymes that catalyse phosphate released from phytic acid and are commonly exploit as additive in order to reduce phytic acid content in feed. Phytate degradation is a lucrative target for investigation due to his negative impact as antinutritional factor, unviable sources of phosphorous and cause of environmental pollution.

4.2.3 - Materials and method

Yeast strains

The yeast strain used in this work was *D. hansenii* Mo40 (Burgaud et al. 2010). For long-term storage, yeast strain was maintained at − 80 °C on 15% (v/v) glycerol and 85% (v/v) YPD media.

Fermentation conditions and media composition

Yeast cells were precultured at 28°C in a rotary shake at 150 rpm in 100 mL bluffed flask containing 20 mL of medium. Optical density was monitored at 600 nm (OD_{600}). Cells were precultured for 24 hours in minimal mineral media, harvested by centrifugation at 5000 rpm and washed tree times with sterile NaCl solution (9 g/L). Then they were used to inoculate the bioreactor at initial OD_{600} 0.05 (almost $5*10^5$ cells/mL).

Aerobic batch cultivations were performed in Applikon system bioreactor with a working volume of 1 l. The temperature was set at 28 °C, the stirring speed at 500 rpm, and the pH, measured by Applisens pH electrode, was adjusted and maintained at 6 or 4.5 by automatic addition of 5 M KOH or 2 M H_2SO_4. The dissolved oxygen concentration (maintained more than 30 % of air saturation) was measured by Applisens polarographic oxygen probe.

Media composition:

YPD: yeast extract 10 g/l, peptone 20 g/l and glucose 20 g/l.

MMP: mineral media were employed for pre-inoculum and for fermentation in bioreactor. Medium composition was glucose 20 g /L, $(NH_4)_2SO_4$ 5g/L, MgSO4* 7 H_2O 0.5 g/L, KH_2PO_4 3 g /L, trace metals (disodic EDTA 15 mg/L, $ZnSO_4$* 4 H_2O 4.5 mg/L, MnCl* 4 H_2O 0.1 mg/L, $CoCl_2$* 6 H_2O 0.3 mg/L, $CuSO_4$* 5 H_2O 0.3mg/L, Na_2MoO_4* 2 H_2O 0.4mg/L, $CaCl_2$* 2 H_2O 4.5 mg/L, $FeSO_4$ * 7 H_2O 3mg/L, H_3BO_3 1 mg/L, KI 0.1 g/L) and vitamins (d-biotin 0.05 mg/L, Ca d(+) panthotenate 1mg/L, nicotinic acid 1 mg/L, myoinositol 25 mg/L, of thiamine hydrochloride 1 mg/L, pyridoxol hydrochloride, p-aminobenzoic acid 0.2 mg/L) as reported in Merico et al 2007 with some modifications.

In pre-inoculum pH 6 was maintained by MES buffer/KOH (2-(*N*-morpholino) ethanesulfonic acid).

To simulate media prepared with seawaters (MMP SS), sea salts at 40 g /L (4%) (S9883) were added to fermentation media.

MMPhy: glucose 20 g /L, $(NH_4)_2SO_4$ 5g/L, $MgSO_4$* $7H_2O$ 0.5 g/L, phytic acid 0.11 g/L sodium salt hydrate (68388 sigma), trace metals and vitamins as in MMP. pH was adjusted at pH 4.5 with the addition of H_2SO_4.

IM (Industrial medium): glucose 33 g/L, xylose 16 g/L, sea salt 40 g /L (4%), yeast extract 2 g/L, corn steep solid 5g/L, urea 2 g/L, KH_2PO_4 3 g /L, H_2SO_4 2 mL/L, $CaCl_2$ 0.1 g/L, NaCl 0.1 g/L. pH was maintained at 4.5 automatically in bioreactor with addiction of 2 M KOH or H_2SO_4 2M .

Medium B: glucose 50 g/L, $(NH_4)_2SO_4$ 1g/L, KH_2PO_4 1g/L, $MgSO_4$ * $7H_2O$ 0.05g/L, NaCl 0.01g/L, $CaCl_2$ 0.01 g/L, yeast extract 1 g/L. pH was maintained at pH 6 with 0.1 M MES buffer/KOH.

Biomass and quantification of compounds

Samples were harvasted from the bioreactor at appropriate intervals and used to monitor the cell growth measuring the optical density at 600 nm with a spectrophotometer, after appropriate dilution. For dry weight determination, washed culture samples were filtered on a 0.45-µm glass microfiber GF/A filter (Whatman) and dried 24 h at 80°C. The concentration of carbon sources, such as glucose and xylose on supernatants were determined by commercial enzymatic kits (Roche, cat. numb. 1 0716251 035, and Megazyme cat number K-XYLOSE 04/18 respectively). All the assays were performed in triplicate and the standard deviations varied between 1 and 5 %. Specific consumption rates of glucose and xylose were calculated during the exponential phase of growth. The yield of biomass was calculated as the total amount of dry weight divided by the consumed carbon sources.

Lipids production

Experiments performed to analyze lipids production were carried on in 500 mL bluffed flask with 100 mL of Medium B. Pre-inoculum was performed in YPD and cells were inoculated at an initial concentration of 0.1 OD_{600} (1 x 10^6 cells/mL) from seed cultures. Lipids yield is reported as g $_{lipids}$/g $_{d.w.}$

Lipids determination

Lipid content was determined via the sulpho-phospho-vanilline colorimetric method (Spinreact) on washed cell pellets (30 OD), suspended in 0.5 mL of cold redistilled water. The assays were performed in triplicate and standard deviations varied between 1 and 5%.

Phytase

Genomic extraction: To isolate genomic DNA, pellets of 30 OD 600nm of cells were resuspended in 0.5 ml of 0.05 M Tris–HCl/0.02 M EDTA at pH 7.5. This suspension was transferred to a precooled tube with an equal volume of glass beads (425–600 µm). Mechanical lysis was performed using a TissueLyser LT alternating 2 min of agitation at 50 Hertz with 1 min in ice for 4 cycles. The supernatant was added with 25 µl of SDS 20% (w/v) and incubated at 65°C for 30 min. Immediately, 0.2 ml of 5 M potassium acetate was added and the tubes were placed on ice for 30 min. Sample were centrifuged at 13000 rpm for 5 min and supernatant was transferred to a fresh microcentrifuge tube. The DNA was precipitated by adding 1 volume of isopropanol. After incubation at room temperature for 5 min, the tubes were centrifuged for 10 min. The DNA was washed with 70% ethanol, and dissolved in 50 µl of TE RNAsi (10 mMTris-HCl, 1 mM EDTA, pH 7.5 RNAsi 100 µg/mL). Samples were incubated at 37°C for 30 min (Donzella et al. 2019).

Phytase sequence of *D. hansenii* Mo40 were obtained in this work. Phytase gene was amplified from gDNA using primers: Forward Phy1 CCG ACC ATG GAT GGT ATC GAT TTC C, Reverse Phy2 CAT CGG ATC CTA ATT GTC ACC GGA. Primers were designed employing *D. hansenii* CBS 767 sequence.

PCR amplification was carried out by denaturing at 98°C for 7 min, followed by 30 cycles of denaturing at 98°C for 10 sec, annealing at 59°C for 30 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 10 min. The produced amplicon was cloned in a plasmid psf URA TPI and sequenced by Mycrosynt company. Mo40 and CBS 767 phytase sequence were aligned and compared using http://multalin.toulouse.inra.fr/multalin/ online tools.

Determination of phytase activity: Cells were precultured for 24 hour in YPD, harvested by centrifugation at 5000 rpm and washed three times with sterile NaCl solution (9 g/L) and inoculated at initial OD_{600} 1 (almost 10^7 cells/mL) in MMPhy. After 48 h of incubation phytase activity was determined. Extracellular enzymatic activity was detected on supernatant, and cell-bound (intracellular) activity using whole cells. The activity was measured by orthophosphate production, following ammonium molibdate blue method as reported in Schimizu 1992 with some modifications. For extracellular activity determination, cell cultures were centrifuged at 13000 rpm and 1 mL of supernatant was added to 4 ml buffer composed by 0.2 M Na acetate/acetic acid, 8 mM phytic acid at pH 4.5. To determine cell-bound activity we used a homogeneous cells suspension. We set up enzymatic activity using a standard amounts of cells 50 OD (corresponding to almost 10 $mg_{d.w.}$ depending on the strain) in final volume of 5 mL. Cells were collected and washed twice with 0.2 M Na acetate/acetic acid pH 4.5 and resuspended in a final volume of 1 mL. Cell suspension was added to 4 ml of buffer 0.2 M Na acetate/acetic acid, 8 mM phytic acid at pH 4.5. All buffer employed to test enzymatic activity were prewarmed at reaction temperature. Blank was assembled using 1 ml of 0.2 M Na acetate/acetic acid at pH 4.5 and 4 mL of 0.2 M Na acetate, 8mM phytic acid and treated as sample.

For enzymatic activity determination 5 ml reaction were incubated in 15 ml tube at 37°C and stirred at 300 rpm. The reaction was immediately stopped (time 0) and stopped after 15, 30, 60, 120 min. Reaction was stopped mixing 0.5 mL of reaction with 0.5 mL TCA 5% solution, samples were centrifuged 3

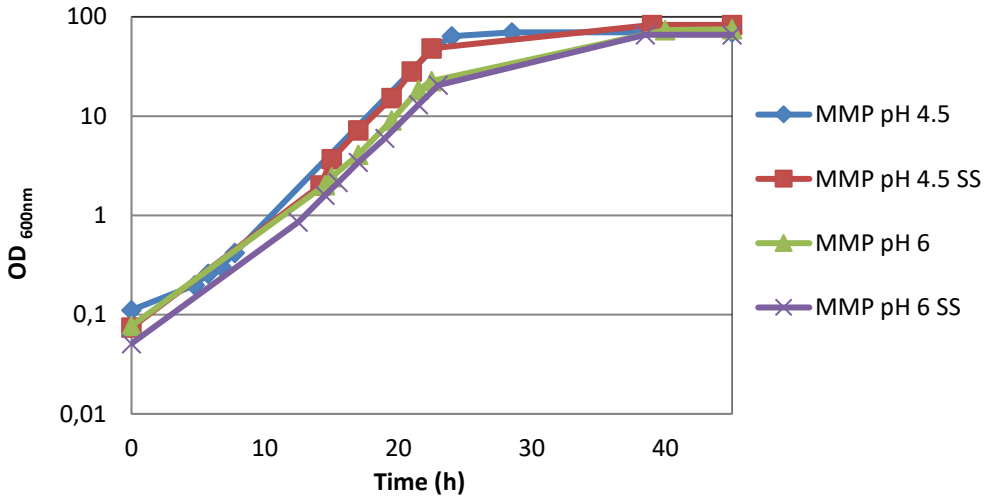
min at 13000 rpm and the supernatant collected. In order to determine orthophosphate concentration, 0.4 mL of supernatant was added to 0.4 mL of molibdate solution. This solution was prepared daily, by mixing solution A and B in a ratio of 4:1 (solution A: 2.6% $N_6H_{24}Mo_7O_{24}$* $4H_2O$ and 5.5% H_2SO_4; solution B: 4.6 %$FeSO_4$*$7H_2O$). The sample was incubated 10 min at 25°C and read against blank at OD_{700}. Phosphate concentration was determined using a standard curve for KH_2PO_4. Unit of phytase is defined as the amount of protein that hydrolyze 1 µmol phosphorus per minute. Specific activity is expressed in mU/mg of cell dry weight. To determine the effect of temperature, samples prepared with prewarmed buffer were incubated at 50°C and 60°C.

4.2.4 - Results and discussion

Optimization of growth conditions in bioreactor

In our previous work we analyzed the effect that the presence of 4% sea salts (SS – the same of seawater), exerts on the growth of two different *D. hansenii* strains isolated from marine environment (Capusoni et al. 2019). Following the results described in Capusoni et al 2019, we decided to proceed focusing our attention on Mo40 strain, which seems to be resistant in presence of high salt concentration. In order to optimize cultural condition suitable for the development of an industrial bioprocess, we analyzed the growth not only in presence of sea salts, simulating the use of seawater-based media, but also at two different pH values: pH 4.5 and pH 6. Low pH permits to reduce risk of bacterial contamination, that are more common at neutral/alkaline pH.

Figure 1. Growth of *D. hansenii* Mo40 strain under different process conditions. SS indicates presence of sea salts.



For comparison, other cultures were arranged on media without salt addition (MMP medium, control cultures). Cultures were performed in bioreactor under controlled aerobic condition, and the initial pH values were maintained during the entire process.

Figure 1 shows the kinetics of growth obtained under different culture conditions. The absence of any lag phase suggested that the marine *D. hansenii* very quickly adapted to the presence of sea salts (pre-inoculum was cultured on medium without sea salts). The high degree of adaptation under different growth conditions is very relevant for an industrial strain (Prista et al. 2005).

Analyzing the data reported in Table 1, it is possible to observe, by comparing MMP cultures, that no negative effect results on growth parameters by lowering the pH of the process. On the contrary, the presence of sea salts (3% NaCl and 1% of other salt species) affects the growth at different pH values.

Table 1. Growth parameters of *D. hansenii* Mo40 cultivated in presence of sea salts 4%(SS) and in Industrial media (IM).

		μ max	Biomass yield	Q Glucosio	Q Xylose
		[h^{-1}]	[$g_{d.w.}/g_{c.s.}$]	[$mmol_{glc}/g_{dw}$ /h]	[$mmol_{xiyl}/g_{dw}$ /h]
pH 4,5	MMP	0.318 ± 0.010	0.611 ± 0.016	2.835 ± 0.098	-
	MMP SS	0.298 ± 0.008	0.552 ± 0.015	2.467 ± 0.088	-
	IM	0.302 ± 0.001	0.627 ± 0.008	2.561 ± 0.144	2.983 ± 0.100
pH 6	MMP	0.315 ± 0.012	0.637 ± 0.007	2.57 ± 0.121	-
	MMP SS	0.346 ± 0.013	0.624 ± 0.009	3.249 ± 0.132	-

In particular, if we focus our attention on the processes performed on media with sea salts (MMP SS), we observe a reduction of 6% in growth rate, 10% biomass yield and 13% in glucose consumption rate at pH 4.5. Analysing data in Table 1 at lower pH the osmotic response, due to the presence of salts, caused a higher metabolic burden, with some negative consequences on growth performance. On the other hand, we need to consider that such reduction could be compensated by the possibility to use conditions like low pH and seawater-based media. The capability to grow and reach high biomass yield under these conditions can be very useful for developing industrial bioprocesses with a reduced ecological foot-print. High osmotic pressure and acidic pH are not suitable for most of microorganisms.

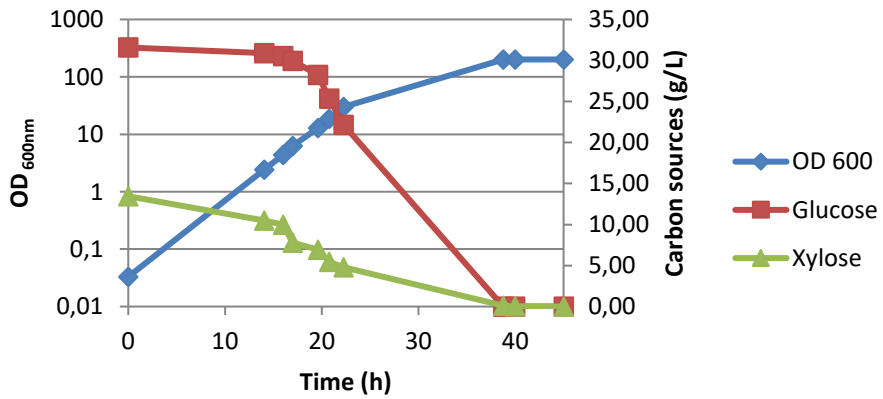
In addition, we observed that the scale-up in bioreactor had a positive effect on the process performance. Growth rate and glucose consumption rate are similar to the one already published in Capusoni et al 2019, but biomass yield showed an increase of 36% (Capusoni et al. 2019 – chapter 4.1.4). In particular this last parameter moved from 0.38 $g_{d.w.}/g_{c.s.}$ detected in flask fermentation to an average of 0.61 $g_{d.w.}/g_{c.s.}$ detected in bioreactor system (Table 1). This improvement is caused by the more efficient aeration system that is found in bioreactor. *D. hansenii* exhibits a respiratory metabolism, and in flask oxygen concentration is easily limiting. This restricted condition has a

strong negative effect on biomass yield and can be fully overcome by a scale-up of the entire process. Indeed, in bioreactor is possible to maintain the dissolved oxygen concentration over 30 % of air saturation.

Simulating an industrial bioprocess

These experiments were performed in order to set-up a process simulating an industrial medium and, more in general, industrial conditions. With this aim, we prepared a medium (IM) with seawater, at pH 4.5. To simulate the use of a lignocellulosic hydrolized, the medium contains glucose and xylose as carbon source. Urea was employed as nitrogen source. This aspect is important since urea is cheaper than nitrogen salts (Sukumaran et al. 2018). Corn steep, which is widely present in industrial media formulation, was also supplemented. The analysis of the kinetic reported in Figure 2 leads to the conclusion that *D. hansenii* was able to efficiently grow on this medium, and no lag phase was observed. As expected, this yeast was able to consume both glucose and xylose (Prista et al 2016). In particular, it is interesting to point out that in *D. hansenii* no glucose repression is observed, indeed both carbon sources were simultaneously metabolized (Figure 2).

Figure 2. Growth of *D. hansenii* on industrial media (IM) .



This capability is very important in order to set-up an industrial bioprocess employing agri-food wastes. This specific kind of substrate contains several sugar species, and the capability to consume all of them at the same time provides several advantages in terms of productivity. Data reported in Table 1 indicate that IM composition did not affect *D. hansenii* fitness. The principal growth parameters like growth rate, glucose consumption rate and biomass yield remained similar to those observed in the process on mineral medium (MMP).

In conclusion, the ability to grow in seawaters-based media even at low pH coupled with the ability to use a wide range of carbon sources from wastes make *D. hansenii* a good candidate to be employed in industrial bioprocesses. This has a positive impact both on costs and on environment, since it reduces the ecological footprint and enables the development of circular economy.

Lipids production

D. hansenii has been reported as lipids accumulating species (Prista et al. 2005). Since few data are available in literature, we decided to focus our attention on this specific feature. Hence the cells were cultured in Medium B, a lipidogenic medium characterized by low nitrogen content, this feature is known to induce lipids accumulation (Donzella et al. 2019). Lipids production was analyzed after 24, 48, 72 h of incubation (Table 2).

Table 2. Mo40 lipids production. Cell were cultivated in Medium B (lipidogenic medium).

Time (h)	Lipids %
24	9 ± 0.9
48	11.5 ± 1.7
72	20.7 ± 2.5

On this medium, *D. hansenii* reached stationary phase after 24 hours of incubation. At this point, lipids started to accumulate until 20.7%$g_{lipids}/g_{d.w.}$ after

72 hours of incubation. Other species of oleaginous yeasts are able to produce lipids with higher yield (Ratledge and Wynn, 2002), but due on its QPS state, it could be interesting to determinate fatty acids composition. Science community and industries are interested in finding alternative ways to produced fatty acids (eg polyunsaturated fatty acids - PUFAs), to be used as feed/food additive (Uemura, 2012). *D. hansenii* could be employed, after optimization process, as cell factory or as sources of genes for lipids production.

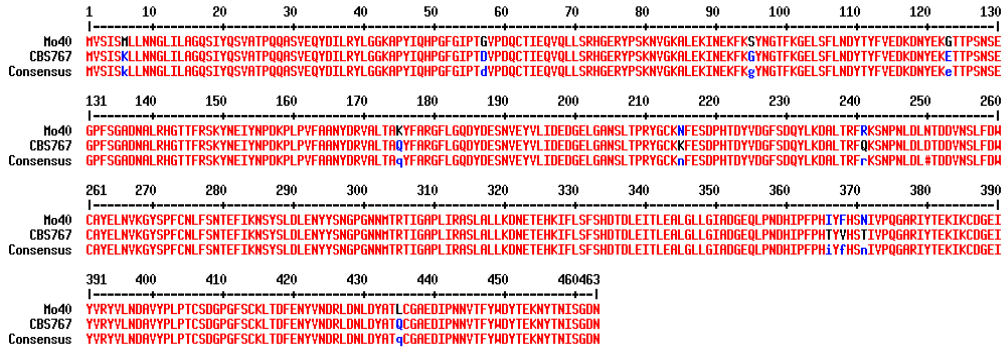
Recently, *D. hansenii* has been studied for probiotic applications (Ochangco et al. 2016). The possibility to have a huge number of cells, thanks to its high biomass yield, with a good lipidic content/profile should be very appealing. Indeed, combining these characteristics it would be possible to produce new generation food supplements.

Phytase activity

Recently, marine yeasts have been exploited as source of enzyme useful in bioprocesses. Salt tolerance showed by these enzymes is often connected with solvent tolerance, that make them more resistant to industrial bioprocess. Preliminary literature data (Olstorpe et al. 2009) reported that *D. hansenii* is able to growth employing phytic acid as sole phosphorous sources. Based on these findings, and since phytases are employed as feed/food ingredients, we decided to investigate the phytase activity of our *D. hansenii* strain.

We started sequencing Mo40 phytase gene in order to compare a marine phytase sequence with its terrestrial counterpart. The reference strain is named CBS 767. Analyzing the two sequences, it is possible to deduce that the two phytases are different (Figure 3). In particular, Mo40 gene presents 46 different nucleotidic substitutions which result in 9 different aminoacids. These modifications could be connected with an evolutionary process that could confer an increased halotolerance.

Figure 3. Sequence alignment between phytase sequence of Mo40 (marine strain) and CBS 767 (*D. hansenii* type strain). The different amminoacids are indicated in blue the conserved one in red.



Phytase could be use in aquacultures (Vohra et al. 2011), but the isolation of an enzyme with an increased salts resistance could be useful for its application also in marine aquaculture systems.

Analyzing the aminoacid sequence, this enzyme belonged to phytase acid class, confirmed by the presence of the conserved motifs RHGERYP (72-79 aa) and HD (334-335 aa). Furthermore, it was possible to speculate that *D. hansenii* phytase could be a cell-bound enzyme. This hypothesis was confirmed by the lack of activity detected on the supernatant and corroborated by the absence of secretion signal sequence. In order to determine phytase activity, *D. hansenii* was cultivated in MMPhy medium. Phytase activity, detected at 37°C and 60°C, was respectively 0.57 mU/$mg_{d.w.}$ and 5.03 mU/$mg_{d.w}$ (Table 3).

Table 3. Phytase activity detected at 37° on cell grown in presence of phytic acid as sole phosphorous surces.

	Cell-bound	Extracellular
60 °C	5,03 ± 0,513	BDL
37 °C	0,57 ± 0,051	BDL

Although these activities are lower in comparison to the ones reported in literature, they permit the growth of *D. hansenii* in presence of phytic acid as sole phosphorous source.

In conclusion, the possibility to employ the biomass of this strain containing a phytase activity could be relevant not only to increase the proteins content of feed, but also to reduce environmental pollution due on the excretion of undigested phytate. In this respect, it could be important to evaluate the capability to degrade phytic acid in seawater, because yeast biomasses are often added to aquaculture feed as sources of proteins (Vohra et al. 2011). This capability could be exploited also for the development of a new probiotic strain. Following this assumption, some research groups retains the phytic acid degradation fundamental for food application (Puppala et al. 2019).

4.2.5 - Conclusion

In this work we studied the optimization of a bioprocess for its scaling-up at industrial level, employing a marine strain of *D. hansenii.* We show that this strain is able to grow at low pH on seawater-based media containing urea as cheap nitrogen source and glucose/xylose mixtures, with high biomass yield. Preliminary characterization experiments indicate that this yeast strain is able to produce lipids and to degrade phytic acid. The above capabilities coupled with its inclusion in QPS EFSA list make this *D. hansenii* strain a good candidate to be employed in food industries.

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4.3 - Marine microorganisms for biocatalysis: selective hydrolysis of nitriles with a salt-resistant strain of *Meyerozyma guilliermondii*

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4.3.1 - Abstract

A screening among marine yeasts was carried out for nitrile hydrolyzing activity. *Meyerozyma guilliermondii* LM2 (UBOCC-A- 214008) was able to efficiently grow on benzonitrile and cyclohexanecarbonitrile (CECN) as sole nitrogen sources. A two-step one-pot method for obtaining cells of *M. guilliermondii* LM2 (UBOCC-A-214008) endowed with high nitrilase activity was established; the resulting whole cells converted different nitriles with high molar conversions and showed interesting enantioselectivity toward racemic substrates. Nitrilase from *M. guilliermondii* LM2 (UBOCC-A-214008) displayed high activity on aromatic substrates, but also arylaliphatic and aliphatic substrates were accepted. Salt-resistant *M. guilliermondii* LM2 (UBOCC-A-214008) was used in media with different salinity, being highly active up to 1.5 M NaCl concentration. Finally, hydrolysis of nitriles was efficiently performed using a bioprocess (yeast growth and biotransformation with resting cells) entirely carried out in seawater.

4.3.2 - Introduction

Important traits for the development of biotechnological process- es are the ability, exhibited by some microorganisms, to tolerate some ofthemost common stresses in industrial processes such as high osmotic pressure, high temperature, unfavorable pHs, and presence of organic cosolvents. The search of microorganisms with improved properties has led to an increased interest of scientific community in marine habitat as location for enzyme bioprospecting activity. This is due to the great potential of marine enzymes compared to the terrestrial counterparts, which is related to the ecological niches where they live (hydrothermal vents, oceanic caves, and high-pressure areas). As marine organisms live in high salinity environments, their enzymes are expected to have peculiar properties, for example high salt tolerance, thermo-stability, barophilicity, and cold adaptivity (Trincone 2011).

Exploitation of extremophile microorganisms from marine environment can represent a reliable alternative to mesophilic sys- tems. Recently, species having these characteristics have been isolated from extreme environments, such as deep-sub-seafloor sediments (Chi et al. 2009; De Vitis et al. 2015; Margesin and Schinner 2001; Rédou et al. 2015; Zaky et al. 2014). Moreover, the use of marine microorganisms opens the way to the use of seawater as alternative to potable water both as fermentation and biotransformation medium, thus improving the greenness of the bioprocess. The use of water as solvent in bioprocesses is highly desirable from the sustainable chemistry point of view and, in this frame, seawater, accounting for 97% of the world's water, can be a promising alternative water resource (Anderson 2012; Domínguez de María 2013; Zambelli et al. 2015).

Nitriles are widely spread in nature and synthetic nitriles are commonly used in organic chemistry as solvents, building blocks in the synthesis of drugs, plastics, resins, dyes, pesticides, and cosmetics (Banerjee et al. 2002). Their conventional chemical hydrolysis requires harsh reaction conditions (acidic or alkaline pH and temperature above 100 °C) and it is plagued by the formation of undesired by products and large amounts of waste (Debabov and Yanenko 2011). Enzymatic hydrolysis of nitrile compounds is an established alternative method to avail a broad spectrum of useful amides and carboxylic acids (Martínková and Křen 2010).

Two possible routes do exist to achieve the hydrolysis of nitriles (Debabov and Yanenko 2011). The first one relies on the action of nitrilases (EC 3.5.5.1) which catalyze the direct cleavage of organic nitriles into carboxylic acid and ammonia. The second route involves two subsequent reactions: the first one is catalyzed by a nitrile hydratase (EC 4.2.1.84) which transforms nitrile into amide that is thus hydrolyzed to carboxylic acid by an amidase (EC 3.5.1.4). Nitrilases can be further divided into three subgroups depending on their substrate specificity (aromatic, aliphatic and arylaliphatic), although some of them are proven to accept a broad range of substrates. Most

of nitrilases are derived from bacteria, filamentous fungi, and plants while yeast nitrilases are less frequently described and exploited, despite more than 60 nitrile-metabolizing yeasts have been isolated (Gong et al. 2012). In addition, the two-step hydrolysis catalyzed by nitrile hydratase and amidase is more frequently described in yeast. In this work, a collection of yeasts isolated from deep subseafloor sediments and deep-sea hydrothermal vents (Burgaud et al. 2015; Rédou et al. 2015) was investigated for the ability to metabolize and use nitriles as nitrogen sources. The prodution of the nitrilase activity in the selected strain was optimized and the resulting biocatalyst was tested for its activity toward different aromatic, aliphatic, and arylaliphatic nitriles. Moreover, the whole process (fermentation and biotransformation) was set up in seawater, demonstrating the feasibility of the green approach.

4.2.3 - Materials and methods

General

All reagents and solvents were obtained from Sigma Aldrich (Milano, Italy) and used without further purification or drying. TLC was performed with Merck silica gel 60 F254 pre-coated plates. HPLC analyses were performed by using a Merck- Hitachi equipped with a UV/Vis detector Merck-Hitachi. Natural seawater (pH 8) was collected from the Camogli beach (Genova, Italy) and micro-filtered prior use; a water salinity of 35 PSU was reported by ARPA (Agenzia Regionale Prevenzione e Ambiente) for this area.

Strains and Growth Conditions

A collection of marine yeasts was previously created from deep sub-seafloor sediments and deep-sea hydrothermal vents (Burgaud et al. 2015; Rédou et al. 2015). All isolates are available in the UBO Culture Collection (http://www.univ- brest.fr/ubocc) (Online resources 1).

For long-term storage, yeast strains were maintained at −80 °C on 15% (vol/vol) glycerol and 85% (vol/vol) yeast peptone dextrose (YPD) medium (10 g/L yeast extract, 20 g/ L peptone, 20 g/L glucose).

Cell growth was monitored by measuring the increase of optical density at 600 nm (OD_{600}) using a spectrophotometer (Jenway 7315; Bibby Scientific Limited, Stone, UK). Liquid cultures were grown at 28 °C under shaking (150 rpm). Cells from pre-cultures grown overnight on YPD were harvested by centrifugation (5000 rpm, 10 min), washed with 0.9% NaCl and inoculated at an optical density of 0.1 into the final culture medium.

The following media were used for yeast cultivation:

YPD medium: 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose.
Yeast nitrogen base (YNB) mineral medium: 20 g/L glucose, 1.7 g/L yeast nitrogen base (YNB) without amino acids and ammonium (Difco, Detroit, MI), 0.1 M MES pH 6.
Corn steep dextrose (CSD) medium: 20 g/L glucose, 0.75 g/L $(NH_4)_2SO_4$, 1 g/L corn steep, 0.5 g/L $MgSO_4$, 1g/L K_2HPO_4, 0.1 g/L CaCl2, and 0.1 M MES pH 6 (fresh water).
Seawater CSD medium was prepared with the same composition of CSD medium but prepared with seawater and filter sterilized.

For induction with shift method, cells grown in YPD were harvested by centrifugation (5000 rpm, 10 min), washed with 0.9% NaCl, and inoculated at an optical density of 10 or 35 (OD10 or OD35) in YNB medium supplemented with cyclohexanecarbonitrile (6–50 mM). The cells suspension was then incubated at 28 °C under shaking (150 rpm) for 3 – 36 h for nitrilase induction.

For induction in CSD medium, after exhaustion of nitrogen (24 h), cells were diluted to OD10 (2 gdry weight/L) with fresh CSD medium lacking $(NH_4)_2SO_4$ and corn steep. Cyclohexanecarbonitrile (12.5 mM) was then

added and the cell suspension was incubated at 28 °C under shaking (150 rpm) for 16 h.

Screening for nitrile utilization

Yeasts were cultured on Petri dishes containing solid mineral medium (YNB) and either benzonitrile or cyclohexanecarbonitrile (0.2 g/L, 0.4 g/L, 0.6 g/L) as sole nitrogen source. The nitrile was added to the agar at 45–50°C, and the homogenous mixture was poured in Petri dishes. Different amounts of yeast cells (10^5,10^4, and 10^3 cells) were spotted on the surface of the agar plates. Then, the plates were incubated at 30 °C for 3 days, and the ability to metabolized nitriles was estimated by colony formation. The control experiment was prepared by spotting yeasts on mineral medium and glucose without the addition of nitrile.

Glucose, nitrogen, and dry weight determination

The concentrations of glucose and inorganic nitrogen were determined in culture supernatants by employing commercial enzymatic kits (Roche, R-Biopharm Italia), after removal of the cells by centrifugation (5 min at 5000 rpm). Total nitrogen (organic and inorganic) concentration in culture supernatants was determined by the Kjeldahl method using a Speed Digester K-376 and a KjelMaster K-375 (Buchi Italia). Dry weight determination was performed after the removal of the cells from the medium by filtration (0.45 µmglass microfiber GF/A filter; Whatman). The filters were washed with three volumes of deionized water and dried overnight at 105°C.

Biotransformations

The induced cells were harvested from the growth/induction media by centrifugation (10 min at 5000 rpm), washed twice with the biotransformation buffer and then resuspended in the same buffer. The nitrilase activity of resting cells was determined in reaction mixtures (5 mL final volume) containing 50 µmol of substrate and an appropriate amount of cells (2 $g_{\text{dry weight}}$/L) in

phosphate buffer (0.025 M pH 7.0). The reaction mixtures were incubated at 28 °C and maintained under orbital shaking at 150 rpm. The effect of pH was determined under standard conditions using 0.025 M sodium acetate buffer (pH 5.0), 0.025 M sodium phosphate buffer (pH 6.0–8.0), 0.025 M Tris-HCl buffer (pH 9.0). Alternatively, microfiltered seawater or phosphate buffer supplemented with NaCl were used as reaction media. The assays with phathalonitrile and homophtalonitrile contained in addition 1% (v/v) of DMSO in order to dissolve the substrates. After different time intervals, samples (0.5 mL each) were taken and the reactions were stopped by the addition of 1 M HCl (0.05 mL). The samples were then extracted with ethyl acetate. The organic phase was collected, evaporated, and analyzed by HPLC. Specific activity was determined when the conversion of the substrate was below 15%. 1 U/mg corresponds to the amount of enzyme that converts 1 µmol of substrate per minute per milligram of cells dry weight.

Analytical methods

The different nitriles, benzamide and their corresponding acids were analyzed by HPLC. For the achiral analysis, a reversed-phase Lichrospher RP18 5 µm (Merck) was used (mobile phase: H_2O/acetonitrile 60:40 + 0.1% trifluoroacetic acid, 1 mL/min, 220 nm). Retention times: benzonitrile, 8.1 min; benzoic acid, 4.6 min; benzamide, 2.9 min; 2-phenylacetonitrile, 7.9 min; 2-phenylacetic acid, 4.3 min; phathalonitrile, 6.4 min; 2 cyanobenzoic acid, 4.0 min; phthalic acid, 2.7 min; 2-(cyanomethyl)benzonitrile, 6.3min; 2-(2-cyanophenyl)acetic ac- id, 4.0 min; and 2-carboxyphenylacetic acid, 2.9 min. Separation of 4-(trifluoromethyl)benzonitrile, 2-(4-(trifluoromethyl) phenyl)acetonitrile and corresponding acids was achieved with a Purosphere STAR RP-18 endcapped 3 µm (Merck) (mobile phase: H_2O/acetonitrile 50:50 + 0.1% trifluoroacetic acid, 0.5 mL/min, 222 nm, 40 °C). Retention times: 4-(trifluoromethyl)benzonitrile, 2. 2 min; 4-(trifluoromethyl)benzoic acid, 1 .4 min;

2-(4-(trifluoromethyl)phenyl)acetonitrile, 2.1 min; and 2-(4-(trifluoromethyl)phenyl acetic acid, 1.3 min.

Separation of the enantiomers of chiral compounds was achieved with a Lux-Cellulose 3 (Phenomenex) (mobile phase: n-hexane/2-propanol 98:2 + 0.1% trifluoroacetic acid, 1 mL/min, 220 nm). Retention times: (R)-2-phenylpropionitrile, 8.6 min; (S)-2-phenylpropionitrile, 8.9 min; (R)-2-phenylpropionic acid, 26.5 min; (S)-2-phenylpropionic acid, 28.8 min; (R)-2-phenylbutanenitrile, 7.7 min; (S)-2-phenylbutanenitrile, 8.6 min; (R)-2-phenylbutyrric acid, 23.9 min; and (S)-2- phenylbutyrric acid, 25.7 min.

For separation of enantiomers of mandelonitrile and mandelic acid, the mobile phase composition was n-hexane/2-propanol 90:10 + 0.1% trifluoroacetic acid. Retention times: (R)-mandelonitrile, 10.3 min; (S)-mandelonitrile, 12.8 min; (R)-mandelic acid, 11.8 min; and (S)-mandelic acid, 13.6 min.

Biotransformations of aliphatic nitriles were monitored by quantifying the corresponding acids in the not-extracted samples using a Rezex ROA organic acid column (mobile phase 0.005 N H_2SO_4, 1 mL/min, 60 °C, 210 nm). Quantification was achieved by using calibration curve prepared with authentic standards. Retention times: cyclohexancarboxylic acid, 42.7 min; isovaleric acid, 31.5 min; malonic acid, 12.9 min; and 2-cyanoacetic acid, 13.7 min

Lyophilized cell preparation

After cultivation and induction, cells were harvested by centrifugation (5000 rpm, 10min), washed once with 0.9% NaCl, and suspended in 10 mL of either deionized water, 1 M sorbitol, or 0.9% NaCl. The cell suspension was then frozen and freeze dried under vacuum for 48 h. The lyophilized preparations were stored under anhydrous environment at room temperature

and rehydrated in the biotransformation buffer for 15 min at 28 °C under orbital shaking at 150 rpm before use.

4.3.4 - Results

Screening for nitrile utilization

A collection of 20 marine yeasts from deep sub-seafloor sediments and deep-sea hydrothermal vents (Burgaud et al. 2015; Rédou et al. 2015) (Online Resource 1) was screened on solid medium containing either benzonitrile or cyclohexanecarbonitrile as sole nitrogen source (range of con centration 0.02–0.06%). Only Me*yerozyma guilliermondii* strains (eight strains) showed the ability to grow under the tested condition (Table 1); nitrilase and amidase activities were previously observed in strains of *M. guilliermondii* (Dias et al. 2000; Zhang et al. 2017). The investigation was then focused on *M. guilliermondii* LM2 (UBOCC-A-214008) that has been previously demonstrated as one of the most halotolerant strains.

Constitutive or induced nature of nitrilase

To test the constitutive or induced nature of nitrilase, *M. guilliermondii* strain LM2 (UBOCC-A-214008) was cultivated on YPD or in the same medium in presence of an inducer (12.5 mM benzonitrile or cyclohexanecarbonitrile), and the presence of nitrilase activity was evaluated as the ability of whole cells (2 $g_{dry\ weigh}$t/L) to convert benzonitrile (10 mM) into benzoic acid. Cells collected after 48 h of growth did not perform any conversion, even when the inducers were present in the cultivation medium, indicating that nitrilase activity in *M. guilliermondii* is not constitutively expressed.

Table 1 Growth of marine yeasts on solid YNB medium containing different nitriles as sole nitrogen source (0.02% - 0.06%). n.d: not detectable

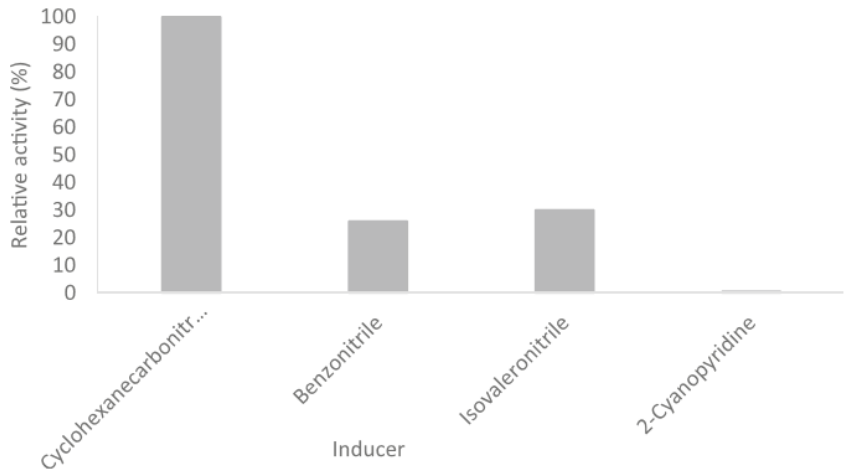
	Microorganism	Benzonitrile	Cyclohexanecarbonitrile
Mo40	*Debaryomyces hansenii*	Nd	Nd
Mo34	*Hortaea werneckii*	Nd	Nd
Mo31	*Candida atlantica*	Nd	Nd
Mo29	*Cryptococcus* sp.	Nd	Nd
Mo35	*Rhodotorula mucilaginosa*	Nd	Nd
Mo38	*Rhodosporidium diobovatum*	Nd	Nd
Mo39	*Candida marinus*	Nd	Nd
Ex7	*Rhodotorula mucilaginosa*	Nd	Nd
Ex15	*Meyerozyma guilliermondii*	Yes	Yes
Bio1	*Candida viswanathii*	Nd	Nd
Bio2	*Debaryomyces hansenii*	Nd	Nd
LM 16	*Rhodotorula mucilaginosa*	Nd	Nd
LM 18	*Rhodotorula mucilaginosa*	Nd	Nd
LM 2	*Meyerozyma guilliermondii*	Yes	Yes
LM 1	*Meyerozyma guilliermondii*	Yes	Yes
LM 3	*Meyerozyma guilliermondii*	Yes	Yes
LM 5	*Meyerozyma guilliermondii*	Yes	Yes
LM 6	*Meyerozyma guilliermondii*	Yes	Yes
LM 7	*Meyerozyma guilliermondii*	Yes	Yes
LM 9	*Meyerozyma guilliermondii*	Yes	Yes

Likewise, cells cultivated on YPD for 24 h and then shifted to a mineral medium (YNB) containing ammonium sulfate (5 g/L) and cyclohexanecarbonitrile (12.5 mM) as inducer showed that after 24 h they were still unable to convert benzonitrile. This result suggested that nitrilase activity could be under nitrogen repression. To demonstrate this hypothesis, cells were cultivated for 48 h in mineral medium YNB containing 12.5 mM cyclohexanecarbonitrile (CECN) as sole nitrogen source and nitrilase activity was tested. These cells (used at 2 $g_{dry\ weight}$/L concentration) completely converted 10 mM benzonitrile after 2 h of bioconversion. These evidences suggested that the induction of nitrilase is effective only in complete absence of other nitrogen sources.

Optimization of nitrilase induction and cell cultivation method

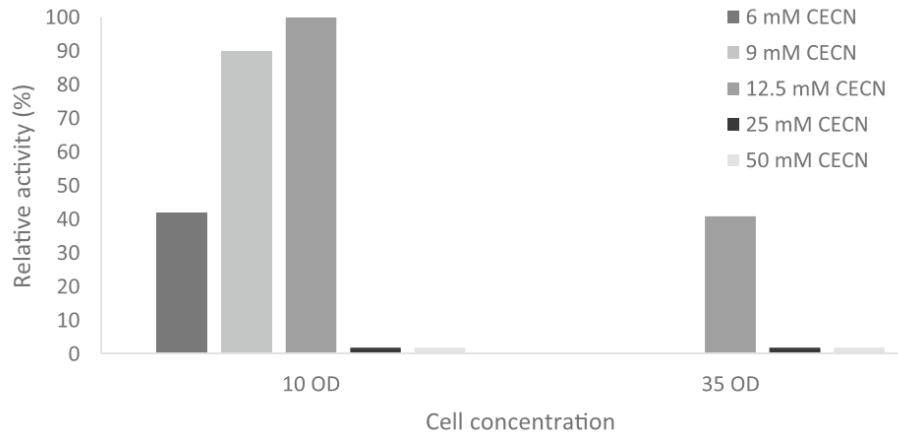
Several studies reporting the use of nitrile utilizing microorganisms describe the induction of the enzyme by growing cells in mineral media containing nitrile compounds as sole nitrogen source (de Oliveira et al. 2013; Dias et al. 2000; Kaplan et al. 2006). However, this strate gy is plagued by the long times required for the growth and by the low biomass production, due to nitrile toxicity that limits the amount of usable nitrogen. Thus, we attempted to set-up a procedure based on the separation between the growth phase (aimed at biomass production), and the nitrilase induction phase. To identify the most efficient inducer, cells cultivated for 24 h on YPD were transferred, at an optical density of 10, to mineral medium in presence of different inducers (benzonitrile, cyclohexanecarbonitrile, isovaleronitrile, and 2- cyanopyridine) as sole nitrogen source, for additional 24 h. The recovered cells proved to possess the highest nitrilase-specific activity (0.08 U/$mg_{dry\ weight}$) when induced by 12.5 mM cyclohexanecarbonitrile (CECN) (Fig. 1).

Fig. 1 Induction of nitrilase activity by different compounds (12.5 mM each), calculated as specific activity on 10 mM benzonitrile.



To optimize the nitrilase expression, an appropriate ratio between the amount of cells and inducer was established. Two concentrations of cells (2 gdry $_{weight}$/L and 7 $_{gdry\ weight}$/ L, corresponding to 10 OD and 35 OD, respectively) were exposed toward increasing concentrations of CECN (6– 50 mM) for 24 h. Figure 2 shows that the optimal CECN concentration for obtaining the highest induced activity was 12.5 mM. In fact, lower concentrations of CECN (6–9mM) provided lower induced activity, whereas concentrations higher than 12.5 mM negatively affected nitrilase activity, even at higher cell concentration, probably because of nitrile toxicity. On the other hand, higher concentration of cells exhibited a lower nitrilase activity when induced in presence of 12.5 mM CECN. In conclusion, the proper cell/inducer ratio was found to be 2 $g_{dry\ weight}$/L of cells (corresponding to 10 OD) and 12.5 mM CECN (0.08 U/$mg_{dry\ weight}$).

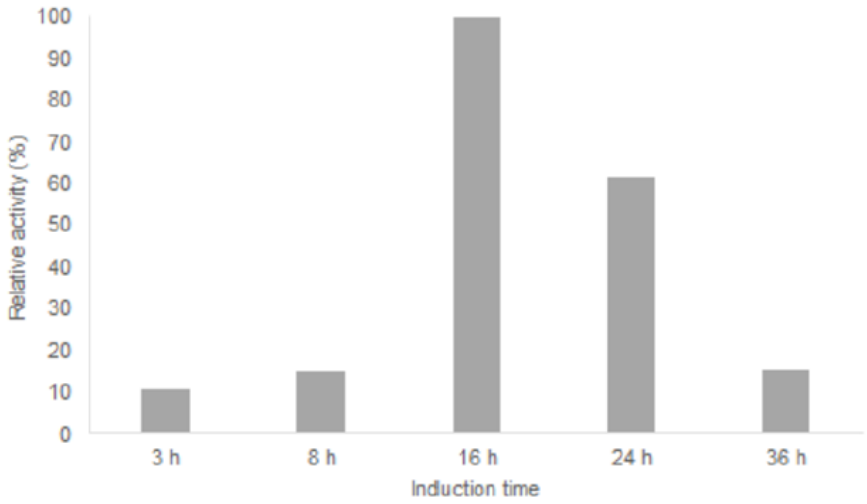
Fig. 2 Effect of inducer (cyclohexanecarbonitrile)/cells concentration ratio on nitrilase specific activity (assessed on 10 mM benzonitrile).



The time required for reaching the highest nitrilase activity was then investigated. Cells collected after different periods of induction in presence of 12.5 mM CECN were analyzed by assaying nitrilase activity. After 16 h from the addition of CECN, a nitrilase activity of 0.12 U/$mg_{dry\ weight}$ was obtained (Fig. 3), but this level subsequently decreased, probably due to the exhaustion of

CECN. These findings prompted us to assess the feasibility of a two-step one-pot culture method that could allow reaching nitrilase-induced biomass production and in short time. To set-up this strategy, cultivations on media containing different carbon to nitrogen ratio were performed, with the aim to obtain in 24 h the growth of biomass and nitrogen exhausted, and then to induce the maximum of nitrilase expression in the following 16 h. During the growth phase, nitrogen and trace elements were supplied as ammonium sulfate and corn steep, which is a suitable industrial source. After the growth phase on CSD medium, (20 g/L glucose, 0.75 g/L $(NH_4)_2SO_4$,1 g/L corn steep, 0.5 g/L $MgSO_4$,1 g/LK_2HPO_4, 0.1 g/L $CaCl_2$, 0.1M MES pH 6), when nitrogen was exhausted, the biomass was induced by addition of fresh medium lacking both nitrogen and corn steep and containing CECN to achieve the proper biomass/inducer ratio (biomass 2 $g_{dry\ weight}$/L and 12.5 mM CECN) (see Materials and Methods section 4.3.3 for details). At the end of this process, the biomass showed 0.06 U/$mg_{dry\ weight}$ of nitrilase-specific activity. In addition, for comparison, we tested also the one-step process, assessing the effect caused by the presence of inducer into the medium since the beginning.

Fig. 3 Induction time of nitrilase specific activity (assessed on benzonitrile) by 2 $g_{dry\ weight}$ /L of cellsand 12.5 mM CECN.

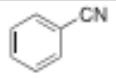
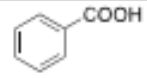
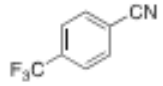
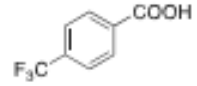
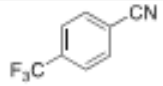
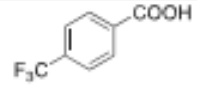
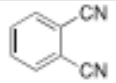
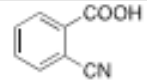
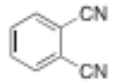
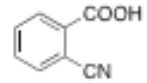
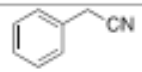
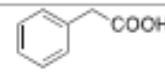
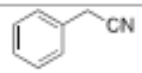
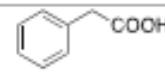
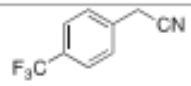
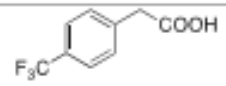
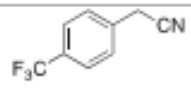
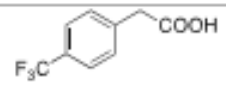
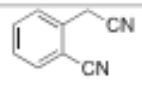
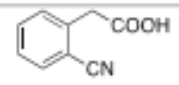
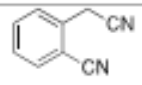
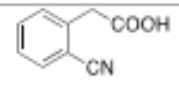


In this case, more than 50% reduction in specific nitrilase activity (0.024 $U/mg_{dry\ weight}$) was obtained. This was probably due to the unfavorable cell/inducer ratio (biomass 4 g/L and 12.5 mM CECN), in accordance with the results showed above (Fig. 2). Interestingly, we observed that the nitrilase-specific activity obtained by the processes performed in CSD medium containing corn steep was lower than that obtained by cultivating the cells in YPD and inducing them in mineral medium YNB. This could be ascribed to the lack of some essential components such as trace elements initially provided by corn steep, but that were not restored when the biomass, for the induction phase, was diluted in CSD medium lacking corn steep. This hypothesis was confirmed by cultivating cells on CSD medium and diluting, for nitrilase induction, with YNB-N-free medium and 12.5 mM CECN. In this case, a higher specific nitrilase activity was in fact obtained (0.1 $U/mg_{dry\ weight}$). In conclusion, an efficient strategy in two steps was developed to obtain biomass and nitrilase induction: the first step implies using the nitrogen sources useful to obtain yeast biomass, the second step at the proper biomass/inducer ratio and in presence of trace elements.

Substrate Scope

Induced whole cells of *M. guilliermondii* were tested for bio-transformation of different aromatic mono- and di-nitriles (Table 2).

Table 2 Hydrolysis of different achiral aromatic nitriles with whole cells of *M. guilliermondii* LM2 (UBOCC-A-214008). Biotransformations were carried out with freshly prepared cells (2 $mg_{dry\ weight}$/mL) suspended in phosphate buffer (0.025 M, pH 7) and 10 mM substrate at 28 °C. Molar conversions were determined by HPLC analysis.

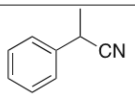
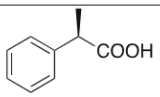
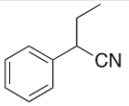
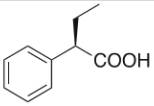
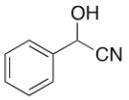
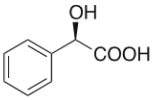
Entry	Substrate	Product	Yield (%)	Time (h)
1			>95	2
2			84	2
3			95	3
4			< 3	2
5			10	24
6			90	2
7			>95	3
8			56	2
9			90	5
10			12	2
11			70	24

Benzonitrile and 4-(trifluoromethyl)benzonitrile were converted rapidly and quantitatively by whole cells of *M. guilliermondii* LM2 (UBOCC-A-214008) (entries 1–3, Table 2), whereas phthalonitrile furnished the corresponding monoacid (2-cyanobenzoic acid) with low yield (entries 4 and 5, Table 2), most likely for the steric hindrance due to the ortho substituent; the negative influence ofthe cyano group in ortho position was confirmed in the biotransformation of 2-(cyanomethyl)benzonitrile, which was selectively converted to 2-(2-cyanophenyl)acetic acid with no formation of the corresponding diacid (entries 10 and 11, Table 2), as previously observed with other nitrilases (Hoyle et al. 1998; Meth- Cohn and Wang 1997). Nitrilase of *M. guilliermondii* catalyzed also the quantitative conversion of 2-phenylacetonitrile and 2-(4-(trifluoromethyl)phenyl)acetonitrile into the corresponding carboxylic acids (entries 6–9, Table 2).

It should be underlined that only $2mg_{dry\ weight}$/mL of whole cells were used in these trials, indicating a high-specific activity of the nitrilase involved in these biotransformations. Experiments were also carried out using benzamide (10 mM) as substrate, but only sluggish activity was observed (< 5% conversion after 24 h), thus showing that the activity involved in the −C ≡N hydrolysis is mostly due to true nitrilase(s).

Whole cells of *M. guilliermondii* LM2 (UBOCC-A-214008) were also used for hydrolyzing racemic nitriles (Table 3); *M. guilliermondii* LM2 (UBOCC-A-214008) enantioselectively produced optically pure (R)- mandelic acid with high yield by dynamic resolution of the racemic substrate (entry 3, Table 3), due to spontaneous racemization of (S)-mandelonitrile, as previously observed with other nitrilases (Zhang et al . 2016).

Table 3 Hydrolysis of different racemic aromatic nitriles with whole cells of *M. guilliermondii* LM2 (UBOCC-A-214008). Biotransformations were carried out with freshly prepared cells (2 $mg_{dry\ weight}$/mL) suspended in phosphate buffer (0.025 M, pH 7) and 10 mM substrate at 28 °C. Molar conversions and enantiomeric excesses were determined by HPLC analysis.

Entry	Substrate	Product	Yield (%)	ee (%)	Time (h)
1			76	75	24
2			80	65	48
3			95	>97	48

(R,S)- 2-phenylpropionitrile was converted with good-to-moderate enantioselectivity (E= 16) to (R)-2-phenylpropionic acid; this substrate is hydrolyzed by nitrilases with very different enantioselectivities: nitrilases from *Synechocystis sp*. and *Neurospora crassa* OR74A converted (R,S)-2-phenylpropionitrile with little enantiopreference (E=0–2) (Mukherjee et al. 2006), whereas the arylacetonitrilase from *Aspergillus niger* CBS 513.88 produced (R)-2- phenylpropionic acid with an e.e value of 90% at 28% conversion (E =24–26) (Petrícková et al. 2012). (R,S)-2-phenylbutanenitrile was hydrolyzed with lower enantioselectivity (E=5–6), which is in accordance with what observed with other nitrilases.

Finally, the biotransformation was also carried out with aliphatic substrates: isovaleronitrile was converted into the corresponding acid with good conversion (92% after 24 h), whereas malononitrile gave 2-cyanoacetic acid with low rates and yield (10% after 24 h). Table 4 summarizes the activity of *M. guilliermondii* LM2 (UBOCC-A-214008) toward the different substrates evaluated.

The comparison of the relative activity shows that nitrilase of *M. guilliermondii* has a marked preference for aromatic nitriles, but arylaceto- and aliphatic nitriles are accepted as well.

Long-term stability of cell-bound nitrilase

Lyophilized cells can be easily produced and maintained, while avoiding costly and time-consuming purifications (Spizzo et al. 2007). The effect of biomass lyophilization on nitrilase activity was then assayed. The induced cells were lyophilized using either H_2O, 0.9% NaCl or 1 M sorbitol as lyophilizing solvents. Upon lyophilization the cells maintained 85% of the starting activity (3 h were required to achieve the complete conversion of 10 mM benzonitrile), without substantial differences among the employed lyophilizing solvents (Fig. 4). Moreover, the lyophilized cells were stable over the time, maintaining the same initial activity after 1-month storage at room temperature under anhydrous conditions (Fig. 4). The lyophilized cells were tested for their activity toward benzonitrile at different pH values (Fig. 5). Nitrilase activity was very poor (< 10%) at pH below 6, while the cells were highly active at pH between 6 and 9.

Fig. 4 Stability of lyophilized cells using different lyophilizing solvents. Conversions of 10 mM benzonitrile after 3 h are reported.

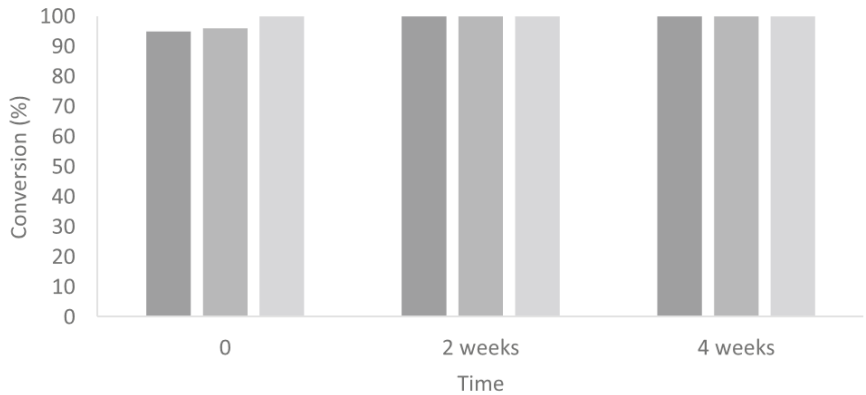


Fig. 5 Activity of lyophilized cells on 10 mM benzonitrile at different pHs. pH 7 is considered as 100%.

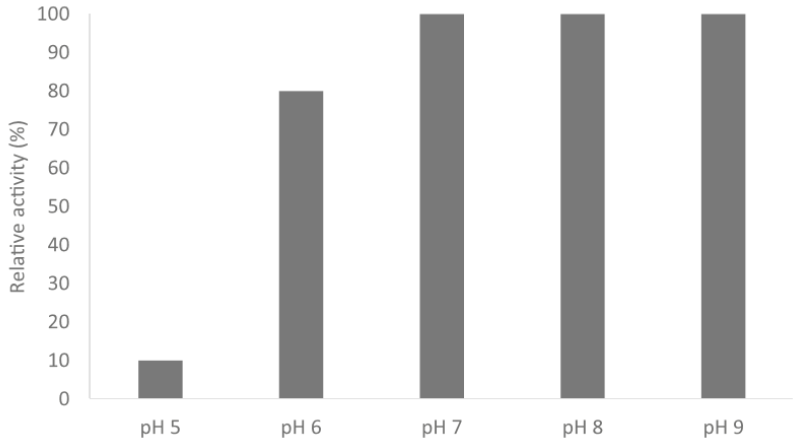
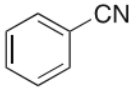
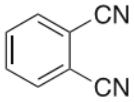
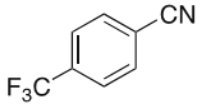
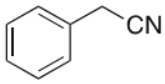
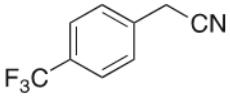
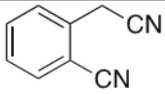
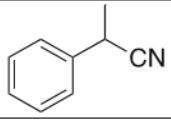
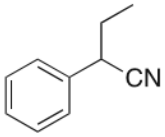
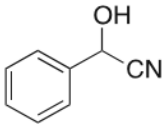
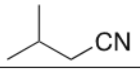
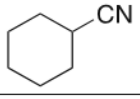


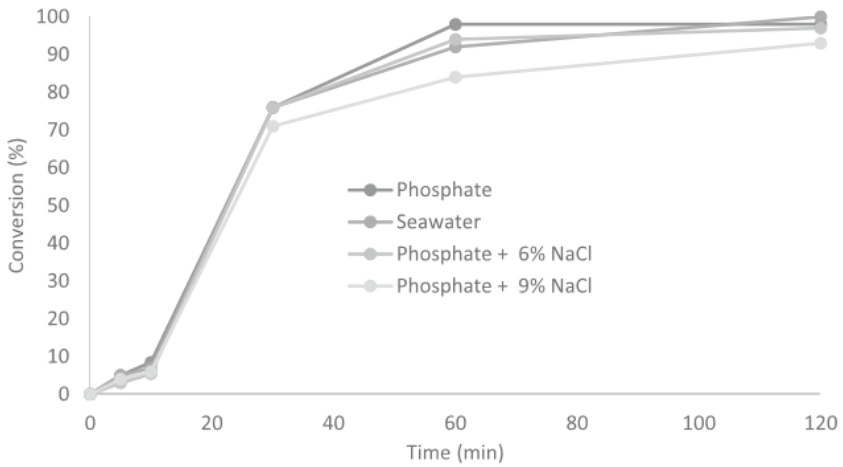
Table 4 Relative activity of resting cells of *M. guilliermondii* LM2 (UBOCC-A-214008) toward different nitriles

Substrate	Relative activity (%)
	100
	1
	90
	80
	78
	14
	8
	6
	7
	15
	4
	8

Seawater Process

The whole process was set up in a seawater-based media. Cells grown and induced in seawater-CSD medium were firstly assayed for the conversion of 10 mM benzonitrile in phosphate buffer and displayed the same activity of cells grown in conventional CSD medium (0.06 U/mgdry weight). Moreover, similar time-courses were observed when the biotransformation was carried out in seawater and in media with increasing concentrations of NaCl (Fig. 6).

Fig. 6 Conversion of 10 mM benzonitrile in media with different salinity *by M. guilliermondii* LM2 (UBOCC-A-214008) grown in seawater- based medium.



Finally, cells prepared in seawater-CSD medium were used for the dynamic resolution of racemic mandelonitrile in seawater. Since the reaction was quite slow using a biocatalyst concentration of 2 $mg_{dry\ weight}$/mL (95% conversion after 48 h), the biotransformation was accomplished on a semi-preparative scale (100 mL) using 20 $mg_{dry\ weight}$/mL of *M. guilliermondii* LM2 (UBOCC-A-214008), achieving the complete conversion into (R)-mandelic acid after 8 h.

4.3.5 - Discussion

Microbial nitrilases are interesting enzymes for catalyzing the hydrolysis of nitriles into carboxylic acids under mild conditions (Gong et al. 2012; O'Reilly and Turner 2003; Thuku et al. 2009). Several yeast species show the ability to metabolize nitriles, although most of them exhibit a nitrile hydratase-amidase system (Brewis et al. 1995; Gong et al. 2012; Rezende et al. 1999). The aim of our study was to characterize nitrilase activity in marine yeast strains. Bio-prospecting of marine microorganisms from deep-sea extreme habitats appears like a promising way to identify new enzymes endowed with resistance toward high ionic strength and polar solvents, including new nitrilases (de Oliveira et al. 2013). Marine yeasts have been recently exploited for biotechnological applications, especially to perform industrial bioprocesses by employing seawater (Serra et al. 2016; Zaky et al. 2014); although pretreatments (e.g., filtration) can affect the overall costs, seawater can represent an alternative medium to the large consumption of freshwater, especially considering the increasing shortage of fresh water in few areas of the world (Domínguez de María 2013). Among the tested species, only *M. guilliermondii* strains showed the ability to use benzonitrile and cyclohexanecarbonitrile as sole nitrogen source. For the establishment of a potential biocatalyst suitable in an industrial bioprocess, the optimization of nitrilase production is an essential goal. This can be achieved by settling cultural conditions that allow obtaining nitrilase-induced cells. Induction experiments were performed in order to find the most efficient inducer, as well as the proper cells/inducer ratio and the shortest time required to reach high nitrilase activity. Nitrilase activity was found under nitrogen repression, being induced exclusively after exhaustion of any other nitrogen source. Among different tested nitriles, cyclohexanecarbonitrile (CECN) induced the highest specific activity after 16 h, at a proper cell/inducer ratio of 2 $g_{dry\ weight}$/L and 12.5 mM nitrile. 2-cyanopyridine that is reputed as a universal inducer in filamentous fungi (Martínková et al. 2009) was not effective in *M. guilliermondii*

LM2 (UBOCC-A-214008) strain. Based on these results, we were able to set up an efficient culture and induction method that works in two steps but in short time, in comparison with other reported methods utilized for yeasts and fungi (de Oliveira et al. 2013;Kaplanet al. 2006; Vejvoda et al. 2006; Rustler et al. 2008; Rezende et al. 1999;Dias et al. 2000). We also showed that the presence of trace elements in the medium is essential not only for biomass production, but also plays a positive effect for reaching high level of nitrilase-specific activity; a similar effect of metal ions has been already observed for *Alcaligenes* sp. ECU0401 nitrilase (He et al. 2010).

M. guilliermondii LM2 (UBOCC-A-214008), optimized for growth and nitrilase activity, converted aromatic substrates with high yields, being also able to transform arylacetonitriles and aliphatic nitriles. This feature allowed for the conversion of chiral α-substituted benzyl nitriles, such as 2-phenylpropio nitrile, 2- phenylbutanenitrile and mandelonitrile with, in all the cases, a strong enantiopreference for hydrolysis of the R- enantiomer, as often encountered with nitrilases. The biotransformation of racemic mandelonitrile with nitrilases is known to be an efficient method to obtain enantiomerically pure (R)-mandelic acid in high yields, since the unreacted (S)-mandelonitrile spontaneously racemizes, thus allowing for a theoretical yield of 100% of (R)-mandelic acid (Yamamoto et al. 1991). The yeast nitrilase system of *M. guilliermondii* LM2 (UBOCC-A- 214008) could thus hydrolyze various nitriles with good activity and high yields, while showing very poor amidase activity, differently from what was previously reported for a different *M. guilliermondii* strain (Dias et al. 2000). Interestingly, the substrate scope displayed by the nitrilase of *M. guilliermondii* LM2 (UBOCC-A-214008) was different than that reported for other strains of the same species, making a difficult comparison in terms of specific activity although the activity of *M. guilliermondii LM2* (UBOCC-A-214008) toward isovaleronitrile and mandelonitrile was lower than what observed with *M. guilliermondii* CGMCC12935 grown on a synthetic medium.

The genome of *M. guilliermondii* ATCC 6260 contains two sequences coding for hypothetical proteins that belong to the nitrilase superfamily. The expression and characterization of these two enzymes would provide new insights on the nitrile hydrolyzing system of *M. guilliermondii* LM2 (UBOCC-A-214008). Long-term stability of the cell-bound nitrilase was proved using lyophilized cells, which could be maintained at room temperature for weeks without significant loss of activity. Nitrile hydrolysis was also performed in media with high salinity; this salt-resistant strain of *M. guilliermondii* was able to convert benzonitrile in a medium containing up to 1.5 M NaCl with the same efficiency observed in conventional buffers.

Finally, a seawater-based biocatalytic process was established, where seawater was used to perform both production of the microbial biomass and biotransformation. *M. guilliermondii* LM2 (UBOCC-A-214008) grown in a seawater-based medium was used for the semi-preparative preparation of(R)-mandelic acid from racemic mandelonitrile in seawater, achieving high conversion (95%) accompanied by high enantiomeric excess (> 98%).

4.3.6 - References

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4.3.7 - Supplemental materials

Microrganisms list

Strain ID	Genus	Species	UBOCC number
Bio1	*Candida*	*viswanathii*	UBOCC-A-208001
Bio2	*Debaryomyces*	*hansenii*	UBOCC-A-208002
Ex15	*Meyerozyma*	*guilliermondii*	UBOCC-A-208004
Ex7	*Rhodotorula*	*mucilaginosa*	UBOCC-A-208010
Mo29	*Cryptococcus*	sp	UBOCC-A-208024
Mo30	*Phaeotheca*	*triangularis*	UBOCC-A-208025
Mo31	*Candida*	*atlantica*	UBOCC-A-208026
Mo34	*Hortaea*	*werneckii*	UBOCC-A-208029
Mo35	*Rhodotorula*	*mucilaginosa*	UBOCC-A-208030
Mo36	*Leucosporidium*	*scottii*	UBOCC-A-208031
Mo38	*Rhodosporidium*	*diobovatum*	UBOCC-A-208033
Mo39	*Candida*	*marinus*	UBOCC-A-208034
Mo40	*Debaryomyces*	*hansenii*	UBOCC-A-208035
LM1	*Meyerozyma*	*guilliermondii*	UBOCC-A-214022
LM2	*Meyerozyma*	*guilliermondii*	UBOCC-A-214008
LM3	*Meyerozyma*	*guilliermondii*	UBOCC-A-214007
LM5	*Meyerozyma*	*guilliermondii*	UBOCC-A-214013
LM6	*Meyerozyma*	*guilliermondii*	UBOCC-A-214014
LM7	*Meyerozyma*	*guilliermondii*	UBOCC-A-214004
LM9	*Meyerozyma*	*guilliermondii*	UBOCC-A-214143

4.4. - Screening for yeast phytase leads to the identification of a novel cell-bound and secreted activities in *Cyberlindnera jadinii* CJ2

Capusoni, C., Serra, I., Donzella, S., Compagno, C.

Manuscript in preparation

4.4.1 - Abstract

Phytate or phytic acid is an antinutritional compound able to chelate proteins and ions, hence food industry is looking for a convenient method which allow its degradation. Phytases are a class of enzymes and their function is to catalyze the degradation of phytic acid and are used as addictive in food-related industrial processes. Due to phytase importance in food industry, our goal was to identify activities of new phytases, in order to select the ones characterized by improved performances. Industrial process requires enzymes characterized by tolerance to high temperature and acid pH. After initial screening on several yeast species, we focused our attention on phytases belonging to three different microorganisms: *Cyberlindnera jadinii*, *Kluyveromyces marxianus* and *Torulaspora delbrueckeii*. In particular *C. jadinii* was the strain with the highest cell-bound activity and the only one that showed extracellular activity. In *C. jadinii* phytase showed optimum of temperature at 50°C, but high activity was detected at 60°C also. The optimum pH was detected at 4.5. The tolerance of *C. jadinii* phytase to high temperature and low pH suggest that this enzyme could be used without any restriction in food-related process.

4.4.2 - Introduction

Enviromental impact of phytate

Phytate ($C_6H_{18}O_{24}P_6$), also known as phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate), is the main source of stored phosphorus in grains, pulse, oil seeds and nuts. (Mullaney and Ullah, 2003). This acid typically represents up to 4-5% of seed dry weight, and 60-80 % of total phosphorous in seed (Sanagelantoni et al. 2018). This compound plays an important role during seed germination and seedling growth, because it serves as storage form of *myo*-inositol and phosphorus. Phytic acid is synthesized in plants starting form glucose-6-P, that is first converted to 1D-

myo-inositol-3-phospate and subsequentially completely phosphorylated (Shi et al. 2005). Furthermore, due to its nature of polyanionic chelating agent, it can be also considered as an antioxidant specie. Indeed, it is able to form stable complexes with Cu^{3+} and Fe^{3+}, preventing formation of reactive oxygen species responsible of cellular damages and carcinogenesis (Graf et al. 1987).

The presence of phytic acid creates problems in breeding, being feeds mainly composed by vegetal materials, rich in this acid. Polygastric animals are able to degrade phytate, thanks to their particular gut microbiota (Nakaschima et al. 2006), *vice versa* this process does not occur in the monogastric ones, like poultry, pigs and fishes. Since phytic acid cannot be metabolized, feeds for monogastric animals are commonly fortified with inorganic phosphorous, increasing final costs of the product (Lei et al. 2013). In addition, accumulation of phytic acid has a negative effect on animal health because it represents an antinutritional and chelating agent, that reduces bioavailability of proteins and ions like Fe^{3+}, Ca^{2+}, Zn^{2+} and Mg^{2+} forming insoluble complex (Reddy et al. 1989). The undigested phytate then accumulates in manure and liquid effluents, leading to phosphorous pollution and water eutrophication. When phytic acid enters in aquatic environment causes cyanobacteria blooms, hypoxia and death of marine/freshwater animals.

For the main reasons described above, science community has focused his attentions on phytate-degrading enzyme (Bae et al. 1999; Pires et al. 2019; Vasudevana et al. 2019). Some of them are commercially available on market in different formulations, like Allzyme®SSF and Natuphos®, isolated from *Aspergillus niger*, and Phyzyme®XP, isolated from engineered *Escherichia coli* (Lei et al. 2013).

Phytase: structure, specificity, localization and applications

Phytases are a class of enzymes that catalyse the hydrolytic degradation of phytic acid to free inorganic phosphorous, to yield lower myo-

inositol phosphate esters and, in some case, free myo-inositol (Rangon et al. 2008). Enzymes described as phytases show different structures: histidine acid phosphatase (HAP), β propeller phytase (BPP) and purple acid phosphatase (PAP - Mullaney and Ullah, 2003). The most known and wide class is HAPs (EC 3.1.3.8). This class is ubiquitous, indeed HAPs can be found in bacteria, yeasts, filamentous fungi but also in upper eukaryotes (Lei et al. 2013). All the proteins belonging to this class maintain two common domains: a conserved N-terminal heptapeptide active site RHGXRXP (38-44) and a C-terminal catalytically active dipeptide HD (325-326 - Mullaney and Ullah, 2003).

Among HAPs exist a variety of specific activity. Wyss and co-workers (Wyss et al. 1999) analyzed several fungal phytases dividing them in two different subclasses: one with broad substrate specificity but lower specific activity on phytate (PhyBp) and the second with narrow substrate specificity but high activity for phytic acid (PhyAp). Curiously some organisms as *Aspergillius niger* possess both forms (Mullaney and Ullah, 2003).

Yeasts are good candidates for phytases production and some of them have been already characterized (Olstorpe et al. 2009, Greppi et al. 2015, Noubariene et al. 2011, Rangoon et al. 2009). In different species these enzymes show different localization: in *A. niger* (Neira-Vielmaa et al. 2017; Vats and Banerjee, 2004), *Saccharomyces cerevisiae* (In et al. 2009; Klosowski et al. 2018), *Candida tropicalis* (Puppala et al. 2018), *Debaryomyces castelli* (Rangoon et al. 2008) phytases are classified as extracellular enzymes and in some case, like in *Kodamea ohmeri* (Li et al. 2009), signal sequences for secretion have been identified. Others yeast phytases are defined as intracellular enzymes like in *Cryptococcus laurenti* (Pavlova et al. 2008) or cell-bound, like in *Wiccheranomyces anomalus,* where the absence of signal sequence confirms its localization (Vohra and Satyanarayana, 2002; Kaur et al. 2010). Although phytases have been reported in a wide number of bacteria, not many of them have been used as

feed supplement since their neutral/alkaline pH optimum and their temperature ranges could preclude their activities in food-related process.

For industrial applications, the ideal phytase should display three characteristics: ability to hydrolyze phytate in the upper digestive tract of the animals, resilience up to 65-90°C and cheap production cost. In particular, to work properly in digestive tract, phytase needs to have a pH optimum between 3.5 and 5.5 and optimum of temperature at 37°C - 40°C. Indeed, gastric pH is ranging from 3.5 in swine to 5.5 in poultry, and gastric temperature is usually ranging from 37°C - 40°C. Furthermore, for industrial application, phytase should actively work at high temperature; this ones are requred in feed production process during pelletting and heat treatment to control of *Salmonella* spoilage (Li et al. 2008; Vasudevana et al. 2019). In addition, it would need to be resistant to protease activity, and to show low sensitivity to ions (Li et al. 2008; Wyss et al. 1999).
In this study we screened 28 yeasts belonging to public and private collections and isolated from different environments. For 3 strains, we investigated in the effects of different temperatures, pH and phosphorous sources on enzymatic activity, in order to better characterize them and to identify the ones displaying the better performances.

4.4.3 - Materials and methods

Yeast strains

The yeast strains studied in this work (Table 1; Figure S1) belong to CBS collection, UBO Culture Collection (http://www.univ-brest.fr/ubocc), DBVPG collection and private collections. The strains were isolated from several environments, mainly marine environment and food matrix. Yeasts were stored in YPD 20% glycerol stocks at -80°C.

Media and growth conditions

YPD: 10 g/l yeast extract, 20 g/L peptone, 20 g/L glucose.

MMPhy: 20 g /L glucose, 5g/L $(NH_4)_2SO_4$, 0.5 g/L MgSO4* $7H_20$, 0.11 g /L phytic acid sodium salt hydrate(68388 sigma), trace metals (disodic EDTA 15 mg/L, $ZnSO_4*4H_2O$ 4.5 mg/L, $MnCl*4H_2O$ 0.1 mg/L, $CoCl_2*6\ H_2O$ 0.3 mg/L, $CuSO_4*5H_2O$ 0.3mg/L, $Na_2MoO_4*2H_20$ 0.4mg/L, $CaCl_2*2H_2O$ 4.5 mg/L, $FeSO_4\ *7H_20$ 3mg/L, H_3BO_3 1 mg/L, KI 0.1 g/L) and vitamins d-biotin 0.05 mg/L, Ca d(+) panthotenate 1mg/L, nicotinic acid 1 mg/L, myoinositol 25 mg/L, of thiamine hydrochloride 1 mg/L, pyridoxol hydrochloride, p-aminobenzoic acid 0.2 mg/L) as reported in Merico et al. 2007 with some modification.

MM-: 20 g /L glucose, 5g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4*\ 7H_2O$, trace metals and vitamins as in MMPhy.

MMP: 20 g /L glucose, 5g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4*\ 7H_2O$, KH_2PO_4 1g/L, trace metals and vitamins as in MMPhy.

MMP/Phy: 20 g /L glucose, 5g/L $(NH_4)_2SO_4$, 0.5 g/L MgSO4* $7H_2O$, 0.11 g /L phytic acid sodium salt hydrate, KH_2PO_4 1g/L, trace metals and vitamins as in MMPhy.

In all the media pH was maintained at 4.5 using H_2SO_4.

Cultivation for phytase screening and determination of activity

Yeast cells were cultivated at 28°C in a rotary shake at 150 rpm in 100 mL in bluffed flask with 20 mL of medium. Optical density was monitored at 600nm (OD_{600}). Cells were precultured for 24 hours in YPD, harvested by centrifugation at 5000 rpm and washed tree times with sterile NaCl solution (9 g/L). Then they were used to inoculate alternatively MMPhy, MMP, MMP/Phy and YPD at initial OD_{600} 1 (almost 10^7 cells/mL). Yeasts grow using phytic acid but to varying extents. In order to be sure to work with yeasts that are actively consuming phytic acid, cells were incubated for 21 hours, or at least until they show 2 duplications, before to be tested for enzymatic activity.

Dry weight determination

For dry weight measurements (DW), samples from different culture conditions were collected (in triplicate at each point). Cells were filtered through a glass microfiber GF/A filter (Whatman), washed with three volumes of de-ionized water and dried at 100°C for 24 h.

Phytase activity determination

Extracellular enzymatic activity was detected on supernatant, and cell-bound activity using whole cells. The activity was measured by orthophosphate production, following ammonium molibdate blue method as reported in Schimizu 1992 with some modifications. For extracellular activity determination, cell cultures were centrifuged at 13000 rpm and 1 mL of supernatant was added to 4 ml buffer composed by 0.2 M Na acetate/acetic acid, 8 mM phytic acid at pH 4.5. To determine cell-bound activity we used a homogeneous cells suspension. We set up enzymatic activity using a standard amounts of cells 50 OD (corresponding to almost 10 $mg_{d.w.}$ depending on the strain) in final volume of 5 mL. Cells were collected and washed twice with 0.2 M Na acetate/acetic acid pH 4.5 and resuspended in a final volume of 1 mL. Cell suspension was added to 4 ml of buffer 0.2 M Na acetate/acetic acid, 8 mM phytic acid at pH 4.5. All buffer employed to test enzymatic activity were prewarmed at reaction temperature. Blank was assembled using 1 ml of 0.2 M Na acetate/acetic acid at pH 4.5 and 4 mL of 0.2 M Na acetate, 8mM phytic acid and treated as sample.

For enzymatic activity determination 5 ml reaction were incubated in 15 ml tube at 37°C and stirred at 300 rpm. The reaction was immediately stopped (time 0) and stopped after 15, 30, 60, 120 min. Reaction was stopped mixing 0.5 mL of reaction with 0.5 mL TCA 5% solution, samples were centrifuged 3 min at 13000 rpm and the supernatant collected. In order to determine

orthophosphate concentration, 0.4 mL of supernatant was added to 0.4 mL of molibdate solution. This solution was prepared daily, by mixing solution A and B in a ratio of 4:1 (solution A: 2.6% $N_6H_{24}Mo_7O_{24}$* $4H_2O$ and 5.5% H_2SO_4; solution B: 4.6 %$FeSO_4$*$7H_2O$). The sample was incubated 10 min at 25°C and read against blank at OD_{700}. Phosphate concentration was determined using a standard curve for KH_2PO_4. Unit of phytase is defined as the amount of protein that hydrolyze 1 µmol phosphorus per minute. Specific activity is expressed in mU/mg of cell dry weight. To determine the effect of temperature, samples prepared with prewarmed buffer (pH 4.5) were incubated at 50°C and 60°C. To determine the effect of pH on enzymatic activity, pH buffers were adjusted at pH 4 and pH 5. 5 and the reaction incubated at 37°C.

Genomic extraction

To isolate genomic DNA, pellets of 30 OD 600nm of cells were resuspended in 0.5 ml of 0.05 M Tris–HCl/0.02 M EDTA at pH 7.5. This suspension was transferred to a precooled tube with an equal volume of glass beads (425–600 µm). Mechanical lysis was performed using a TissueLyser LT alternating 2 min of agitation at 50 Hertz with 1 min in ice for 4 cycles. The supernatant was added with 25 µl of SDS 20% (w/v) and incubated at 65°C for 30 min. Immediately, 0.2 ml of 5 M potassium acetate was added and the tubes were placed on ice for 30 min. Sample were centrifuged at 13000 rpm for 5 min and supernatant was transferred to a fresh microcentrifuge tube. The DNA was precipitated by adding 1 volume of isopropanol. After incubation at room temperature for 5 min, the tubes were centrifuged for 10 min. The DNA was washed with 70% ethanol, and dissolved in 50 µl of TE RNAsi (10 mMTris-HCl, 1 mM EDTA, pH 7.5 RNAsi 100 µg/mL). Samples were incubated at 37°C for 30 min (Donzella et al. 2019).

Strain identification

C. jadinii CJ2 isolated from sugar cane bagasse was characterized for the first time in this work. For strain identification gDNA was isolated and purified following the method described by Donzella et al. (2019). gDNA was amplified with PHUSION taq polymerase employing universal primers for amplification on D1/D2 domain NL1 GCA TAT CAA TAA GCG GAG GAA AAG, NL4 GGT CCG TGT TTC AAG ACG G 0.2 µM each, 200 µM DNTP and MgCl2 2.5 mM. PCR amplification was carried out by denaturing at 98°C for 7 min, followed by 30 cycles of denaturing at 98°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The produced amplicon was sequenced by Mycrosynt company.

Phylogenetic and bioinformatics analysis

Phylogenetic analysis was performed using Mega X 10.1.7 (www.megasoftware.net). Aminoacidic sequences were available on https://www.ncbi.nlm.nih.gov/. Phytase sequence of *D. hansenii* Mo40 and Bio2 were obtained in this work. Phytase gene was amplified from gDNA using primers: Forward Phy1 CCG ACC ATG GAT GGT ATC GAT TTC C, Reverse Phy2 CAT CGG ATC CTA ATT GTC ACC GGA. Primers were designed based on *D. hansenii* CBS 767 sequence. PCR amplification was carried out by denaturing at 98°C for 7 min, followed by 30 cycles of denaturing at 98°C for 10 sec, annealing at 59°C for 30 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 10 min. The produced amplicon was cloned in a plasmid, namely psf URA TPI and sequenced by Mycrosynt company.

All aminoacidic sequences were aligned using MUSCLE and a maximum likelihood tree was built. Analysis of signal secretion sequence was performed employing SignalIP-5.0 available on http://www.cbs.dtu.dk/services/SignalP/.

4.4.4 - Results and discussion

Growth in presence of phytic acid as sole phosphorous source

28 yeast species belonging to *Debaryomyces*, *Cyberlindnera*, *Schizosaccaromyces*, *Kluyveromyces*, *Saccharomyces*, *Brettanomyces*, *Candida*, *Torulospora*, *Rhodosporidium*, *Meyerozyma*, *Hanseniaspora*, *Pichia*, *Lachacea*, *Kazakistania* and *Rhodotorula* genera were characterized on their ability to grow using phytic acid as sole phosphorous source (Table 1, Figure S1). Strain CJ2 was identified in this work as *Cyberlindnera jadinii*. All the strains were cultivated in bluffed flasks on medium MMPhy, and growth was monitored at 28°C for 72 hours. In parallel, as negative control, growth on MM- was carried out, and no appreciable growth was detected (data not shown). We avoided to perform characterization on solid media due on ambiguous results reported in literature (Olstrope et al. 2009).
The strains were precultured in YPD media for 24 hours and inoculated in MMPhy medium at initial OD_{600} 1. All except *Kluyveromyces lactis* Y1356 were able to grow using phytic acid as sole phosphorous source but with variable extent (Table 1). Some strains, like *C. jadinii* and *Meyerozyma guilliermondii* are able to exceed 20 OD after 24 h of incubation, and reach 26 and 25 OD respectively after 48 h.

Other strains, like *Kluyveromyces marxianus, Brettanomyces bruxellensis* and *T. delbrueckeii* reached lower OD values (14, 10 and 8.56 OD respectively) after 72 h of incubation. On the contrary, *K. lactis* CBS 2359 duplicates only 2 times reaching 4 OD (complete analysis is reported in Table 1 - Figure S1). These differences could be due on yeast capability to get phosphate from phytic acid, indicating that the efficiency of phytase activity is strain dependent, as well as the mechanism of phytic acid hydrolysis. Literature reports that in some yeasts, like *Debaryomyces castelli*, phytase is able to completely hydrolyze phosphate from phytic acid (Rangoon et al.

2008). Other phytases, like in *A. niger, Saccharomyces cerevisiae, K. ohmeri* are., on the contrary, not able to perform a complete hydrolysis of this acid, leaving some phosphate groups not bioavailable (Rangon et al. 2008 - Li et al. 2009).

Table 1. Results of the screening (BDL: below detection limit, N A: not available)

			Biomass	Phytase activity		
Yeasts	**Strains**	**Isolation**	**72 h OD 600 nm**	**cell-bound**	**extracellular**	**Reference**
Debaryomyces hansenii	MI 1	Creamery	5,5	BDL	BDL	N A
Ciberlindnera jadinii	CJ2	Sugar cane bagasse	28	YES	YES	This work
Schizosaccharomyces pombe	Y709	N A	3.7	BDL	BDL	N A
Kluyveromyces lactis	CBS 2359	Creamery	4	YES	BDL	Wésolowski et al. 1982
Kluyveromyces lactis	Y1356	N A	1	BDL	BDL	N A
Kluyveromyces marxianus	Y1058	N A	14	YES	BDL	Hangman et al. 2014
Hanseniaspora uvarum	UMY 514	Grapes	8	YES	BDL	N A
Hanseniaspora uvarum	UMY 571	Grapes	14	YES	BDL	N A
Saccharomyces cerevisiae	CENPK 113 7D	LAB STRAIN	7,78	BDL	BDL	Otero et al. 2010
Saccharomyces cerevisiae	LALVIN T73	Lallemand starter	8,36	BDL	BDL	https://www.lallemandwine.com/
Brettanomyces bruxellensis	CBS2499	Wine	10	YES	BDL	Piskur et al. 2012
Lachancea thermotolerans	CBS 6340	Mirabelle-plum conserve	8	YES	BDL	Zhou et al. 2018
Torulaspora delbrukei	CBS1466	N A	8,56	YES	BDL	Kurtzman et al. 2003
Candida humilis	CBS 5658	Bantu beer	8,6	BDL	BDL	Kurtzman et al. 2011
Candida milleri	CBS 6897	Sourdough for bread	11	BDL	BDL	Yarrow et al. 1978
Rhodosporidium azoricum	DBVPG 4620	Soil	12	BDL	BDL	Capusoni et al. 2017
Zygosaccharomyces kombutchaensis	CBS 8849	Kombucha tea	7	YES	BDL	Kurtzman and Fell. 2003
Kazakistania unispora	CML133	Fermented maize bran	3	BDL	BDL	Decimo et al. 2017
Meyerozima guilliermondi UBOCC-A-214008	LM2	Sediment	26	YES	BDL	Rédou et al. 2015
Meyerozima guilliermondi UBOCC-A-214143	LM9	Sediment	20	YES	BDL	Rédou et al. 2015
Pichia guilliermondi UBOCC-A-208004	Ex15	Siliceous sponge	21	YES	BDL	Burgaud et al. 2010
Rhodotolura mucillaginosa	LM16	Sediment	10	BDL	BDL	Rédou et al. 2015
Rhodotolura mucillaginosa	LM18	Sediment	7,9	YES	BDL	Rédou et al. 2015
Candida atlantica UBOCC-A-208026	Mo31	*Bathymodiolus azoricus*	25	YES	BDL	Burgaud et al. 2015
Candida marina UBOCC-A-208034	Mo39	sponge	4,2	BDL	BDL	Burgaud et al. 2015
Debaryomyces hansenii UBOCC-A-208002	BIO2	gastropod gills	11	YES	BDL	Burgaud et al. 2010
Debaryomyces hansenii UBOCC-A-208035	Mo40	coral	8,2	BDL	BDL	Burgaud et al. 2010
Rhodotorula diobovatum UBOCC-A-208033	Mo38	sponge	12,5	YES	BDL	Burgaud et al. 2010

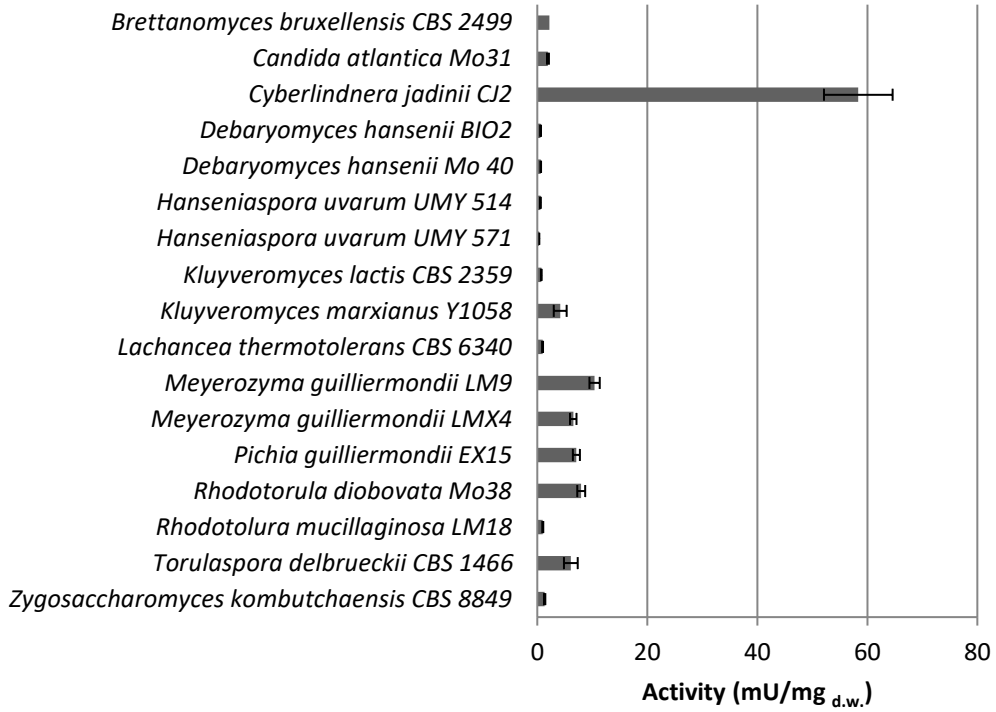
Screening for specific phytase activity

In order to identify an enzyme with characteristics suitable in feed industry (a phytase able to work in the upper digestive tract of monogastric animals, with temperature range between 37-40°C and pH range between 3.5 - 5.5), we screened phytase activity at 37°C and pH 4.5 (Lei et al. 2013).

Extracellular and cell-bound activity was determined on cells grown using phytate as sole phosphorous source on MMPhy medium at 28°C. To better compare phytase activity in various strains, we decided to express phytase activity as mU/mg $_{d.w.}$, instead of mU/mL as often reported in literature. In this way we avoided the bias due to the different ability of strains to grow in presence of phytate (Table 1), and then to reach different amount of biomass/mL. In this way we could also correctly compare cell-bound and extracellular activities.

Under these conditions, only 16 from the 28 tested strains showed a detectable cell-bound enzymatic activity (Table 1 and Figure 1). Extracellular phytase activity was present only in *C. jadinii* (26.25 mU/mg$_{d.w.}$), and this strain was also identified as the one with the highest cell-bound activity (58.36 mU/mg$_{d.w.}$ - Table 1, Table 2).

Figure 1. Specific cell-bound phytase activity (mU/mg $_{dw}$) detected using whole cells.



Lower levels of cell-bound activity were detected in *Kluyveromyces marxianus* (4.17 mU/mg$_{d.w}$), *Torulospora delbrueckeii* (6.1 mU/mg$_{d.w.}$), *M. guilliermondii* (10.49 mU/mg$_{d.w.}$) and *Rhodotolura diobovata* (7.99 mU/mg$_{d.w.}$ (Figure 1).

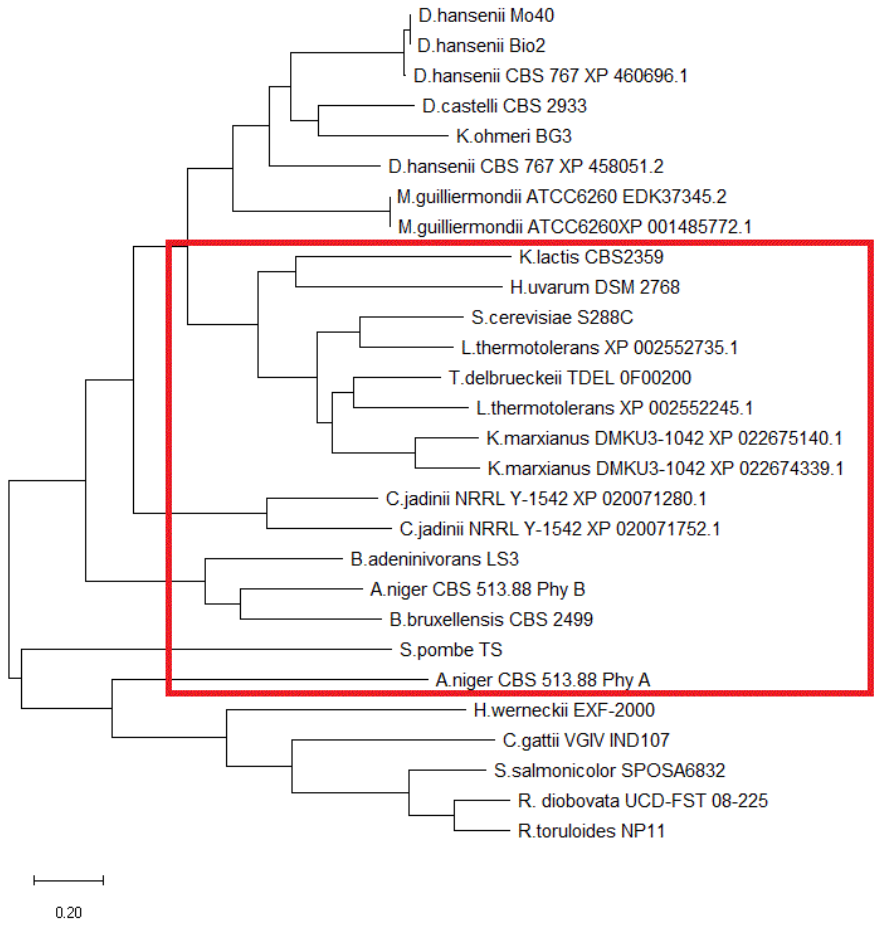
Analysis of conserved domain

Aminoacidic sequences of phytases were recovered from NCBI database, except for *D. hansenii* Mo40 and BIO 2, that we sequenced in this work. All strains analyzed have a protein that shows a good homology with a phytase, and some like *M. guilliermondi*, *C. jadinii* and *K. marxianus* have even two sequences encoding for this enzyme. Only *K. unispora* did not possess a phytase gene. Probably this strain is able to make a duplication on MMPhy medium, because the presence of a phosphatase that is able to

cleave few phosphate from phytic acid, leading to a slight biomass increase (Figure S1, Table 1).

Generally, HAP phytase maintains two common domains: a conserved N-terminal heptapeptide active site RHGXRXP (38-44) and a C-terminal catalytically active dipeptide HD (325-326) (Mullaney and Ullah, 2003). In our strains, the C-terminal domain was always conserved.

Figure 2: Phylogenetic tree showing homology between phytase aminoacidic sequences. The red rectangle includes sequences that contain a signal sequence for secretion (detected using SignalIP-5.0)



N-terminal domain was maintained in all strains except in *R. toruloides*, where the active site shows mismatch XHGHRXP, leading us to conclude that all the analyzed phytases belong to HAP family. These sequences were used to build a phylogenetic tree (Figure 2). In the red box we included the sequences that contain a secretory signal peptide. The cleavage site was identified employing the online tool SignalIP-5.0. Literature data reported that *K. ohmeri* (Li et al. 2009) contained this sequence, but this tool is not able to recognized it. How it is reported in Figure 2, all the sequences showing a signal peptide can be clusterised, suggesting that extracellular localization can be phylogenetically related. In conclusion we identified extracellular activity only in *C. jadinii,* probably because we used culture supernatants without any step to concentrate this activity. It is possible speculates that in the other strains extracellular phytase activity was too low to be detected. Corroborating this hypothesis in other studies extracellular activity has been detected in concentrated samples (Olstrope et al. 2009, Hellstrom et al. 2015, Rangoon et al. 2009).

Regulation of phytase expression

To perform this analysis, we selected three strains, *C. jadinii*, *K. marxianus* and *T. delbrueckeii* that showed higher phytase activity, and furthermore, that can be employed in food related bioprocess. Indeed *C. jadinii* and *K. marxianus* are included in QPS EFSA list (Quality Presumption as Safe - European Food Seafty Autority - Koutsoumanis et al 2019) and *T. delbrueckeii* is a wine starter with commercial name (BIODIVA™ - lallemand).

In these strains, phytase activity was investigated by cultivating cells in presence of different phosphate sources: phytic acid only (MMPhy media), phosphate salt (KH_2PO_4 which is included in MMP media), organic phosphate (YPD media) and on a mixture of phytic acid and phosphate salt (MMP/Phy media).

This allows to understand the regulation of phytase expression based on the available phosphate (Table 2). On MMPhy, *C. jadinii*, *K. marxianus* and *T. delbrueckeii* reached 25, 10 and 7 OD respectively, while in MMP and in MMP/Phy the final biomass amounts were 25, 18 and 7 OD respectively (data not shown). All togheter these results suggest tha *C. jadinii* growth without any restriction independently from the source of phosphate employed.

C. jadinii was the sole yeast that in our screening showed both cell-bound and extracellular activity. When cells were incubated in media containing phytic acid as the sole phosphorous sources, we detected cell-bound activity at 58.36 mU/$mg_{d.w.}$ (Table 2 – column named MMPhy) and extracellular activity at 26.25 mU/$mg_{d.w.}$ (data not shown). These activities were not appreciable in presence of phosphate (MMP and MMP/Phy), suggesting that the presence of KH_2PO_4 (inorganic source of phosphate) inhibits the enzyme expression. In addition, the concomitant presence of phytic acid and KH_2PO_4 (MMP/Phy media) is not able to induce phytase activity (Table 2). When *C. jadinii* was cultivated in presence of organic phosphate (YPD media) a reduced cell-bound activity was detected: it decreased from 58.36 mU/$mg_{d.w.}$, measured in MMPhy, to 2.02 mU/$mg_{d.w.}$ detected in YPD (Table 2).

Table 2. Effect of phosphate source on phytase activity. Specific activity (mU/$mg_{d.w.}$) was determined at 37°C, pH 4.5. Cell-bound activity was detected using whole cells.

	Activity mU/mg *dw*			
	YPD	MMPhy	MMP	MMP/Phy
C. jadinii	2.02 ± 0.4	58.36 ± 6.24	BDL	BDL
T. delbrueckii	2.39 ± 0.29	6.1 ± 1.25	BDL	BDL
K. marxianus	1.71 ± 0.35	4.17 ± 1.17	BDL	BDL

In this condition, extracellular activity is under the detection limit. The basal-activity detected in YPD is due not only to absence of inorganic phosphate but also to the presence of the organic one.

The same behaviour was observed also in T. *delbrueckeii* and *K. marxianus*. The highest activities (6.1 mU/$mg_{d.w.}$ and 4.17 mU/$mg_{d.w.}$ respectively) were detected in presence of the sole phytic acid and decreased in YPD (Table 2). As observed in *C. jadinii,* also in *T. delbrueckeii* and in *K. marxianus* the presence of phosphate salt inhibits the expression of phytase, being no activity detected in cells growing in MMP and in MMP/Phy.

In conclusion, a good level of phytase activity can be expressed only when cells grow using phytate as sole phosphorous source. On the contrary, the presence of KH_2PO_4 completely inhibits the expression of phytase activity. No activity was detectable in MMP medium, where KH_2PO_4 is the sole phosphate source; curiously this happens also in MMP/Phy medium, with the concomitant presence of KH_2PO_4 and phytic acid. This indicates that the sole presence of phytic acid is not enough to induce phytase activity, and lead us to speculate that also lack of inorganic phosphate is requested. The presence of a low activity when the cells grow in a medium not containing phosphate salt, namely YPD, corroborates this hypothesis.

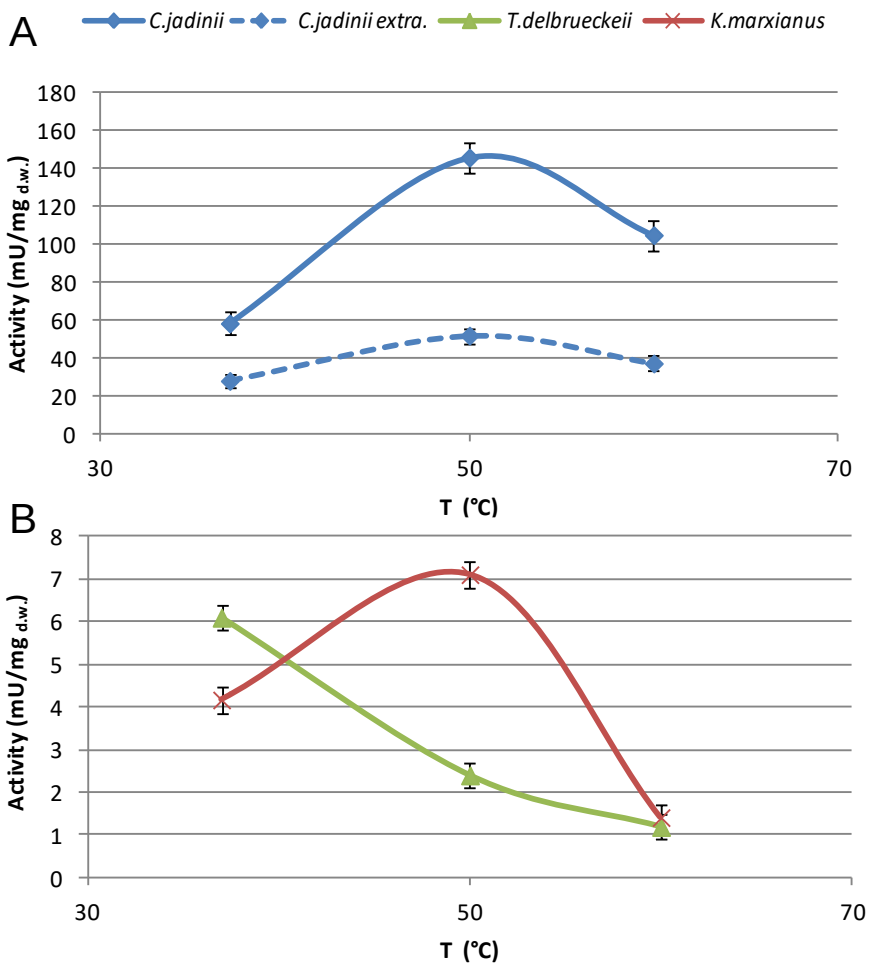
An analogous phenomenon has been observed previously by Olstrope and colleagues in 2009. They saw that in some yeasts like *Wickeranomyces anomalus*, *Candida lambica* and *S. cerevisiae*, phytase activity was repressed in presence of inorganic phosphate, but this does not occur in species like *Blastobotrys adeninivorans* and *Cryptococcus laurentii* (Olstrope et al. 2009, Pavlova et al. 2008). Understanding which role plays phosphate (organic/inorganic) concentration on phytase activity is pivotal for set up industrial process.

Characterization of phytase activity

Phytases, suitable for food-related processes, need to be able to work in upper digestive tract of monogastric animals and need to be resilient during the feed production process. To work in digestive tract, a good phytase, should exhibit a pH optimum between 3.5 and 5.5 and activity at 37°C. The resilience at high temperature is requests because heat treatments are commonly adopted to contains *Salmonella* spoilage and during pelleting processes. Pelleting of feed reduces the troubles with dusts, making feed easier to be consumed for the livestock. This treatment permits also incorporation of ingredients in the feed to “lock” the feed mixture. Unfortunately heat treatment could reduce phytase activity, and for this reason it is important to select a thermo-stable enzyme (Vasudevana et al. 2019).

In order to select enzymes for feed application purpose, the effects of temperature and pH on enzymatic activity were investigated (Figure 3 - 4). The optimal temperature for *C. jadinii* was found to be 50°C. When the assay was performed at this temperature, cell-bound activity reached 146 mU/$mg_{d.w.}$ and extracellular activity was 51.95 mU/$mg_{d.w.}$. At 60°C the values for cell-bound and extracellular activity were lower: 105.2 and 37.2 mU/$mg_{d.w.}$ respectively (Figure 3 – Panel A). The situation was found similar for *K. marxianus*. In this case, the optimum of temperature was observed at 50°C, with activity of 7.11 mU/$mg_{d.w.}$. On the contrary, in *T. delbrueckeii* the highest activity was detected at 37°C, 6.1 mU/$mg_{d.w}$ (Figure 3 – Panel B).

Figure 3. Effects of temperature on phytase activity, measured at pH 4.5. Panel A report cell-bound (full line) and extracellular (dotted line) activities detected *on C. jadinii*. Panel B the right report cell-bound activity detected on *T. delbuekeii* and *K. marxianus.*

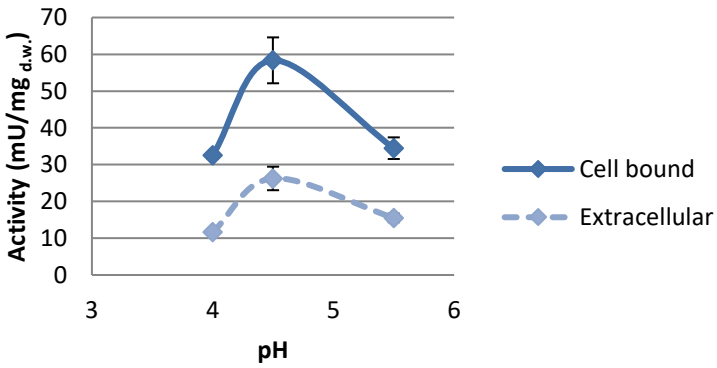


The data reported in Figure 3 lead us to hypothesize that phytase activity in *C. jadinii* could be suitable for industrial purpose and, for this reason, in this work we concentrate our attention on it. Indeed, at 37°C *C. jadinii* phytase activity is higher in comparison with *K. marxianus* and *T. Delbrueckeii*. In

addtiono, the high phytase activity detected at 60°C suggests that this enzyme could be resilient at the higher temperature usually set up during feeds production. Furthermore, Cruz and colleagues demonstrate that biomass of *C. jadinii* can partially replace feed protein content (generally consisted in soybean meal, fish meal, rapeseed meal) in swine and poultry formulation (Cruz et al 2020a, Cruz et al 2020b). In this contest, it could be interesting to investigate if *C. jadinii* dry cells would conserve phytase activity. The possibility to have active enzymes in feed could be very appealing in order to decrease phytate content. This phenomenon had been observed in *W. anomalus*, whose biomass containing phytase was added to acquaculture feed (1000 U/Kg feed), with results comparable to commercial phytase (Vohra et al. 2011).

With the aim to investigate the effect of pH on *C. jadiniii* phytase activity, we analyzed it at three different values: 4, 4.5 and 5.5. For this experiment we selected a temperature of 37°C (Figure 4).

Figure 4: Effect of pH on phytase activity detected in *C. jadinii*. Activity was measured at 37°C.



Even if this temperature is not the optimum found for *C. jadinii* enzymatic activity, it is the one requested by feed industry (gastric temperature - Lei et al. 2013). As reported in Figure 4, the pH optimum for *C. jadinii* phytase is 4.5

and, as reported by Lei and colleagues, this is an important characteristic for the development of enzyme as feed additive.

Comparing our results with the data reported in literature, it is possible to observe that phytase activity found in *C. jadinii* could be promising for future applications (Table 3). To the best of our knowledge, *C. jadinii* cell-bound activity is one of the higher observed on cells grown in mineral media with phytate as sole phosphorous sources. Higher enzymatic activity reported in *S. cerevisiae*, *W. anomalus* and *in Candida tropicalis* have been observed in different conditions. *W. anomalus* was cultivated in fed batch on agri-food by-products and phytase activity of *S. cerevisiae* and *C. tropicalis* was observed on cells grown in rich media.

Table 3: Phytase activity in several yeasts. Abbr: MYGP (Malt extract, Yeast extract, Glucose, Peptone), YPG (Yeast extract, Peptone, Glucose). pH and temperature marked with * indicate optimum value.

	Media	pH	T°C	Cell bound	Reference
				mU/mg dw	This work
C. jadinii CJ2	minimal media phytate	*4,5*	*37*	58,36	This work
		*4,5**	*50**	146	This work
T. delbrueckii CBS 1466	minimal media phytate	*4,5*	*37*	6,1	This work
K. marxianus Y1058	minimal media phytate	*4,5*	*37*	4,17	This work
		4,5	*50**	7,11	This work
C. laurentii AL27	minimal media phytate	*5*	*37*	15.7	Pavlova et al. 2008
C. tropicalis NCIM 3321	MYGP	4,5*	*70**	236	Puppala et al. 2018
	MYGP (different concentration)	4,5*	*70**	1014	Puppala et al. 2018
S. cerevisiae NCIM 3662	MYGP	4,5*	*50**	45	Puppala et al. 2019
	MYGP (different concentration)	4,5*	*50**	164	Puppala et al. 2019
W. anomalus	minimal media phytate	4	*60*	6	Vohra and Satyanarayana, 2001
	cane molasses (fed batch)	4	60	176	Vohra and Satyanarayana, 2004

In conclusion, we think that the results found for *C. jadinii* phytase activity could represent a good starting point to set-up optimization of cultural conditions in order to improve phytase production (Table 3).

As reported by Puppala et al (2018 - 2019) with a statistical media optimization approach phytase production can be easily increased. In *S. cerevisiae* modulation of media components, mineral concentration (like addition of magnesium sulphate, manganese sulphate and ferrous sulphate) and scale up in 10 L fermenter could increase phytase activity from 45 $mU/mg_{d.w.}$ to 164 $mU/mg_{d.w}$. In *K. marxianus* phytase activity could be easily increase adding to fermentation media cheap substrates rich in phytic acid like rice bran (5.1 to 8.6% - Pires et al 2019). Similar behaviour can be observed in *W. anomalus*, where the presence of cane molasses in media can increase enzymatic activity from 6 $mU/mg_{d.w.}$ up to 176 $mU/mg_{d.w.}$ reducing enzyme production cost from 0.25 £ to 0.006 £ per 1000 U (Vhora and Satyanarayana, 2004). In our case, performing media optimization could be the right approach in order to increase phytase productivity reducing the enzymes production costs.

4.4.5 - Conclusion

The combined effect of phytate as antinutritional factor, unviable sources of phosphorous and as causes of environmental pollution makes phytase a lucrative target for investigation. This enzyme is commonly exploited as feed additive in monogastric animal diet. A screening performed on several yeast strains led to identify a novel enzymatic activity in *C. jadinii*. This strain showed both intracellular and cell-bound activity. To our knowledge this was the first time that *C. jadinii* phytase was described in literature. Our results suggest that this enzyme is suitable as feed additive. Indeed, it could work in upper digestive tract and its activity is detectable also at 60°C, suggests that phytase of *C. jadinii* could be resilient to feed production process.

4.4.6 - References

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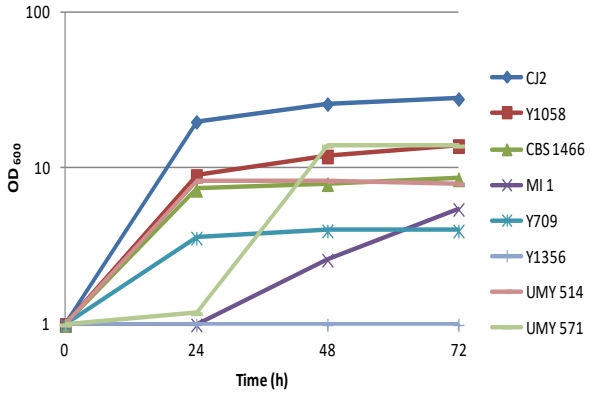
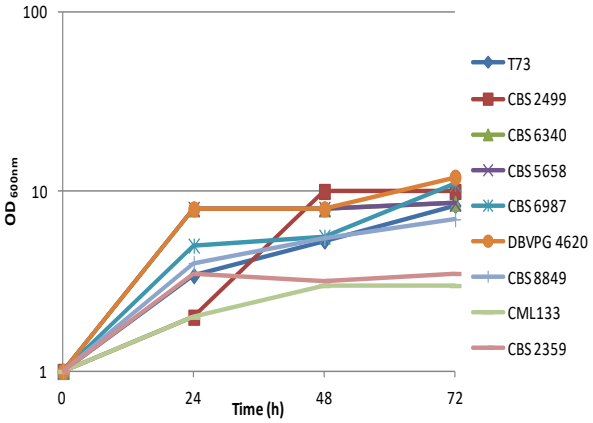
4.4.7 - Supplemental materials

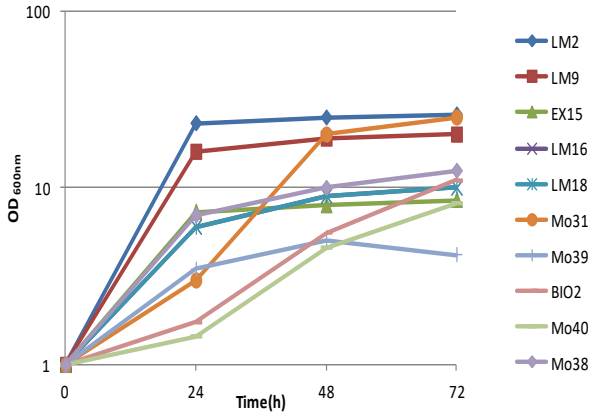
Figure S1: Growth curves detected on MMPhy media.



C. jadinii : **CJ2,**
K. marxianus**: Y1058**
T. delbrueckeii**: CBS 1466,**
Debaryomyces hansenii**: MI1**
Schizosaccharomyces pombe**:Y709**
Kluyveromyces lactis**: Y707,**
K. lactis**: Y1356,**
Hanseniaspora uvarum**: UMY514,**
Hanseniaspora uvarum**: UMY571**



S. cerevisiae **:T73,**
B. bruxellensis **: CBS 2499,**
Lachancea thermotolerans **:CBS6340**
Candida humili**: CBS 5658**
Candida milleri**:CBS6987**
Rhodosporidium azoricum **:DBVPG 4620**
Zygosaccharomyces kombutchaensis **: CBS 8849**
Kazakistania unispora :**CML 133**
K.lactis **:CBS 2359**



M. guilliermondii **: LM2,**
M. guilliermondii**: LM9,**
Pichia guilliermondii**: EX15**
Rhodotolura mucillaginosa**: LM16**
Rhodotolura mucillaginosa**: LM18**
Candida atlanti**: Mo31**
Candida marinus: **Mo39**
D. hansenii: **BIO2**
D. hansenii **: Mo40**
Rhodosporidium diobovatum **:Mo38**

5 - GENERAL CONCLUSION

To date science community is focused on the development of new processes characterized by a reduced ecological foot-print. The possibility to perform a seawater-based process could help in this scenario, because it permits to save a huge amount of freshwater. NCYs, and in particular yeast isolated from marine environment, are good candidates for this purpose. To increase the know-how about their physiological traits, osmotic stress response has been investigated in an halotolerant marine yeast, namely *D. hansenii*. I found that presence of high salt concentration leads to a decreased membrane permeability which is strictly dependent on membrane depolarization. This phenomenon reduces ions permeability and can negatively affect the uptake of charged substrates during bioprocesses.

D. hansenii growth was analysed on different media under several growth conditions (eg presence/absence of sea salts and different pH), to identify the more suitable parameters to perform a sustainable bioprocess. Base on my research, I set-up a new fermentation protocol in seawater-based media containing mixtures of sugars and cheap nitrogen sources, to obtain high biomass productivity with reduced production cost.

NCYs could play an important role in bioprocesses not only as biomass, but also as source of protein. During my PhD project I have identified two different enzymes with promising features for industrial application: a nitrilase isolated form a marine strains of *M. guilliermondii* and novel phytases isolated from *C. jadinii.*

Bioconversions performed with *M. guilliermondii* nitrilase represent a sustainable alternative to produce carboxylic acids from nitriles, avoiding traditional chemical synthesis. This latter protocol requires harsh reaction conditions and produces side products causing yield losses. A biocatalytic approach gives, on the contrary, the opportunity to obtain pure carboxylic acids, from nitriles compounds, with a good enantiomeric excess in mild reaction condition. Therefore, I developed a new sustainable seawater-based

process to produce mandelic acid. This acid is an important molecule that finds several applications in pharmaceutical industry.

In the last part of my PhD project, I characterized *C. jadinii* phytase activity. The production of this enzyme is a lucrative target of investigation, because phytic acid is not only an antinutritional factor but it causes also environmental pollution. In particular, this enzyme possesses interesting features that makes it suitable for feed application.

6 - IMPLICATIONS AND FUTURE DIRECTIONS

The possibility to investigate in academia mechanisms involved in stress response, gives the opportunity to increase the know-how on NCYs. At this point the technological transfer becomes fundamental for the success of industrial process.

In the first part of my PhD project I investigated osmotic stress response in *D. hansenii* using flow cytometry. My results suggest that presence of high salts concentration reduce ions permeability decreasing the uptake of charged substrate. This aspect needs to be taken in account during the experimental design of an industrial bioprocess, and could be susceptible of further investigations. A deeper knowledge of *D. hansenii* physiology allows us to set up a sustainable seawater-based bioprocess with high biomass productivity.

The next steps could be deeply analyze this biomass in order to identify other useful properties related to exploitation as food supplement (eg vitamin, antioxidant production). So far, preliminary experiments suggest that this strain is able to produce a huge amount of B2 vitamin (Capusoni et al unpublished data). The production of these value-added molecules combining with *D*. *hansenii* inclusion in QPS EFSA (Qualified Presumption of Safety - European Food Safety Authority) makes this yeast suitable for application as new-concept probiotic.

NCYs are good candidate for enzyme production. During my PhD I studied nitrilase activity of marine strain of *M. guilliermondii*. This enzyme is able to catalyze reaction in seawater-based process. To date I performed only production of mandelic acid in seawater, but would be interesting to investigate other bioconversion in order to increase the greenness of nitrile hydrolysis process.

Moreover, I identified and characterized a novel phytase isolated from *C. jadinii*. This enzyme possesses suitable characteristics to be employed as feed additive. Commonly, these proteins are supplemented to animal feed as enzyme mixtures. Recently, *C. jadinii* biomass has been employed as source

of proteins in the diet of swine and poultry. Hence given this contest, it could be interesting to investigate if *C. jadinii* dry cells would conserve phytase activity. The possibility to have active enzymes in feed formulation could be very appealing in order to decrease phytate content.

Finally, data collected in my PhD thesis suggest that NCYs are an interesting research field. Flow cytometry experiments reveal us the utility to investigate on the mechanism related to halotolerace. To better exploit, in bioprocesses, the peculiarities of NCYs is necessary to pursue the studies; not only deeply understanding mechanisms involved in increased tolerance behaviour (for example thermotolerance and barotolerance), but also indentifying new yeast species with promising features suitable for industrial application.

7 - ACTIVITIES LIST

Workshop and Congress

XXIV Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology. Florence, Italy, September 2019.

7th Conference on Physiology of Yeast & Folamentous Fungi (PYFF). Milan, Italy. June 2019.

XXIII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology. Oristano, Italy, September 2018.

Courses of UNIMI PhD catalogue catalogue curses

Bioprocesses for the valorisation of agrifood by-products and residues.

Environmental microbiomes: from microbial ecology to ecosystem services.

Sensing technologies and chemometrics.

Sustainability concepts in food technology – methodological approaches and case studies.

Tranferable skills

Open access – open data e il mondo delle pubblicazioni

Research integrity

Placement

La valutazione della ricerca

“Come scrivere un progetto di ricerca: parte 1. Le 100 cose che avrei voluto sapere quando ero un dottorando”

Tutelare e valorizzare sul mercato i risultati della ricerca

GrantmanshipI

Communication of new media I (2 hour)

Communication of new media II (2 hour)

Fake news

Data protection e attività di ricerca scientifica

Valorizzare creando impresa (parte 1)

Valorizzare creando impresa (parte 2)

CV e tecniche di selezione

Sostenibilità e innovazione

Publications

Donzella, S. , Cucchetti, D., Capusoni, C., Rizzi, A., Galafassi, S., Gambaro, C., Compagno C. 2019. Engineering cytoplasmic acetyl-CoA synthesis decouples lipid production from nitrogen starvation in the oleaginous yeast *Rhodosporidium azoricum*. Microb. Cell Fact.18,99.

Serra, I., Capusoni, C., Molinari, F., Musso, L., Pellegrino, L., Compagno, C.2019. Marine Microorganisms for Biocatalysis: Selective Hydrolysis of Nitriles with a Salt-Resistant Strain of Meyerozyma guilliermondii. Marine Biotechnology. 21, (2),229-239.

Capusoni, C., Arioli S., Donzella, S., Guidi, B., Serra, I., Compagno, C. 2019. Hyper-osmotic stress elicits membrane depolarization and decreased Permeability in halotolerant marine *Debaryomyces hansenii* strains and in *Saccharomyces cerevisiae*. Front. Microbiol., 82, (15),4673-4681.

Capusoni, C., Rodighiero, V., Cucchetti, D., Galafassi, S., Bianchi, D., Franzosi, G., Compagno C. 2017. Characterization of lipid accumulation and

lipidome analysis in the oleaginous yeasts *Rhodosporidium azoricum* and *Trichosporon oleaginosus*, Biores Technol. 238, 281-289.

Posters

Capusoni, C. Application of not conventional yeasts in food related bioprocesses. XXIV Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology. Florence, Italy, September 2019.

Capusoni, C., Arioli, S., Donzella, S., Guidi, B., Serra, I., Compagno, C. Hyper-osmotic stress elicits membrane depolarization and decreased permeability in halotolerant marine *Debaryomyces hansenii* strains and in *Saccharomyces cerevisiae*. 7th Conference on Physiology of Yeast & Folamentous Fungi (PYFF). Milan, Italy. June 2019.

Serra, I., Capusoni, C., Compagno, C. Marine halotolerance yeast for biocatalysis. 7th Conference on Physiology of Yeast & Folamentous Fungi (PYFF). Milan, Italy. June 2019.

Capusoni, C. Marine yeasts as “Cell factory” for the production of value added molecules. XXIII Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology. Oristano, Italy, September 2018.

Patents

PTC/IB2018/055301 - Rodighero V., Franzosi G., Compagno C., Capusoni C. Variant oleaginous yeast and its use for the production of lipids.

Tutoring activities

Giorgia Frabetti (matricola 880491 Bachelor degree in Biotechnology) AA 2018-2019 Tutor: Concetta Compagno, Cotutor: Claudia Capusoni.

Silvia Donzella (matricola 879551 Master degree in Molecular Biotechnology and Bioinformatics) AA 2016-2017 Tutor: Concetta Compagno, Cotutor: Claudia Capusoni.

Institutional responsibilities

Representative of PhD students within the Board of the Department (DeFENS) of the University of Milan.