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DOTTORATO DI RICERCA
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Metabolic studies on the nitrophile yeast *Rhodotorula glutinis* DSBCA06

Dottorando: Dott. Enrico Civiero
Coordinatore: Prof.ssa Alessandra Pani
Tutor: Prof. Enrico Sanjust

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ABSTRACT

Nitrogen is the most abundant element in atmosphere and fundamental component of proteins, nucleic acids and other essential molecules. In the past century the industrial use of nitrogen compounds has grown exponentially causing widespread pollution. Nitrogen pollution has wide-ranging impacts including contributions to global warming, acid rains and eutrophication.

Reduction of nitrogen use in industry and agriculture coupled whit remediation treatments could represent a solution.

To this purpose we isolated from environmental samples a nitrophile strain capable of removing nitrogen compounds efficiently from the medium. Through the molecular characterization, we identified the strain as a *Rhodotorula glutinis* that we called DSBCA06.

We examined the main metabolic features of the strain, also to determine the best growing conditions. At the same time, the ability of the strain to grow in presence of high nitrite concentrations was assayed, being a relevant feature poorly studied earlierfor other environmental yeasts. The ability of the strain to grow in presence of heavy metal cations was also tested, showing a noticeable tolerance.

The cost of bioremediation treatments is often a problem. One of the way to obviate this is to produce valuable secondary metabolites, capable of positively impact the cost of the processes. In this context the ability of the strain to produce carotenoids, natural molecules with antioxidant properties used for food production, cosmetic and pharmaceutical industry, has been evaluated.

The strain *Rhodotorula glutinis* DSBCA06 showed interesting features suggesting its possible use in bioremediation or industrials process for production of secondary metabolites such as lipids and carotenoids.

SOMMARIO

L’azoto è l’elemento più abbondante nell’atmosfera, componente fondamentale di proteine, acidi nucleici ed altre essenziali molecole.

Nel secolo scorso l’uso industriale dell’azoto sotto forma di differenti composti è cresciuto in maniera esponenziale causando inquinamento.

L’inquinamento da composti azotati si manifesta in diversi modi contribuendo al riscaldamento globale, al fenomeno delle piogge acide e a quello dell’eutrofizzazione.

La riduzione dell’utilizzo dei composti azotati nell’industria e in agricoltura, accoppiata a trattamenti di (bio)risanamento, potrebbe rappresentare una soluzione al problema.

Con questa idea, abbiamo isolato da campioni ambientali un ceppo nitratofilo estremamente efficiente nel rimuovere composti azotati dal mezzo di coltura.

Dopo la caratterizzazione molecolare, è stato possibile classificarlo come un lievito appartenente alla specie *Rhodotorula glutinis* che abbiamo identificato con la sigla DSBCA06.

Abbiamo poi esaminato le principali caratteristiche metaboliche come le fonti di carbonio che era capace di utilizzare (pure e rinnovabili) e l’intervallo di pH di crescita al fine di determinare le migliori condizioni per il lievito. Nello stesso tempo, abbiamo valutato anche la capacità del ceppo di crescere in presenza di elevate concentrazioni di nitrito, in considerazione del fatto che in pochi altri casi questo aspetto è stato studiato in lieviti ambientali.

Abbiamo anche effettuato prove sulla capacità del ceppo di resistere ad elevate concentrazioni di metalli pesanti presenti nel mezzo di crescita sui quali il ceppo ha mostrato una notevole tolleranza.

Il costo dei trattamenti che prevedano l’uso di tecniche di biorisanamento può essere spesso un problema. Una delle possibilità di limitare il problema dei costi è provare ad accoppiarli con la produzione di metaboliti secondari che abbiano un valore economico, così da ridurre i costi del processo. In questo contesto è stata

valutata l’abilità del ceppo di produrre carotenoidi, molecole con proprietà antiossidanti usati nella produzione alimentare e nelle industri cosmetica e farmaceutica.

Il ceppo *Rhodotorula glutinis* DSBCA06 ha mostrato interessanti caratteristiche che lasciano prevedere un suo possibile impiego nel campo del biorisanamento o nella produzione industriale di metaboliti secondari come lipidi e carotenoidi.

INTRODUCTION

Nitrogen

Nitrogen is the most abundant element in atmosphere. Seventy-eight per cent of the world's atmosphere is composed of di-nitrogen gas (N_2),which is relatively un reactive(Welbaum, Sturz, Dong, & Nowak, 2004).

Nitrogen is a fundamental component of proteins, nucleic acids and other essential molecules. Nitrogen is absolutely essential for life, being the fourth most abundant element in living organisms (behind hydrogen, oxygen, and carbon) (Maia & Moura, 2014). Nitrate (NO_3^-) is a part of the nitrogen cycle, found naturally in the environment and an important plant nutrient.

In the past century the industrial use of nitrogen compounds has grown exponentially and Humans have learnt to acquire reactive nitrogen through the industrial Haber-Bosch process, which captures di-nitrogen gas and converts it into ammonia (NH_3).

This process dramatically increased use of nitrogen compounds as fertilizers, plastics, explosives, among many other products, without considering that most of the nitrogen is leaked back into the environment.

Perturbations affecting global nitrogen cycle received less attention than disturbance to the carbon cycle, but the nitrogen cycle is at least as, if not more, out of balance.

Water pollution by nitrates is a concern because of introduction of intensive farming methods, with increased use of chemical fertilizers and higher concentrations of animals in smaller areas. Agriculture is the greatest cause of nitrogen pollution; as the use of nitrogen fertilizers increases field productivity,

unfortunately, most of nitrate and nitrite are washed away by water and reach the surface and ground waters.

High concentration of nitrogen organic compounds and the chemical fertilizers used in agriculture frequently causes severe environmental pollution. Among the N-fertilizers, about 80% of the demand are met by urea, which is highly water soluble and prone to losses. In rice cultures when any N compound is applied, it is lost through leaching, denitrification, volatilization and runoff. Of the total N loss, leaching contributes about 30-50%, mostly as nitrate; denitrification, about 10-30% as N_2, and volatilization, about 2-30% as ammonia (Ghosh & Bhat, 1998).

Nitrogen pollution wide-ranging impacts include contributions to global warming, acid rains and eutrophication.

Nitrates Directive

In order to protect waters, European Commission issued the Nitrates Directive in 1991.

The *Nitrates Directive (*Council Directive 91/676/EEC) forms an integral part of the *Water Framework Directive* with the aims of protectingground and surface waters across Europe against pollution caused by nitrogen compoundsfrom agricultural sources (http://ec.europa.eu/environment/water/water-framework/index_en.html).

Countries of EU community were forced to identify surface and groundwater affected by pollution or at risk of being so. These areas were identified as the so-called “Nitrate Vulnerable Zones” with the use of procedures and criteria detailed in the Directive. These establish and promote a code of good agricultural practice to be implemented by farmers on a voluntary basis

(http://ec.europa.eu/environment/marine/good-environmental-status/descriptor-5/index_en.htm).

The directive established a limit for nitrate concentration in surface and in ground waters (50mg/L) and a limit for the use of nitrogen compounds and livestock manure as fertilizers (170 kg/ha).

Member States must monitor water quality, applying standardized reference methods to measure the nitrogen-compound content; if needed, additional measures have to be taken.

European Commission makes a report every four years and the last published is in 2011.

In this report is possible to see that the information on N-discharge into the environment has not been provided by all 27 Member States. However, according to the available data, a decrease in discharge has been observed but agriculture remains the biggest source of nitrogen discharged into the environment, as in the previous reporting periods.

Nitrate and nitrite risk

Nitrite is widely consumed - in minute amounts and very low concentrations - from the diet by animals and humans. However the largest contribution to exposure results from the *in vivo* conversion of exogenously derived nitrate to nitrite. Because of its potential to cause to methaemoglobin (MetHb)(Ghosh & Bhat, 1998).

In waters with an elevate nitrite concentration, we have the same toxic action on aquatic animals, particularly on fish and crayfish; it is due to the conversion of oxygen-carrying pigments to forms that are incapable of carrying oxygen, causing

hypoxia and ultimately death. In fish, entry of nitrite into the red blood cells is associated with the oxidation of iron atoms ($Fe^{2+} \rightarrow Fe^{3+}$), functional hemoglobin being converted into methemoglobin that is unable to bind molecular oxygen (Camargo & Alonso, 2006)

Further, it is assumed that nitrate is reduced to nitrite in the gastro-intestinal tract due to microbial activity and the nitrite formed reacts with secondary amines and amides producing carcinogenic N-nitroso compounds. (Ghosh & Bhat, 1998).

Forages and contaminated water have been shown to contain high levelsof nitrate and represent the largest contributors to nitrite exposure for food-producing animals.

Basidiomycetous Yeasts

In the last decades, biotechnologies gradually predominated in many scientific fields. Often these technologies provide the use of microorganisms for bioremediation, chemical reactions and drug synthesis.

Yeasts play an important role in biotech applications. These eukaryotes have many interesting features and their life is often related with ours.

In particular, since ancient times Ascomycete yeasts have been used in biotechnology. The most typical example is *Saccharomyces cerevisiae*, which for its use in production of fermented beverages and foods is considered a pivotal event in human history and for advances in biotechnology.

Saccharomyces cerevisiae is present in human history since a long time. This yeast is the most common known and studied but it is not the only yeast used in biotechnology. Ascomycota (in particular *Penicillum* genus) are used in food productions (cheese) and mostly for the production of penicillin (antibiotic drug).

In recent times research about yeasts developed quickly, increasing their potential biotech applications, being the simplest eukaria model organisms.

Yeasts at the moment are the major producers of biotech products worldwide; many types of yeast have fundamental importance in scientific research, food, medical, and agricultural industries.

Most of the yeasts used and studied for biotechnology are Ascomycetes, important for production of proteins, food and fodder, heterologous proteins and enzymes, and as models and fundamental organisms for the study of genes and their function in mammalian and human metabolism, and in disease processes.

In contrast, the basidiomycetous yeasts have not commonly been recognized to have historical beneficial roles for humans (Johnson, 2013); knowledge about basidiomycetous yeast is limited and in this context *Basidiomycota* phylum is now a new frontier for research.

During the past five decades basidiomycetous yeasts have been shown to have beneficial attributes mainly for the production of enzymes used in pharmaceutical and chemical synthesis, for production of certain classes of primary and secondary metabolites as carotenoids, for bioremediation process and for their ability to degrade environmental pollutants and xenotoxicants.

Basidiomycetous yeasts are unfortunately also involved in food spoilage and economic losses, especially the genera *Cryptococcus* and *Rhodotorula* (Johnson, 2013).

Many species of Basidiomycetes utilize recalcitrant substrates, including pentoses such as xylose and arabinose, sugar alcohols, and to a limited degree tannin and lignin components present in lignocellulosics, providing a mean of biomass utilization.

Basidiomycetes features

The Basidiomycetes form a unique group of fungi with several key features, including the formation of a distinct sexual state characterized by basidia and basidiospores, a unique cell wall composition, generally highly oxidative catabolic capabilities with ability to degrade recalcitrant natural substrates and xenobiotics, and the ability of certain species to form brilliant carotenoids.

Basidiomycetous yeasts are recognized as fungi with asexual reproduction by budding or fission. Yeasts do not form their sexual states within or upon a fruiting body. The life cycle of basidiomycetous yeasts include heterothallic and homothallic systems, and the teleomorphic species form basidia.

Basidiomycetous yeasts were not recognized as such until the twentieth century. The definitive demonstration of yeasts with basidiomycetous sexual reproduction was shown in *Rhodotorula*, leading to the new teleomorph species *Rhodosporidium* (Banno, 1963, 1967), and subsequent discovery of sexual states in other yeast species (Newell & Hunter, 1970). Until 2001 possibly only 1-5% of the existing species have been discovered; and our understanding of their phylogeny is emerging as a result of recent methods and studies in molecular biology and ultrastructure (Hibbett & Thorn, 2001).

Examination of physiological properties is the primary method for differentiating species as described by Barnett (1990) and Kurtzman and Fell (1998). Routine tests include fermentation and growth with carbon sources, growth on nitrogen compounds, requirements for vitamins, growth at various temperatures, hydrolysis of urea, and formation of starch-like compounds. Because few basidiomycetes ferment at rates that result in visible reactions, fermentation tests are usually limited to glucose (Hibbett & Thorn, 2001).

By virtue of their ecology and habitat, many basidiomycetous yeasts produce valuable enzymes and end-products. Many of these products enable protection against radiation, reactive oxygen species and pollutants, and allow these yeasts to grow aerobically on recalcitrant substrates. Basidiomycetous yeasts are important in environmental remediation, including metal adsorption and probably radionuclide extraction from the environment(Dae Haeng Cho & Kim, 2003).

The knowledge on the phylum is increasing highlighting some economically important Basidiomycota.

Phaffia rhodozyma (teleomorph of *Xanthophyllomyces dendrorhous*) is a biological source for astaxanthin, an economically important pigment used in aquaculture. There is a growing market for astaxanthin as marine fish farms account for 10 to 15% of the seafood business (Johnson, 2013)

Cryptococcus laurentii, C. curvatus, Rhodotorula glutinis, R. gracilis, R. graminis, R. mucilaginosa, Trichosporon cutaneum, T. pullulans, are able to accumulate up to 40 % of its dry weight as fatty acids (Gill, Hall, & Ratledge, 1977) and *T. pullulans* accumulates more than 65% of its biomass as lipid

Rhodotorula

Rhodotorula is a genus of imperfect yeasts within the family of anamorphic yeasts *Cryptococcaceae*. This is part of the *Sporidiobolus* clade which represents the red-pigmented teliosporic yeasts *Rhodosporidium* and *Sporidiobolus* with phragmometabasidia (Hibbett & Thorn, 2001).

The vegetative form of the organism is spheroid or oval or elongate and contain conspicuous fat globules when growth in 5% malt extract. Ballistoconidia are not

formed. The colonies are often reddish, pink, orange or yellow in consequence of pigment synthesis, mainly carotenoids.

Some cultures of *Rhodotorula spp*. are smooth and moist to mucoid, but others are pasty or dry and wrinkled, with variable growth at 37ºC and a diameter between three to five microns (C. P. Kurtzman, Fell, & Boekhout, 2011).

Reproduction is through multilateral or polar budding. Strains of some species form pseudo or true hyphae. Ascospores or ballistospores are not formed. And undergo sexual reproduction with mycelial clamp connections and teliospores (Hernández-Almanza et al., 2014).

Rhodotorula yeasts have an exclusively oxidative pathway of energy metabolism; they are all non–fermentative. The possible carbon sources change from species to species (C. P. Kurtzman et al., 2011), but usually these yeasts are considered unable to assimilate inositol as the sole carbon source, whereas most of organic acids and alcohols can be utilized.

Rhodotorula species are capable of utilizing non-carbohydrate substrates as well as carbohydrates. *Rhodotorula* isolates capable of biotransforming polycyclic aromatic hydrocarbons are known. Some esters of testosterone, such as acetate, propionate, enanthate, caprate, undecanoate, isobutyrate and isocaproate (some of them are used as drugs) are transformed by *R. mucilaginosa*.

Rhodotorula species are isolated in many different environments and conditions; species of this genus were isolated from bark-beetles, tree exudates, various types of vegetables and plants, soils and fresh waters. They are also frequently encountered in coastal sediments. *Rhodotorula* spp. are found in crabs, clams and insects (Molnár, Wuczkowski, & Prillinger, 2008). Species of this genus are harmless components of the microflora of blood-sucking mosquitoes.

Rhodotorula spp. have been also found in alpine environments (Margesin, Fonteyne, Schinner, & Sampaio, 2007).

In humans, *Rhodotorula* species occur rarely in the oral cavity in healthy individuals, but are found in the microflora of patients with oral cancer or psoriasis.

Rhodotorula glutinis

A large part of information about the species *Rhodotorula glutinis* is found in the book "the Yeasts" write by C.P. Kurtzman and al.

R. glutinis was described for the first time by F.C. Harrison in 1958. After growth in malt extract and in malt agar the species presents main features of the genus; the cells are ovoidal or spherical and the colour changes from orange to red.

The colony surface varies from smooth, often with fine transverse striations, to wrinkled; the appearance is from highly glossy to semi-glossy. The texture varies from mucoid to pasty to slightly tough. This specie belongs to Basidiomycota phylum; Urediniomycetes class and Sporodial order.

On Sabouraud-Dextrose Agar, cultures of *Rhodotorula* species grow rapidly at 30°C; they are coral pink, smooth, and moist to mucoid, and growth at 37°C is variable (Hernández-Almanza et al., 2014).

R. glutinis is particularly important for food industries because their biotechnological potential and safety implications.

R. glutinis is the type species of the genus, being worldwide distributed and isolated from a wide variety of substrates. It is probably the most prevalent species in the genus. The species is not considered to be a human pathogen except for immune-suppressed and immune-compromised subjects.

Rhodotorula spp. are indeed know for very interesting abilities:

Strains of *R. glutinis* K–24 produces a highly viscous polysaccharide (Fukagawa, Yamaguchi, Yonezawa, & Murao, 1974); strains of this specie were tested on oil-polluted environment (Csutak, Stoica, & Vassu, 2012). Indeed these molecules has also been used in chemico-physical processes to remediate hydrocarbon or heavy metal contaminated sites, such as in *in situ* soil flushing and *ex situ* soil washing for remediation of unsaturated zone or pump and treat for aquifer remediation (Ruggeri et al., 2009).

The possibility of using *R. glutinis* in environments and on substrates contaminated by heavy metals is supported by various publications: *R. glutinis* R-1 showed multiple tolerance towards aluminium and manganese ions (Nguyen, Senoo, Mishima, & Hisamatsu, 2001). *Rhodotorula* sp. Y11, a red-pigmented yeast, isolated from mine soil, could survive till 2000 mg/L cadmium and is found to absorb cadmium with high efficiency (Z. Li, Yuan, & Hu, 2008). *R. glutinis* KCTC was characterized for Pb^{2+} biosorption from aqueous solution (Dae Haeng Cho & Kim, 2003).

R. glutinis is known to be an oleaginous yeast (having at least 20% oil by dry weight), being able to accumulate lipids up to 40% in particular growth conditions. For this reason, in many studies, it is considered a viable candidate for biodiesel production (Sitepu et al., 2014).

Identification of the genus

In the past identification of species was made which morphological, nutritional and physiological tests and often, required days or weeks to be completed (Yeeh, 1999). Detection, identification and classification of yeasts have undergone a

major transformation in the past decade tanks to the use of new techniques. Indeed, genetic methods are now preferred to perform molecular microbial taxonomy (Biswas, Yokoyama, Nishimura, & Miyaji, 2001; Seifert, 2009).

Now we have a database (barcode) of easily determined gene sequences from domains 1 and 2 (D1/D2) of large subunit rRNA and from the internal transcribed spacer (ITS), allowing many laboratories to accurately identify species and this led to double the number of known species of yeasts over the past decade (Cletus P. Kurtzman, 2014).

The *Rhodotorula* genus, as is currently defined, includes 34 species comprising a polyphyletic group of organisms. *R. glutinis* is one of the 34 species, classified taxonomically in the super-kingdom *Eukaria*, kingdom Fungi, sub-kingdom *Dikaria*, phylum *Basidiomycota.*

Pathogenicity of Rhodotorula

Only in very rare reports *Rhodotorula* could be associated with human opportunistic infections,usuallyin elderly and in immunodepressed patients (Lanzafame, De Checchi, Parinello, & Cattelan, 2001; Mondello, 2010).

It is often present in the oral cavity as a commensal in patients with infections.

R. glutinis infections are related to fungaemia in children and fungal infections can occur in neutropenic patients due to *R. rubra*; furthermore some species cause respiratory allergy. *Rhodotorula* species are associated with disruption of the natural barrier of the skin, including catheterization of urinary, venous and arterial systems. Immunodeficiency increases the prevalence and severity of mycosis caused by fungi such as *R. rubra*. Fungaemia in patients infected with human

immunodeficiency virus (HIV) often presents as a community-acquired infection, which is frequently due to newly emerging opportunistic *R. rubra*.

Rhodotorula is found in patients with acute myeloblastic leukaemia under bone marrow transplant; *R. glutinis*is responsible for infections during leukopenia; *R. mucilaginosa*-induced meningitis in HIV-infected patients can be characterized by severe headache and high body temperature (Mohd Nor, Tan, Na, & Ng, 2015).

Rhodotorula spp. are often isolated from living animals (Bond, 2010), some of these species are potential human pathogens. However, no sufficiently reliable criteria can be established to prove the association with disease in humans. In reptiles, dermatomycosis has been detected. *Rhodotorula* is involved also in bovine mycotic mastitis and can be isolated from milk samples from normal, clinical and subclinical mastitis quarters from dairy herds (Ksouri, Djebir, Hadef, & Benakhla, 2014). It is known that serum IgG antibody concentrations against *R. glutinis* in horses change seasonally.

R. glutinis is fully resistant to itraconazole, but sensitive to a pradimicin derivative and *R. minuta* and *R. rubra* infections are successfully treated with amphotericin, miconazole and 5-fluorocytosine.

Nitrate, nitrite, and Rhodotorula

In a collaboration period with the Department of Biochemistry and Molecular Biology of La Laguna University (ULL), Spain, under the supervision of Prof. J. M. Siverio, we have applied on our strain some of investigation techniques that have been used over the years to study nitrogen assimilation of *Hansenula anomala* (CECT 1 1 12), *H. wingei* (CBS 2432) *and H. polymorpha* (CBS4732).

R. glutinis was shown to be able to assimilate nitrate as sole nitrogen source, like other yeasts studied by the team of ULL (Barnett, Payne, & Yarrow, 1983).

Nitrate is reduced to nitrite by nitrate reductase (NR), and nitrite in turn to ammonium by nitrite reductase.

Nitrate reductase and nitrate uptake system appear to be the main points of regulation in the nitrate assimilatory pathway.

Regulation of nitrate assimilation takes place mainly at the level of NR, which has evolved a variety of regulatory mechanisms in different organisms. Nitrate assimilation balance (with nitrate and nitrite efflux) is unknown, as well as the proteins involved.

In yeasts, nitrate acts as an inducer once it enters the cell, and therefore, intracellular nitrate levels play a key role in regulating nitrate assimilation genes. In this framework, nitrate and nitrite effluxes from the cell could play an important role in net nitrate/nitrite uptake and also in keeping nitrite below toxic levels.

Nitrite efflux has been observed in most organisms, including *H. polymorpha* growing in nitrate, indicating a clear imbalance between nitrate uptake and reduction to nitrite and its further transformation to ammonium.

In general, nitrate assimilation requires aerobic energy metabolism; nitrate is the inducer of NR synthesis while reduced nitrogen sources (nitrite and ammonium) are repressors.

So far (A. H. Ali & Hipkin, 1985; Hipkin, Flynn, Marjot, Hamoudi, & Cannons, 1990) it has been shown that NR appears in cells cultured in nitrate-containing media and is absent in ammonium-cultured cells.

In Basidiomycota yeasts the rapid inhibition of nitrate assimilation by ammonia was not the result of an inhibition of nitrate reductase (NR) activity. Nitrite also inhibited nitrate assimilation. NR in cell-free extracts of *S. roseus* was NADPH specific and its activity was repressed in cultures containing ammonia and de-repressed during nitrogen starvation. Nitrate stimulated the appearance of NR in these cultures.

Several questions remain unanswered concerning the regulation of NR expression and repression in yeasts .Particularly the complex interactions among activating/repressing mechanisms (involving the effects of nitrate, reduced nitrogen forms, and carbon sources) are not still completely clear (Gonzalez & Siverio, 1992).

Metabolites from Rhodotorula *spp.*

Substances produced by *R. glutinis* and *R. rubra* have 'killer' effects on other microorganisms. *Rhodotorula* species isolated from water or sediment samples are known to have lethal effects against the majority of ascomycetous and basidiomycetous species. *R. glutinis* isolated from phylloplane is found to produce antibacterial compounds inhibitory to both *Pseudomonas fluorescens* and *Staphylococcus aureus* (McCormack, Wildman, & Jeffries, 1994).

Some species of the genus are known for the ability to accumulate elevate amounts of lipids; *R. graminis* is able to use a broad range of carbon sources for lipid production, and is able to resist some of the inhibitors commonly released during hydrolysis of lignocellulosic materials (Yeeh, 1999).

R. glutinis is a strong synthesizer of lipids. *Rhodotorula spp.* can produce over 20% of their biomass as fat and the yields can approach 70% (dry weight) of cell

mass under specialized culture conditions. The yeasts can produce a lipid yield of 40% from molasses and 67% from sugar cane syrup. *n*-Alkanes, starch, waste cellulose hydrolysates, molasses, peat moss hydrolysate, ethanol, glucose, lactose and xylose are all substrates for lipid synthesis. The major fatty acids synthesized are oleic, linoleic and palmitic acids.

The composition of extracellular, insoluble glycolipids can be influenced by the addition of precursors (long-chain lipids and hydrocarbons) to culture media.

Exopolysaccharides

Fungal polysaccharides (PSs) are classified into different groups according to structure (linear and branched), sugar composition (homo- and hetero-polysaccharides), type of bonds between the monomers (β-(1 → 3), β-(1 → 6), and α-(1 → 3)) and their location in the cell (cell wall PSs, exoPSs, and endoPSs). Exopolysaccharides (EPSs) play different biological functions, for example in the protection against environmental stress factors and in interactions with other organisms. EPSs obtained from Ascomycota and Basidiomycota fungal cultures are known for their antioxidant, immunostimulating, antitumor, and antimicrobial properties (Osińska-Jaroszuk et al., 2015).

PSs are water-soluble macromolecules increasing the viscosity of the medium under the influence of different physical and chemical agents (Pavlova & Grigorova, 1999). Biopolymers with industrial application are bacterial and fungal products like xanthan, dextran and scleroglucan.

The exopolysaccharides from yeast cells are more easily separated than in bacterial system and thus they are attractive for large-scale production (Peterson et al. 1989). The types of polymers reported for yeast producers include mannans,

glucans, glucomannans, galactomannans, phosphomannans (Pavlova & Grigorova, 1999).

Rhodotorula genus possess the ability to synthesize other bioactive substances extracellularly. Strains of *R. rubra* cultivated on synthetic substrates containing carbohydrates can synthesize exopolysaccharides (G. Frengova, Simova, & Beshkova, 1997).

Rhodotorula glutinis is known to produce, when grown in yeast nitrogen base medium (YNB) (Gorin, Horitsu, & Spencer, 1965), exocellular polysaccharides. Monosaccharide composition of the synthetized biopolymers is known to be predominantly D-mannose and in smaller quantities fucose, glucose, and galactose (D. H. Cho, Chae, & Kim, 2001).

Carotenoids

Carotenoids belong to the group of yellow or red pigments that occur widely in plants, animals and humans. They are the most common class of pigments in nature being synthesized in plants and in some microorganisms.The number of known natural carotenoids was about 80 in 1960, around 500 in 1980 (Feltl, Pacakova, Stulik, & Volka, 2005), and currently about 750 known natural carotenoids which, in various combinations, are responsible for the yellow, orange, red and purple pigmentation in plants, microorganisms and animals.

Carotenoids are only introduced with diet into human and animal organisms, since they are incapable of *de novo* synthesis.

Among pigments of natural origin, carotenoids seem to play a fundamental role. Their presence in human diet is considered positive because of their action as pro-vitamin, antioxidant or possible tumor-inhibiting agents.

Carotenoids have been intensely studied because of their highly physiological importance: they always accompany chlorophyll and assist photosynthesis and phototaxis as auxiliary light absorbers and, on the other hand, protect plants and microorganisms against excessive irradiation. Furthermore, they strongly interact with reactive oxygen species acting in plant and animal organisms as potent free radical quenchers, singlet oxygen scavengers and lipid antioxidants; some of them are vitamin A precursors. Changes in their contents and structure can also act as markers of environmental damage (Feltl et al., 2005)

Yeasts from the genus *Rhodotorula* are able to synthesize different pigments of high economic value like β-carotene, torulene, and torularhodin, and therefore represent a biotechnologically interesting group of yeasts.

The economic significance of carotenoids is increasing because of their commercially use as dyes for food, feed, and cosmetic products, as nutritional supplement due to their pro-vitamin A character and in pharmaceutical products for their anticancer and antioxidant properties (Ungureanu, Ferdes, & Chirvase, 2012). Carotenoid production in *Rhodotorula* yeasts has advantages over other microorganisms such as algae and other fungi. Specific growth rate is high and large quantity of cell biomass is relatively easy to obtain at lab and pilot plant scale. Cells can be cultured in conventional bioreactors and biomass can be used directly as feed or as additive in pharmaceutical products. Besides, yeasts can adapt to different environmental conditions and grow under a wide variety of carbon and nitrogen sources. However, the low production rate of pigment in these microorganisms limits its industrial application (Moliné, Libkind, & Van Broock, 2012).

Carotenoids are long, aliphatic, conjugated double bond systems, usually composed of eight isoprene units with the molecular formula $C_{40}H_{56}$. The central portion of the molecule contains four isoprene units, two of which are joined tail-to-tail and open chain or ring structures form the ends of this chain. These hydrocarbons are called carotenes. A great majority of natural carotenes have double bonds in the all-trans position, where R is an open-chain structure or a ring system. Only a few natural carotenes exhibit a cis-trans configuration.

Another part of the carotenoid group are oxygenated derivatives of carotenes with various combinations of e.g. hydroxy-, epoxy-, alcohol-, aldehyde-, keto-, lactone-, carboxylic acid-, ester-, or phenolic functions. These compounds are called xanthophylls. The oxygen-containing functional groups are located at the chain ends, not within the multiconjugated system. No heteroatoms other than oxygen have so far been found in natural carotenoids.

About one half of natural carotenoids are chiral, usually containing one to six chiral centres. Physico-chemical attacks (light, temperature, oxidants, substituents, etc.) at carotenoid molecules have profound effects on the structure and configuration of the products and thus also on theirphysico-chemical properties. Trans-cis shifts have especially strong effects on the overall shape of the molecule and thus also on its properties.

Most *Rhodotorula* species produce different types of carotenoid pigments, four of which were identified in almost all species: torularhodin (3', 4'-Didehydro-β, ψ-caroten-16'-oic acid), torulene (3',4'-Didehydro-β,ψ-carotene), γ-carotene (β,ψ carotene), and β-carotene (β,β-carotene). Pigments are synthesized via the mevalonate pathway (Disch, Schwender, Müller, Lichtenthaler, & Rohmer, 1998). Mevalonic acid is transformed in isopentenyl pyrophosphate units and then

successively condensed to form phytoene. Successive transformations of phytoene leads to each of the aforementioned pigments. The proportion of each carotenoid depends on the strain and culture conditions (P. Buzzini)

Due to the presence of a long carbon chain with conjugated double bonds, most of carotenoids are lipophilic, practically insoluble in water but soluble in organic solvents such as acetone, ethyl ether, chloroform, ethyl acetate, dimethylsulfoxide, petroleum ether, hexane and toluene.

The conjugated double bonds rappresent the chromophore portion of the molecule and determine the absorption in the visible spectrum.Most carotenoids absorb at three wavelengths whose value increases in proportion to the number of conjugated double bonds, for example β-carotene, which possesses 11 conjugate double bonds, is orange-yellow and shows absorption maxima at 450 and 477 nm, and a shoulder at 425 nm in hexane.

Carotenoids follow the Lambert and Beer's law, and their absorbance is directly proportional to the concentration in a given solvent and this allows the spectrophotometric quantification, but the absorption measured is strictly dependent on the solvent used.

Carotenoid biosynthesis

In general, terpenoid biosynthesis can be divided into four parts.

i) synthesis of the isoprene unit, isopentenyl pyrophosphate;

ii) assembly of isopentenyl pyrophosphate into (C5)*n* isoprenoid backbone;

iii) cyclization of (C5)*n* isoprenoid backbone into the carbon skeletons;

iv) formation of individual terpenoids.

The first step in the terpenoid biosynthesis pathway is the synthesis of the isoprene chain. Mevalonic acid is obtained from acetyl-CoA by mevalonic acid pathway, using the well-know 3-hydroxy-3-methyl-glutaryl-CoA as the key intermediate. The first step consist in a condensation of 3 Acetyl-CoA molecules to produce one 3-hydroxy3-methyl-glutaryl-CoA molecule, that is in turn reduced in the cytoplasm by the enzyme HMG-CoA reductase. Mevalonate undergoes a phosphorylation, forming the mevalonate pyrophosphate (Figure 1).

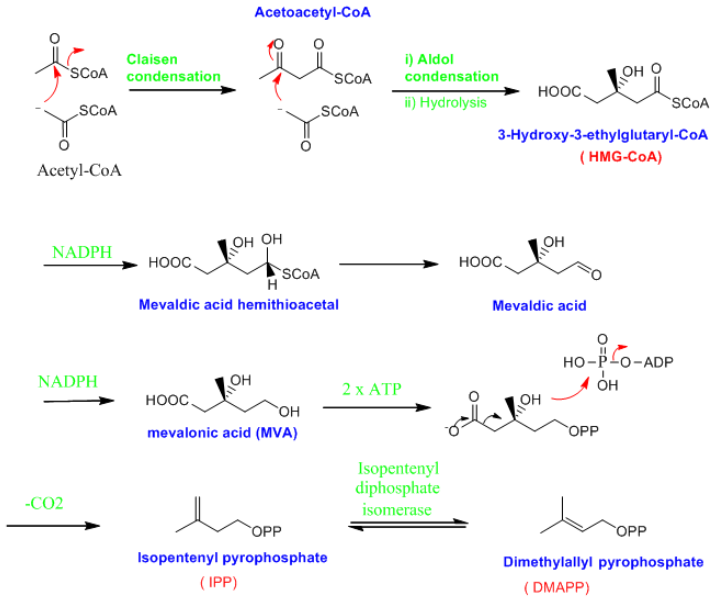


Figure 1: Mevalonic Acid Pathway
(from http://ictwiki.iitk.ernet.in/wiki/index.php/Terpenes)

Simultaneous elimination and decarboxylation reactions occur, yielding 3-isopentenylpyrophosphate (IPP). This is the isoprene unit that will be isomerized into 3,3-dimethylallyl pyrophosphate (DMAPP) that is the alkylating agent in the biosynthesis of isoprenoid. The described pathway is the main way in animals, fungi, plants and in some bacteria, but alternative pathways are known in other bacteria and in phototrophic organisms.

IPP and DMAPP are universal building blocks for the synthesis of various classes of terpenes. The enzyme, isopentenyl pyrophosphate isomerase catalyses the reaction between IPP and DMAPP in presence of metal ion to produce geranylpyrophospahte (GPP, C10), farnesyl pyrophosphate (FPP, C15), geranylgeranylpyrophosphate (GGPP, C20), squalene (C30) from IPP and DMAPP; this is a precursor for mono-, sesqui-, di-, tri-, tetra-, and poly-terpenes.

In the presence of isopentenyl pyrophosphate isomerase, pyrophosphate group is activated and acts as leaving group to generate an allylic-tertiary carbocation. The carbocation acts as an electrophile and is attacked by the double bond of IPP, a second substrate, to generate a new carbocation which on stereospecific loss of proton produces various precursors for the synthesis of terpenes. This enzymatic reaction involves removal of the pro-R H atom and formation of new C=C double bond.

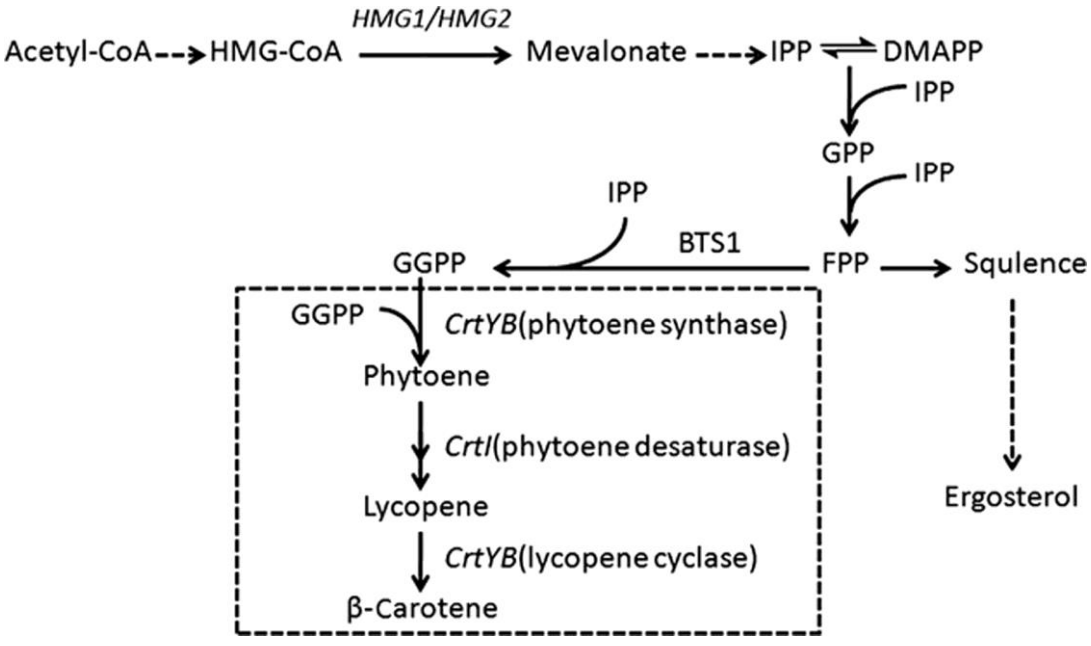


Figure 2: Pathway for the biosynthesis of carotenoids

The next step is the cyclization of GPP, FPP, or GGPP to mono-, sesqui-, or di-terpenoids respectively. This is achieved by enzymes known as terpene cyclases, a large family of enzymes that use GPP, FPP or GGPP as substrates for the formation of mono-, sesqui-, di-terpenoid products. The enzyme terpene cyclase uses Mg^{2+} or Mn^{2+} as a co-factor during catalysis.

The dimerization of GGPP leads to the first C40 compound (phytoene, Figure 2), that is the starting block for all carotenoids synthesis.

Carotenoids in Rhodotorula

R. glutinis is widely known as a β-carotene producing yeast (Pietro Buzzini & Martini, 2000). It is potentially useful for industries since it is able to grow on various inexpensive agricultural raw materials such as sugar cane juice, sugar cane molasses,peat extract, whey, grape must, beet molasses, hydrolyzed mung bean waste flour, soybean and corn flour extracts (Aksu & Eren, 2007; Bhosale & Gadre, 2001; Pietro Buzzini & Martini, 2000; G. Frengova et al., 1997). It has an advantage over algae, fungi and bacteria due to its unicellular and relatively high growth rate with utilizing low cost fermentation media (Malisorn & Suntornsuk, 2008).

Carotenoid pigments accumulation in most yeasts starts in the late logarithmic phase and continues in the stationary phase; the presence of a suitable carbon source is important for biosynthesis during the non-growth phase. (GinkaI Frengova & Beshkova, 2009).

Different autors have investigated carotenoids production by *R. glutinis* in different condition: as a function of initial pH, temperature, aeration rate, initial sugar (glucose, molasses sucrose and whey lactose),ammonium sulfate

concentrations and activator (cotton seed oil and Tween 80) addition (Aksu & Eren, 2007). An optimum of pH 6 and 30°C were reportedfor total carotenoids production. The yield was significantly enhanced with increasing aeration rate. An initial ammonium sulfate concentration of 2 g L^{-1} gave the maximum carotenoids production.

According to El-Banna (El-Banna, El-Razek, & El-Mahdy, 2012) the production of carotenoids depends on the C/N ratio, the type of carbon and nitrogen source, the presence of mineral salts and the temperature of growth. In relation to the C/N ratio these authors report an increase in production of biomass and a parallel decrease in the production of carotenoids with increasing C/N, suggesting the use of high fructose corn syrup to maximize the production of biomass and sucrose to maximize carotenoids yield.

Other autors observe that most of the volumetric production of carotenoid is obtained in *R. glutinis* for C/N equal to 10. (Bhosale & Gadre, 2001)

Latha et al. (2005) distinguish the effect of monosaccharides, disaccharides and pentoses on the production of carotenoids reporting that fructose leads to a greater quantity of carotenoid compared to glucose and galactose. Among the disaccharides, sucrose resulted the best choice for biomass yield and for carotenoids production. It has also been observed that L-arabinose does not determine a good growth and yield of carotenoids; D-xylose allows good growth but it does not allows a good yield of carotenoids while D-ribose allows to obtain both a good biomass yield and carotenoids production.

The type of carbon source also affects the proportion of the products pigments: It has been reported that the administration of glucose, fructose or sucrose maximizes the production of β-carotene (69%), torulene (63%) or (60%),

respectively (Bhosale and Gadre (2001). Fructose or glucose increase the percentage of β-carotene, while sucrose and glucose syrup increase the percentage of torulene and torularhodine (El-Banna et al., 2012).

Carotenoids production depends also on the nature of the nitrogen source. The ratio of β-carotene, torulene and torularhodine can be different on the basis of the type of nitrogen source used.

It has been reported that organic nitrogen sources (Bhosale & Gadre, 2001) including the acid casein hydrolyzate, urea and yeast extract have a positive effect on the production of carotenoids but data reported by different authors are not in agreement.

The temperature is a very important factor for carotenoid production and influences the different products pigments ratio.

Aksu (Aksu & Eren, 2007) reported an optimum of temperature for total carotenoids production as 30°C, and most of the authors carried *R. glutinis* cultures out between 25 and 35°C.

Frengova (G. Frengova et al., 1997) and Buzzini (1999) observed that in the range between 20 and 25°C the synthesis of β-carotene and torulene is higher than that of other carotenoids while in the range between 30°C and 35°C the synthesis of torularhodine is higher.

R. glutinis is an aerobic microorganism; for this reason the effect of ventilation is very important for cell growth and for the production of carotenoids (Bhosale & Gadre, 2001). Aksu and Eren (2007) have shown that the increase of the aeration rate has a positive effects on cell growth and consequently on the yield of carotenoids.

Carotenoids are also important because of their antioxidant action, so their production could be greatly influenced by the presence of exogenous stress factors during the fermentation (addition of NaCl, H_2O_2) that can determine an increases on the production of β-carotene.

Several authors have reported a different production of pigments produced upon addition to the culture medium of metal ions such as copper, zinc, iron, calcium, and aluminum (Bhosale & Gadre, 2001; Buzzini, 1999) that may activate or inhibit the enzymes involved in biosynthetic paths (Buzzini, 1999), or induce the formation of active oxygen species.

Also the light can positively influence pigment production: the white light increases the production of torularhodine and β-carotene (Bhosale & Gadre, 2001; Sakaki, Nakanishi, Tada, Miki, & Komemushi, 2001), while UV has been proven useful to increase the production of carotenoids in *R. glutinis (Marova, Carnecka, Halienova, Breierova, & Koci, 2010).*

The addiction to the the culture medium of intermediates of the citric acid cycle and solvents such as ethanol, methanol, ethylene glycol stimulate the synthesis of carotenoids (Bhosale, 2003)

Ethanol, for example, activates hydroxy-methyl-glutaryl CoA reductase and consequently increases the production of carotenoids, and being ethanol a pro-oxidant, it would result in an increase in reactive oxygen species oxygen and consequently a greater production of carotenoids due to the presence of exogenous stress factors.

Aim of the work

The research presented in this PhD Thesis focused on the isolation of a *R. glutinis* strain from plates with elevates nitrogen concentrations (10 mM $NaNO_3$). The elevate tolerance towards nitrogen compounds has induced our research group to study the strain for potential development to bioremediation tool and industrial applications.

Accordingly, the nitratophile yeast *R. glutinis* DSBCA06 has been studied to define its nitrogen-based metabolism, optimize the assimilation of nitrogen compounds, and evaluate the potential usefulness of the strain in bioremediation applications. The produced biomass could be applied to reduce oxidized nitrogen polluting compounds and to convert them into proteins, less environmentally impacting and with potential application as fertilizers or as animal feed.

Besides we evaluated at the same time the production of secondary metabolites, with significant added value, to develop a cost-efficient tool for their production even starting from waste.

MATERIALS AND METHODS

Typical agar media used in the study

Yeast Nitrogen Base agar (YNB)

In each liter of medium were present: 1M Buffer K_1K_2 (KH_2PO_4, K_2HPO_4) pH 7 50mL, Mix 100x vitamins and oligoelements (composition for one litre: biotin 0,2mg, folic acid 0,2mg, copper sulfate 4mg, potassium iodide 10 mg, p-amino benzoic acid 20 mg, calcium pantothenate 40 mg, inositol 200 mg, niacin 40 mg, pyridoxine 40 mg, riboflavin 20 mg, thiamine HCl 40 mg, boric acid 50 mg, ferric chloride 20 mg, manganese sulfate 40 mg, sodium molybdate 20 mg, zinc sulfate 40 mg) 10mL, KH_2PO_4 0,2 g, NaCl 0,02 g, magnesium sulfate 0,1 g, calcium chloride 0,02 g, sodium nitrate 1.6998 g, agarose (Sigma05040) 15 g, only for solid plates).

Glucose Yeast Peptone agar (GYP)

Agar plates were prepared with Glucose Yeast Peptone (GYP): for one liter, glucose 20 g, yeast extract 10 g, peptone from casein 10 g, phosphate buffer pH 6 10 mL, agarose ((Sigma05040) 15 g, only for solid plates).

Isolation of the strain R. glutinis DSBCA06

Several YNB agar plates ($NaNO_3$ enriched as the sole nitrogen sources) were exposed to air overnight in various location in Cagliari, Sardinia. After 4 days' incubation at 25°C, the colonies that were growing faster were passed through multiple streaks in the same medium until isolation.

Molecular characterization of R. glutinis DSBCA06

DNA extraction

Genomic DNA was extracted from *R. glutinis* DSBCA06 cells using Wizard genomic DNA purification kit (Promega) according to the manufacturer's instructions.

DNA sequencing

Purified DNA was then sequenced though external service (BMR Genomics). Samples of 200 ng (conc. 30 ng/µl) of genomic DNA we used. The service "identifungo plus" (http://www.bmr-genomics.it/seq_index.html#genescan) was based on the analysis of ribosomal DNA sequences ITS1 and ITS2 (Internal Transcribed Spacer), and D1 and D2 sequences from subunit 26S from rDNA regions. Sequencing gave back 1500 bases (6 sequences), usually sufficient for identification at the species level.

The sequences contained in the amplifier of our strain were compared with those of the database NT of NCBI.

Physiological characterization and optimization of growth conditions

Evaluation of different growth conditions

Liquid cultures were prepared in Glucose Yeast Peptone, and kept at 25 °C in a rotary shaker at 150 rpm for 18 h.

The cells were removed by centrifugation, washed twice in physiological solution and suspended in Yeast Nitrogen Base.

The cultures were prepared in 100 mL glass bottles containing 25 mL of YNB minimal medium and inoculated to an initial OD_{600} of 0.050. Unless otherwise stated, glucose was the sole carbon source, being sterilized and supplied at an initial concentration of 20.0 g/L. Bottles were closed with plastic or cellulose caps. Cultures were grown at 25 °C in a rotary shaker at 150 rpm. At regular times, oxygen pressure, OD, carbon consumption, nitrite disappearance, and pH in the medium are measured and compared in both systems.

Several different conditions were tested. Three different nitrogen sources were evaluated: 10mM $NaNO_2$, 10mM $NaNO_3$ and 5 mM $(NH_4)_2SO_4$, unless otherwise stated.

Different carbon and energy sources were evaluated: glucose, sucrose, fructose, maltose, mannose, mannitol, lactose, galactose, acetate, glycerol, gluconate, ethanol, and citrate. The selected C source was sterilized and supplied at an initial concentration of 20.0 g/L.

Influence of pH was studied adjusting media using 1M K_1K_2 Buffer (KH_2PO_4, K_2HPO_4) for pH 6 and pH 7 values, acetate buffer for pH 4 and 5 and

pyrophosphate buffer for 8 and 9 pH values. All buffers were at final concentration 50mM.

Determination of total nitrogen

Biomass disruption was made by the use of an oxidant mixture of 0.6960 g potassium peroxydisulfate ($K_2S_2O_8$) (<0,001% Nitrogen), 0.2250 g boric acid, and 0.1050 g sodium hydroxide in 15 mL water (Valderrama, 1981). The resulting total nitrogen was determined as nitrate.

Analytic determinations

Determination of nitrate and nitrite was make with modified Griess assay (Miranda, Espey, & Wink, 2001).

Ammonia was determined by using phenol/hypochlorite method according to literature (Weatherburn, 1967).

Glucose was quantified by the enzymatic kit GAGO-20 (Sigma, St. Louis, MO), according to manufacturer instructions (Boaduo, Katerere, Eloff, & Naidoo, 2014).

Dry weight measurement

Cellulose nitrate filters (Sartorius Stedim, 0,45 μm) were kept at 100°C for one hour and weighed before filtration, using Millipore system (All-Glass Filter Holder Assembly with funnel, fritted base, cap, clamp, 47mm).

After the use, filters were kept for 24h at 100°C and rapidly weighed. Sample weights were determined by difference.

Operational features for bioremediation applications

Growth in renewable carbon sources

Liquid cultures were prepared as described above using as carbon sources:

beet molasses 2% *p/v*

Olive mill wastewater (OMW) 2%*p/v*

Black liquor (BL), obtained from *Quercus suber* bark, collected in Giara di Tuili (Sardinia, Italy) 1%*v/v*

cork factory wastewater (CFW) 2%*p/v*

soybean oil 2% *v/v*

corn oil 2%*v/v*

paraffin 2% *v/v*

Tolerance to heavy metals

Liquid cultures were as above in the presence of Cd, Co, Hg, Ni and Pb (chosen for their presence in Sardinia in polluted mining sites). They were tested at the limiting concentrations defined from Italian laws "D.lgs. 152/2006: 03.04.2006 (S.O. n. 96 Gazzetta Ufficiale 14 aprile 2006, n. 88)" soil values limits (table1):

metal	Legal limits mg/Kg *		Concentrations	
cadmium acetate	2	15	0,0075mM	0,056mM
cobalt acetate	20	250	0,08mM	1mM
mercury acetate	1	5	0,0031mM	0,015mM
nickel chloride	120	500	0,5mM	2,1mM
lead acetate	100	1000	0,264mM	2,64mM

Table 1: concentrations of heavy metals in green areas D.lgs. 152/06

Bio-reactor scaling-up

Two *"Applikon"* bio-reactors (total volume 2.3L) were used equipped with *Bio controller ADI 1032* for temperature, pH and oxygen control, and with *Stirrer controller P100 ADI 1032* for the control of the stirring motor. Working volume in each fermenter was 1 litre.

Anabolic features of R. glutinis

Carotenoids assay

Chemicals

Standard of all-trans-β-carotene and all solvents used, of the highest available purity, were purchased from Sigma-Aldrich (Milan, Italy). All the chemicals used in this study were of analytical grade.

Analyses of carotenoids

The dried cell residues were dissolved in methanol (1 mL) and aliquots of these solutions were injected into the HPLC system.

Analyses of carotenoids were carried out with an Agilent Technologies 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector (DAD) (Agilent Technologies) Carotenoids, detected at 450 nm (all-trans-β-carotene and its isomer) and 485 nm (torularhodin and torulene), were measured with the use of an Inertsil ODS-2 column, 150 × 4.6 mm, 5 µm particle size (Superchrom, Milan, Italy), and MeOH as the mobile phase, at a flow rate of 1.2 mL/min. The temperature of the column was maintained at 37 °C. The identification of carotenoids was made using all-trans-β-carotene as external standard compound and the conventional UV-Vis spectra, generated with the

Agilent OpenLAB Chromatography data system. Carotenoid peaks were also assigned on the basis of literature data ((Moliné et al., 2012)). Carotenoids quantification was performed using all-trans-β-carotene calibration curve at 450 nm (linear, with correlation coefficient = 0.9985).

Nitrogen metabolism by R. glutinis *DSBCA06*

Nitrate reductase activity

Liquid cultures were prepared as described above.

After 18 hours the cells were recovered by centrifugation, washed and suspended in Yeast Nitrogen Base without other nitrogen sources for 30 minutes, to an OD_{600} value of 3.0 A.U.. After starvation, the chosen nitrate source was added at medium at 5mM concentration.

Samples were collected at regular times to measure NR activity.

After 3h ammonium sulphate was added at 5mM concentration, and samples were collected at regular times to measure NR inhibition. The cells were stored at –20 °C until used.

Preparation of cell-free extracts and NR assay.

Approximately 50 mg of cells (wet weight) were mixed with 200 extraction buffer (100 mM-potassium phosphate, pH 7.4, 20 µM FAD, 1mM-EDTA,1 mM-PMSF and cOmplete™ EDTA-free protease inhibitor cocktail (Roche 11836170001) (1 tablet for 10mL buffer)) and 1 g glass beads (0.5 mm diameter) and vortexed for 1 minute and after 1 minute on ice, repeated 3 times.

300µL extraction buffer were added, and the mixture was vortexed again for 10 s. The mixture was then centrifuged for 5 min at 3000g, and the supernatant immediately analyzed. NR was assayed in a final volume of 0.4 ml (assay volume for 20µL or 40 µL extract.), containing 50 mM-potassium phosphate buffer, pH 7.4, 20 mM-potassium nitrate and 0.2 mM-NADPH, plus 10 mM potassium sulfite as nitrite reductase inhibitor, FAD 0.04 mM. The assay mixture was incubated at 30°C for 15 min. Nitrite was then determined in the supernatant with Griess assay (Miranda et al., 2001).

Determination of proteins concentration in cell extracts

Protein concentrations were quantified using the Bradford method (Bradford, 1976) by means of the commercial kit Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, EEUU) using Bovine serum albumin as the standard.

To determine the protein concentration of our samples, 10 or 20 µL of cell extract, to which were added water till volume of 200 µL, were used. Finally, 800 µL of Bradford reactive (dil. 1:6) were added to each tube.

Samples were then incubated for 5 min at room temperature, before measuring the absorbance at 590 nm.

Nitrate assimilation

Liquid cultures were prepared as described above.

At OD_{600} value 1.0 A.U., cells were recovered by centrifugation, washed twice and suspended in Yeast Nitrogen Base without nitrogen sources for 30 minutes; after this time 5mM $NaNO_3$ was added at medium.

Samples were collected at regular times along two hours; nitrate and nitrite present in medium were measured to determine nitrate assimilation (Miranda et al., 2001).

Nitrite assimilation in short time

Liquid cultures were prepared as above.

After 18 hours cells were recovered by centrifugation, washed and suspended in Yeast Nitrogen Base without other nitrogen sources for 60 minutes to an OD_{600} value of 1.0 A.U.. After this time 5mM nitrite was added to the medium. Samples were collected a regular time to measure NiR activity.

RESULTS AND DISCUSSION

Chapter 1: Isolation of strain Rhodotorula glutinis DSBCA06

Selective enrichment cultures were prepared in Yeast Nitrogen Base (YNB) medium solidified with agar with $NaNO_3$ as the sole nitrogen source. Sodium nitrate was added to a final concentration of 20mM. Petri dishes were exposed to air overnight, closed, and incubated at 25ºC for 72 h. Colonies were obtained in pure culture by repeated streaks on YNB agar plates. Among themost rapidly growing YNB colonies, the pink one were then chosen to perform further studies and named DSBCA06 strain.

Pink colonies were examined. (Figure 3 and 4) show regular and pink colonies. Isolated colonies are coral pink, usually smooth, moist to mucoid yeast-like in appearance; after several days' single colonies are indistinguishable.

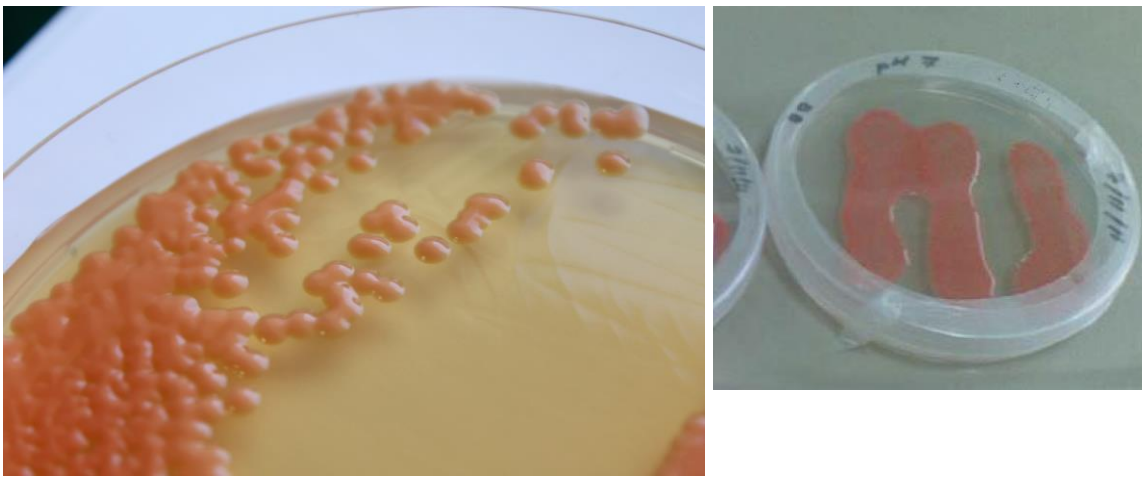


Figure 3: *Rhodotorula glutinis* in GYP and YNB

On agar GYP cultures, colonies are at first pink and mucoid, after three days tending to orange. Sometimes are reticulate and corrugated.

Under optical microscopy cell morphology shows spherical to oval budding yeast-like cells, 2.5-6.5 um in size (Figure 5).

Figure 4: Colonies of *R. glutinis*

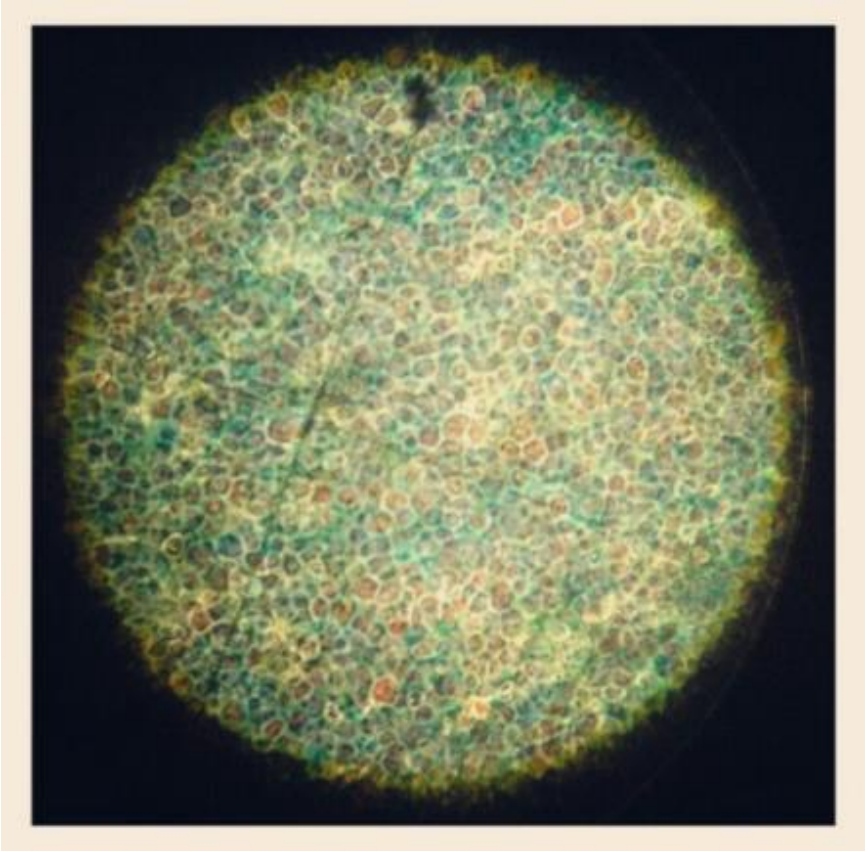


Figure 5: *R. glutinis* (optical microscope)

Chapter 2: Molecular characterization of R. glutinis DSBCA06

In mycology, only from the 1990s molecular taxonomy techniques began to assert itself against phenotypic taxonomy.

Inspired by molecular bacterial taxonomy, the initial phylogenetic and molecular identification of fungi relied on nuclear ribosomal genes. The classic paper by White et al. (1990) included universal primers still widely used for amplifying three main components of the fungal ribosomal operon: the large subunit (LSU, variously referred to as the 26S or 28S, and including two variable subregions called D1 and D2); the small subunit (SSU, or 18S), separated by the ITS, comprising two sections (ITS1, ITS2) that bracket the conserved 5.8S region figure 6 (Seifert, 2009).

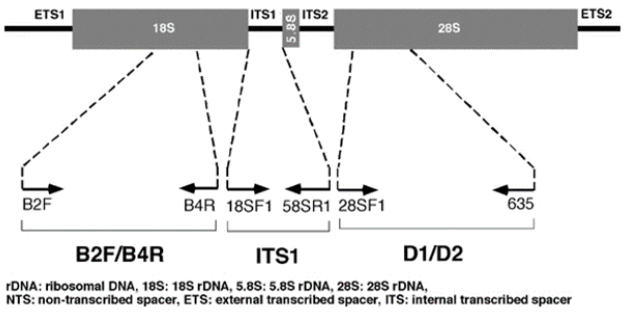
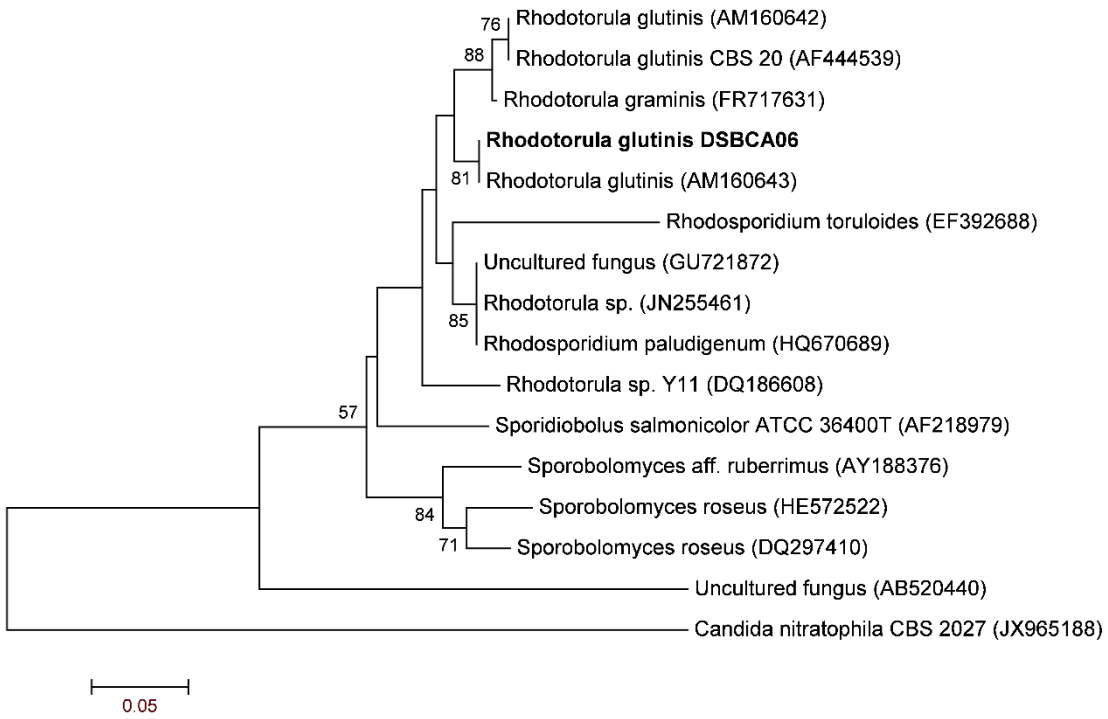


Figure 6: ITS1, ITS2 and D1/D2 barcoding regions

Other projects for barcoding fungi and yeasts have been made over the last 20 years with different DNA barcode-like Internet database, which have got data for different sequences as COX1, COX 1 and 2. Species resolution obtained with COX1 barcodes was inferior to both the ITS and the D2.

In 2007, 37 mycologists from 12 countries met at the Smithsonian to establish criteria for barcoding of fungi and decided for the use of ITS with a second marker for detecting species. Most used is D1-D2 region of the LSU (Seifert, 2009).

The strain DSBCA06 has been attributed to the *Rhodotorula* genus by ITS and D1/D2 region analysis (Schoch et al., 2012; Seifert, 2009)The phylogenetic position of the strain was updated based of the recent description of novel species within the genus.



Identification was made with the databases UNITE, INSD (GenBank, EMBL, DDBJ), and EnviRh.

The strain with the highest degree of homology with our strain, in the sequences examined is *R. glutinis* HB1215 isolated from guts of several insect pests on maize. In a paper (Molnár et al., 2008)is possible to find an example of the difficulties encountered along characterization of yeasts with the use of a single sequence.

Chapter 3: Physiological characterization and optimization of growth conditions

R. glutinis DSBCA06 was then tested in liquid cultures to evaluate its metabolic requirements and the optimal growth conditions. This phase is crucial in the perspective of *R. glutinis* application as bioremediation tool or for wastewater treatment.

Figure 7: *R. glutinis* DSBCA06 growth in different conditions

3.1 Growth in different nitrogen sources

In order to evaluate the ability of the strain to assimilate different nitrogen sources, growth curves were built in YNB in the same conditions for carbon source (glucose 2%), shaking (150RPM), temperature (25°C), and pH (7 50mM potassium phosphate buffer, pH 7) but with different nitrogen source.

10 mM $NaNO_3$, 10 mM $NaNO_2$ and 5 mM $(NH_4)_2SO_4$ were included in the study.

It is well-known that assimilation of nitrogen compounds requires a more complex enzymatic pattern for ammonium forms, whereas more energy and time for nitrite and nitrate (Crawford & Glass, 1998).

In Figure 8 we can see that the growth on ammonium is faster than on other nitrogen sources, leading to more biomass. OD_{600} values nearly double in ammonium (final 22.51) than in nitrate (11.52) whereas in nitrite an intermediate result (16.9) was obtained.

Nitrogen compounds disappearance followed the same pattern, being ammonium the fastest assimilated (in 20h no ammonium detectable in the medium) (figure 8).

Nitrite and nitrate were removed from the medium slower, with a similar velocity during exponential phase, arriving to complete removal after 40-50 hours.

After that the growth did not stop at the same time for nitrite and ammonium, suggesting that the uptake and use of nitrogen do not occur at the same times and probably *Rhodotorula* is able to store considerable quantity of nitrogen sources within the cell.

In the second chart pH and oxygen saturation in the medium are reported for nitrite and ammonium curves (figure 9). We have chosen curves that had higher OD_{600} value, assuming that they also had higher oxygen consumption.

We can see that, although our system utilized passive aeration, oxygen did not drop under 50%saturation and, under these conditions, it could not be considered a limiting factor.

The medium pH values remain constant for both curves.

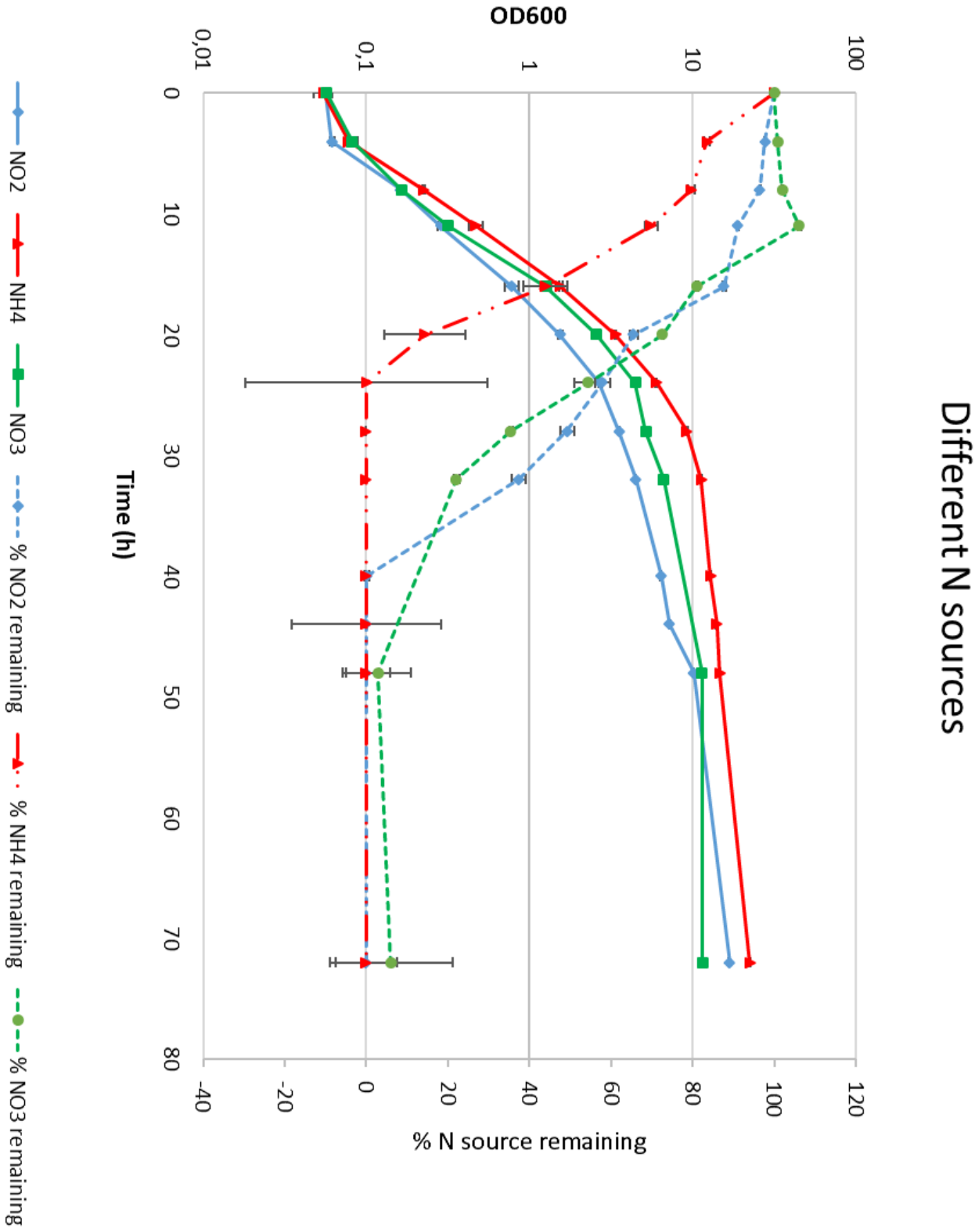


Figure 8: Growth on different nitrogen sources

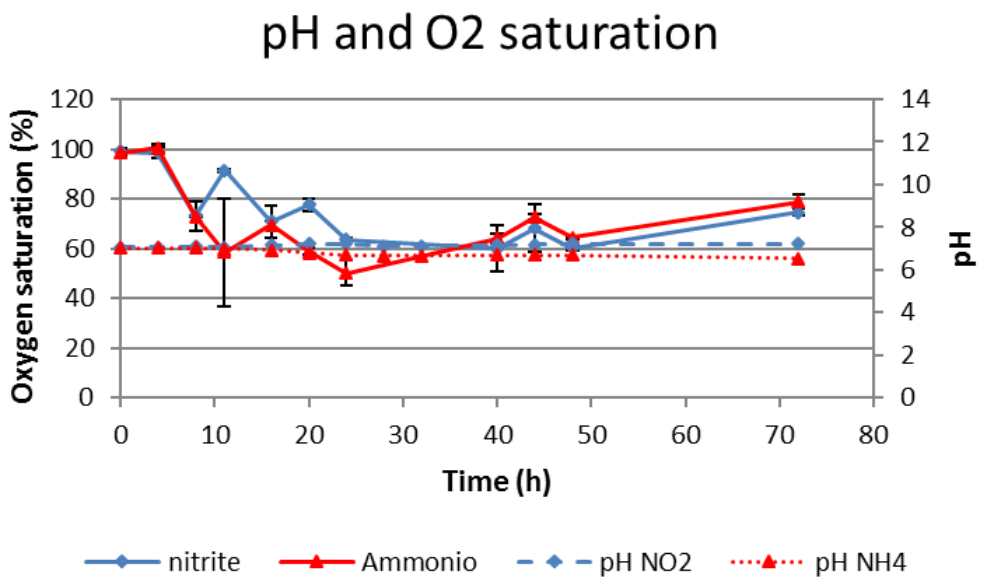


Figure 9: Oxygen saturation and pH changes in NH_4^+ and NO_2- curves

Several yeasts are able to assimilate nitrate as a nitrogen source, and the physiology and enzymology of nitrate assimilation of some species, in genera such as *Sporobolomyces*, *Hansenula* and *Rhodotorula*, has been deeply studied (A. Ali & Hipkin, 1986; Gonzalez & Siverio, 1992; Siverio, 2002; Sollai, Zucca, Rescigno, Dumitriu, & Sanjust, 2012).

Nitrite, an intermediate in the reductive assimilation of nitrate nitrogen into ammonium-nitrogen, is also a potential source of nitrogen for these organisms and appears to play an important role in the regulation of nitrate assimilation (Gonzalez et al., 1994). However, if the nitrate assimilation is well known, nothing has been reported until now about nitrite.

Nitrite uptake has been studied in cyanobacteria and algae, whereas comparatively little is known about the uptake and assimilation of nitrite by yeasts. Moreover, nitrite is toxic to many cells at the concentrations well tolerated by yeasts (KUBISI, Ali, & Hipkin, 1996).

For this reason, we have chosen to continue our studies focusing on nitrite as the main nitrogen source.

3.2 Growth with different carbon sources

Culture broths with different carbon sources were prepared to identify the better energy sources for isolated strain DSBCA06.

R. glutinis DSBCA06 has been grown in YNB 10mM $NaNO_2$ medium that differed only for the sole carbon source at 25°C, at pH 7 and 150 RPM of stirring.

Carbon sources tested were fructose, glucose, mannose, galactose (monosaccharides), sucrose, maltose, lactose (disaccharides), mannitol (reduced sugar), sodium acetate, citrate, gluconate, glycerol and ethanol. All carbon sources were 2% *p/v* concentration.

Absorbance and nitrite present in broths were measured during the growth, and the results are reported in figure 10.

Among tested carbon sources, the strain showed negligible growth after 48h in lactose, galactose, acetate, citrate and gluconate.

The best results were obtained with glucose, fructose, sucrose and mannose. In figure 8 we can see growth and nitrite removal expressed in percentage, using the culture in glucose as the reference.

Very slow growth was detected using glycerol, although in the literature this substrate is often used as a carbon source for others strains of *Rhodotorula*.

The nitrite removal followed the same pattern as described above for biomass production.

The obtained results suggest the use of glucose as the carbon source of the strain, since it is the most inexpensive sugar among the ones with the more promising results.

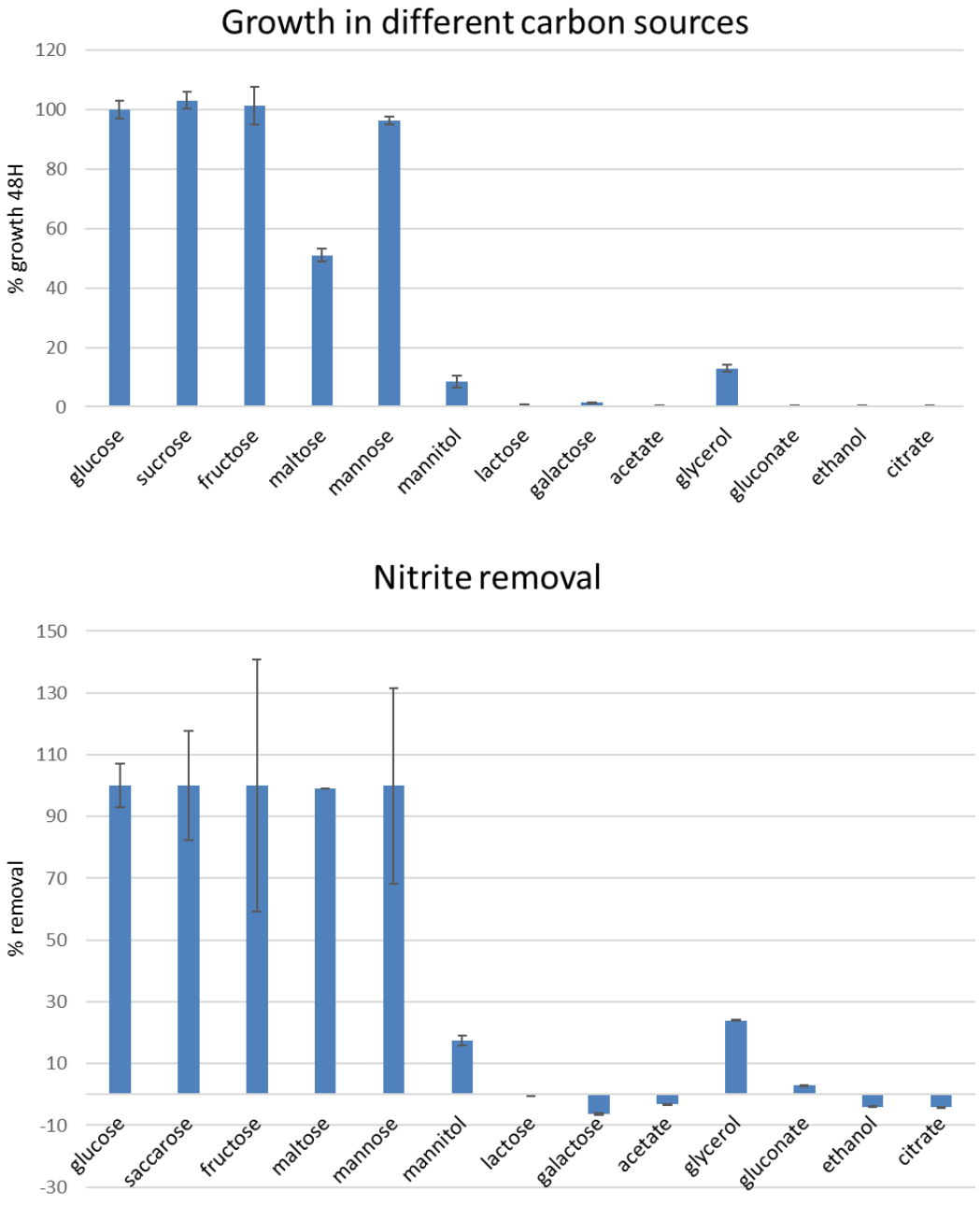


Figure 10: growth on different carbon sources and nitrite removal

3.3Influence of pH

The influence of different pH values was studied, since pH influences directly growth, metabolism, and secondary metabolites production.

For these reasons, determination of the optimum pH range is quite important.

We used different buffers for different pH ranges: sodium acetate buffer for pH 4 and 5, potassium phosphate buffer for pH 6 and 7, and sodium pyrophosphate buffer for pH 8 and 9. We measured the growth after 48h, and the results are reported in Figure 11.

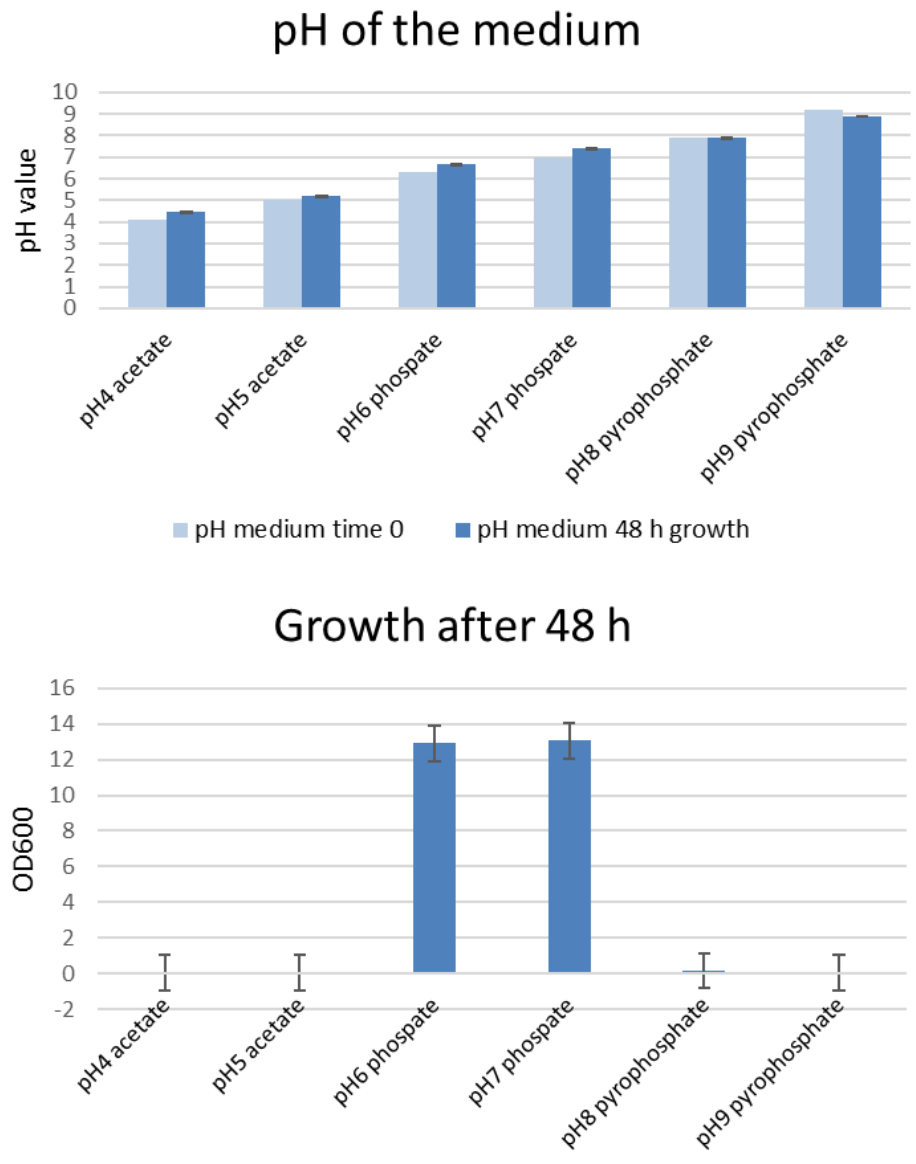


Figure 11: growth on different media under different pH values

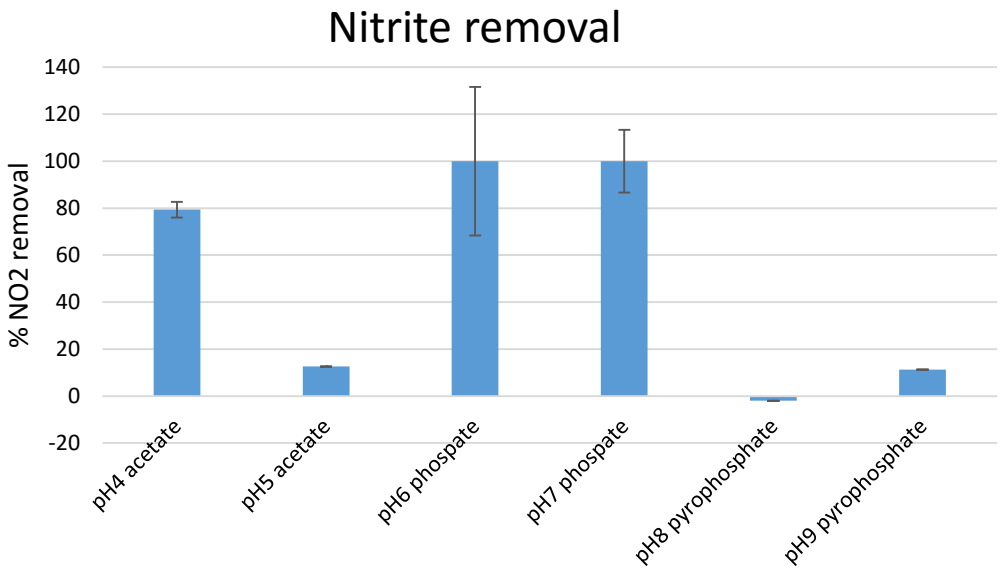


Figure 12: nitrite removal in media with different pH values

Nitrite removal was highest around neutrality, suggesting to keep 7 as the pH for further optimization studies (figure 12).

3.4 Tolerance to nitrite

R. glutinis DSBCA06 was then tested about its ability to tolerate different nitrite concentrations.

The obtained results are reported in Figures 13, 14, 15.

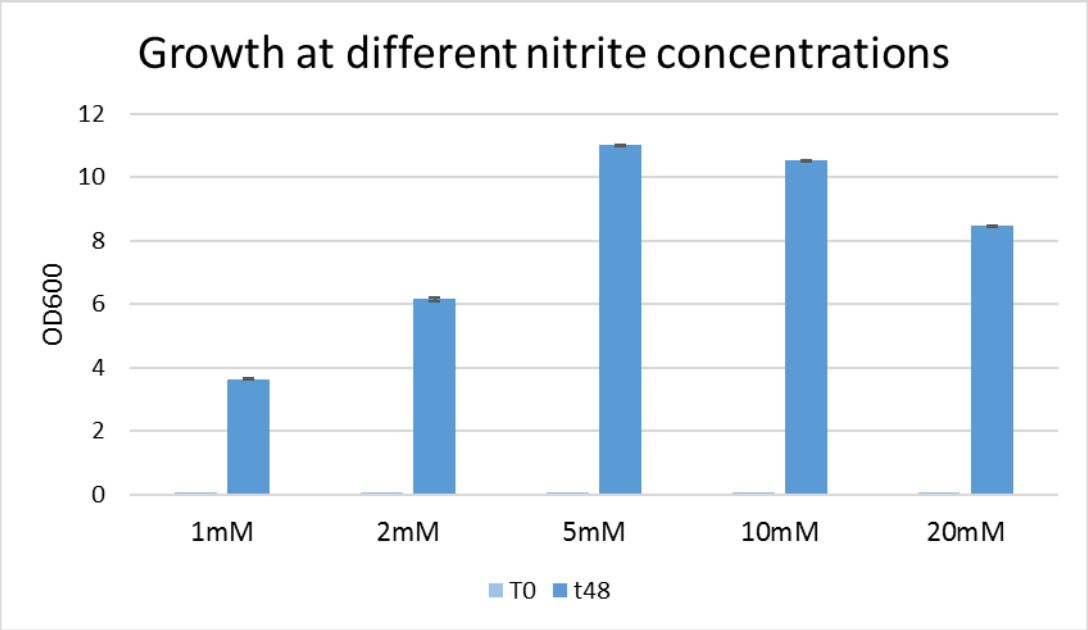


Figure 1: growth in different nitrite concentrations

R. glutinis DSBCA06 is able to tolerate up to 20 mM initial nitrite, with small decrement in growth in the range 5-20 mM. Higher concentrations led to complete growth inhibition.

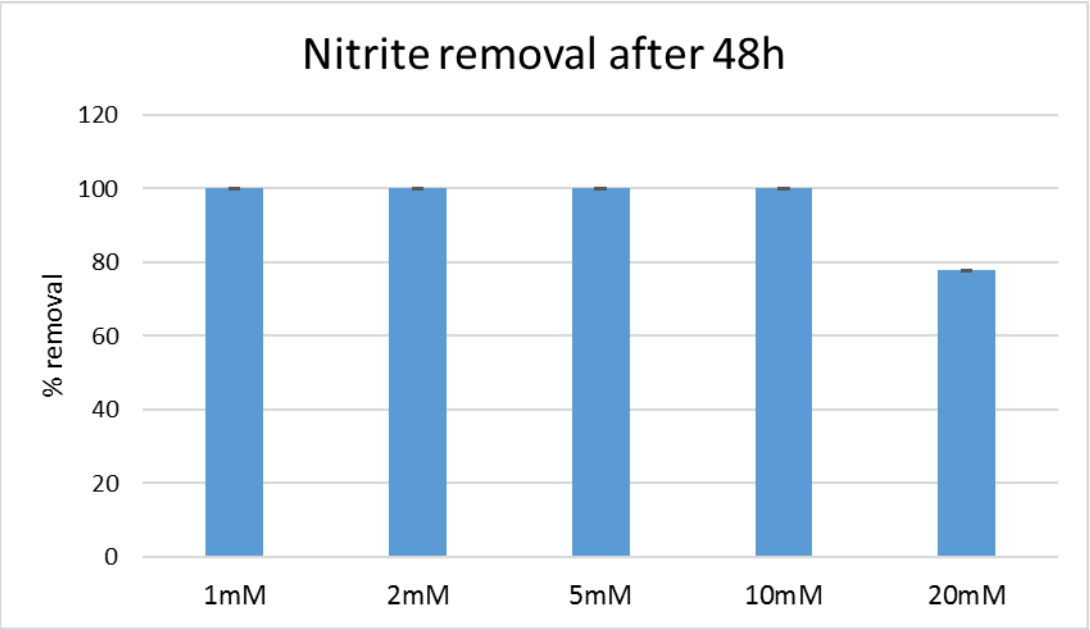


Figure 14: Nitrite removal at different concentrations

During the growth, almost complete nitrite removal was observed in 48 h up to 10 mM initial nitrite. Whereas in the presence of 20 mM nitrite only 80% of removal was measured.

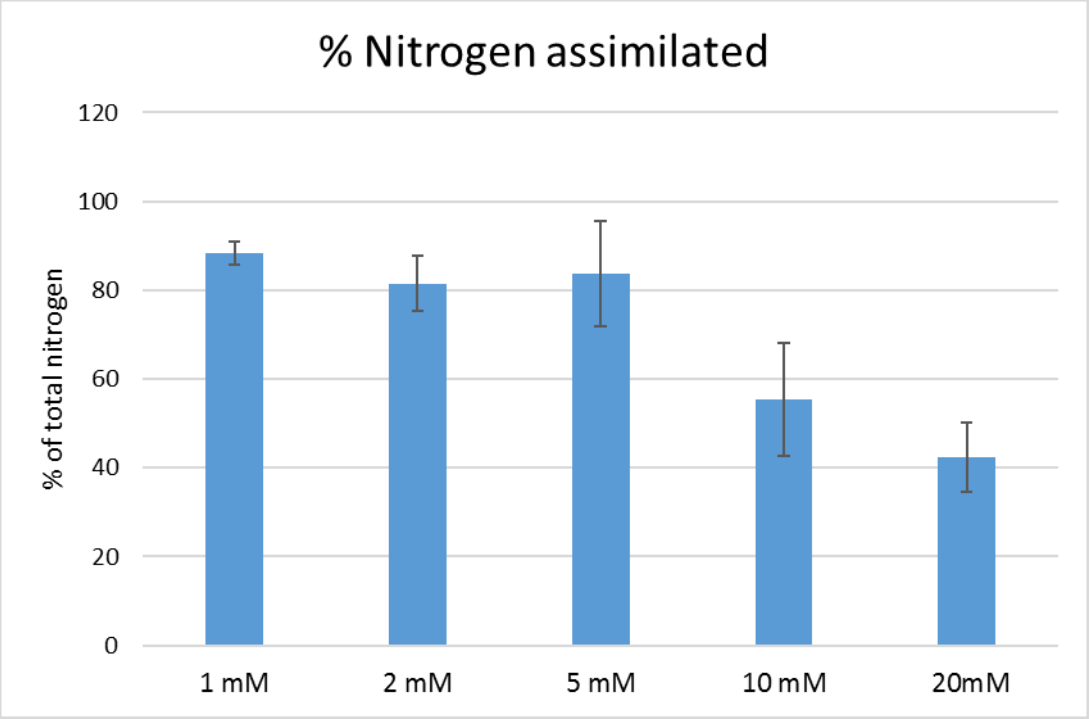


Figure 15: Nitrogen assimilated by *Rhodotorula glutinis* DSBCA06

Using elemental analysis, the removed nitrite was shown to be almost completely assimilated as organic nitrogen (Figures 14-15). At the same time a noticeable increase in yeast biomnass was observed.

Only when higher initial nitrite concentrations were used (10 and 20 mM) only about 50% of initial nitrogen was recovered as organic compounds.

According to these results, we chose 10 mM as the optimal initial nitrite concentration for the rest of the study.

These results are very promising in the perspective of application of the strain as bioremedetion tools, since it is able to tolerate high nitrite concentration (much higher than the polluting level usually present even in the most polluted environments), and to convert it in non toxic organic nitrogen, that in turn could present potential secondary application (i.e. fertilizer or animal feed).

Chapter 4: Operational features for bioremediation applications

The development of a bioremediation tool requires some particular features, in order to allow the growth of the selected strains under harsh conditions, usually different form laboratory controlled batches.

Particularly, we decided to investigate the ability of *R. glutinis* DSBCA06 to use renewable carbon sources, and its tolerance to heavy metals. Besides, preliminary experiments of scaling-up in 3 L bio-reactor were performed.

4.1. Growth in renewable carbon sources

It has been estimated that raw materials account for 10 to 30% of the total production costs in most biotechnological processes. A strategy to reduce costs of microbial metabolites is the use of low-cost raw renewable materials (Mukherjee, Das, & Sen, 2006)

This series of experiments was performed to evaluate the ability of *R. glutinis* DSBCA06 to grow in presence of elevate concentrations of nitrogen compounds using a variety of cheap renewable substrates from edible and non-edible plant-derived oils, industrial wastes (black liquor, molasses).

The use of cheap raw materials and wastes will contribute to the reduction of process costs for possible large-scale industrial application of the strain, such as the development of a tools to remove environmental nitrogen compounds or to treat industrial wastes. Besides, the anabolic features of *R. glutinis* DSBCA06 (*vide ultra*) suggest that bioremediation systems could be coupled with the

production of commercially interesting molecules such as lipids for biodiesel production, and carotenoids.

As shown in Figure 17, *R. glutinis* DSBCA06 is able to grow efficiently on molasses, on a wide spectrum of plant-oils, and on olive mill wastewaters. However, it did not show a significant biomass increase after 3 days in Black liquor, paraffin and on cork factory wastewaters.

Overall, *R. glutinis* DSBCA06 displays important features for the future development of economically efficient industrial-scale biotechnological processes. Furthermore, yeast growth and potential production can be supported by low cost renewable substrates.

At research level, it is rare to use complex substrates as each one may contain a wide range of impurities and the composition of the substrate can be highly variable depending on what part of the world the substrate is grown in, on soil type, on climate, etc. It implies high in-process variability, even with careful planning of experimentation (e.g., using experimental design methods), and more replicate fermentations will be required to obtain clear results.

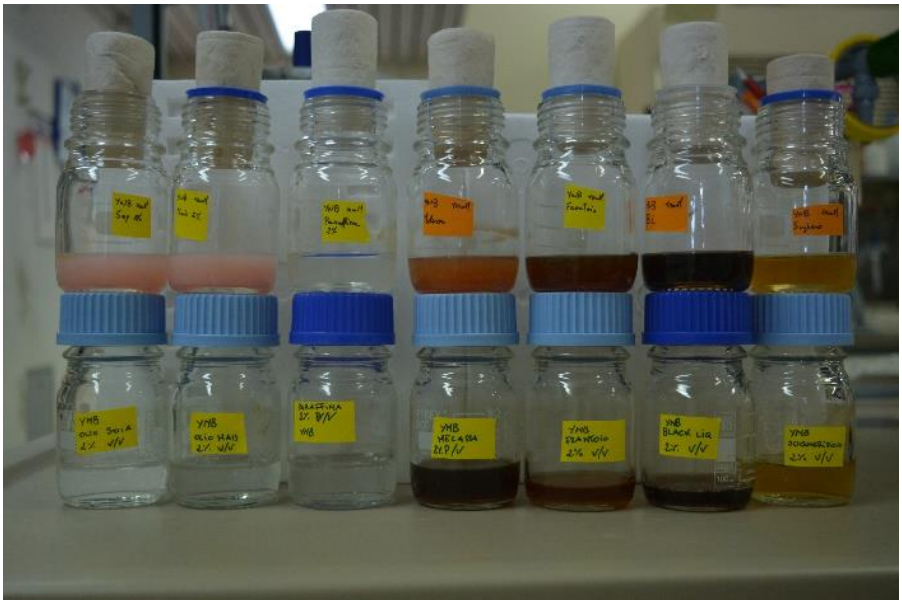


Figure 2: Cultures in different renewable carbon sources

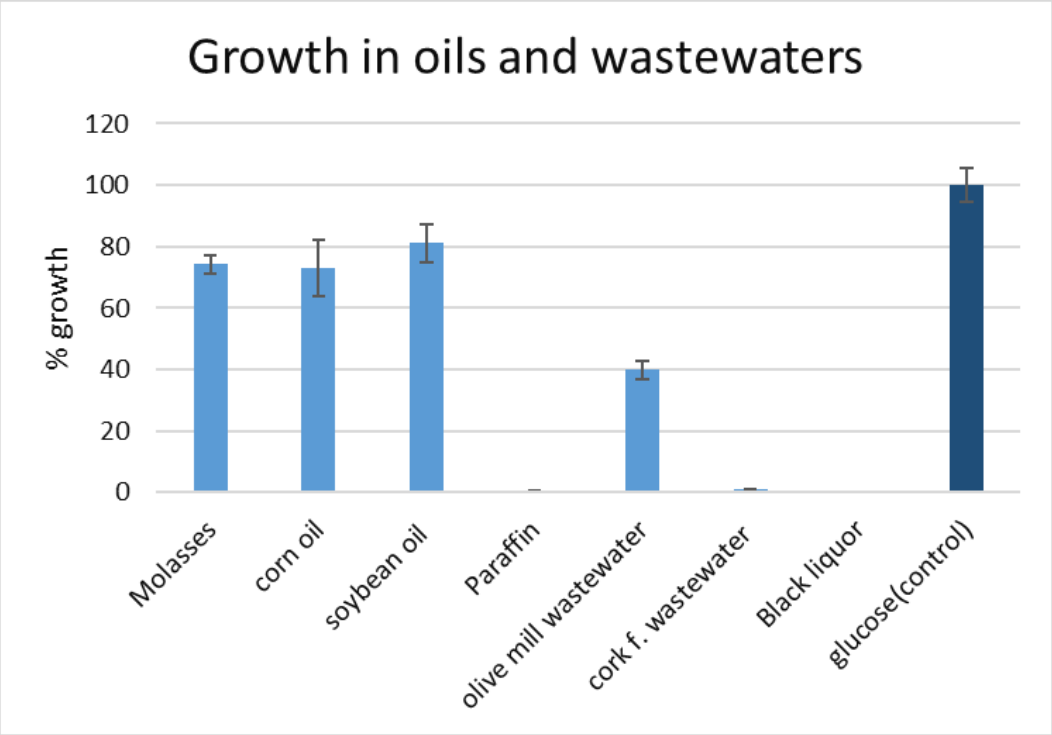


Figure 17: Growth on different renewable wastes

4.2 Tolerance towards heavy metals

One important feature for bioremediation tools can be the tolerance to heavy metals (X. Li, Poon, & Liu, 2001), since usually environmental samples or industrial wastes can be contaminated.

Heavy metals have a toxic effect on cells (Geoffrey M Gadd & Griffiths, 1977; Goyer & Clarkson, 1996)but several strains of bacteria and yeasts are able to survive in soils and wastewaters with elevate concentration of heavy metals ions. In fact, bacteria and fungi are normally involved in many reactions of biogeochemical metal cycles; balance between mobilization and immobilization varies depending on the microorganisms involved, on physical and chemical environmental conditions. Mobilization of metals in soils can be the result of bio-leaching processes, while the immobilization can result from processes of inclusion in the production of biomass or exopolymers. Furthermore, the

metabolism of microorganisms can change the oxidation state of a metal by varying the bioavailability (Geoffrey M. Gadd, 2004) or bringing it to a less toxic oxidation state (Barkay & Schaefer, 2001; White, Sayer, & Gadd, 1997) This type of response seems to promote the microorganisms and between these, mostly yeasts, as the hinge elements for bioremediation treatments *in situ* or *ex situ* as also for the industrial treatments that use biomasses. Many types of yeast, in fact, have not only a high resistance to heavy metals but also have strong bioaccumulation in vacuoles. This ability varies from species to species and depending on pollutant concentration, availability of nutrients, pH of the soil and availability of energy sources required also from the uptake of metal ions (Blackwell, Singleton, & Tobin, 1995).

In Sardinia we have large sites polluted from heavy metals; mining dumps have always constituted an environmental concern (Costantini S., 2004). The most evident processes of degradation started after mines closure, with the cessation of activities and protection measures. Millions of tons of polluted material leaked from flotation basins to get out in soils, rivers, lakes and in the sea. With an eye to future potential application scenarios, we tested our strain about its ability to grow in the presence of several heavy metals. We used metals that are common in mining areas. In YNB medium with elevate concentrations of nitrite (10mM) *R. glutinis* DSBCA06 was able to grow in presence of cadmium, cobalt and lead (Figure 18). Only Mercury completely inhibit the growth of the yeast strain. These are very promising features in the perspective of the development of large-scale bioremediation processes.

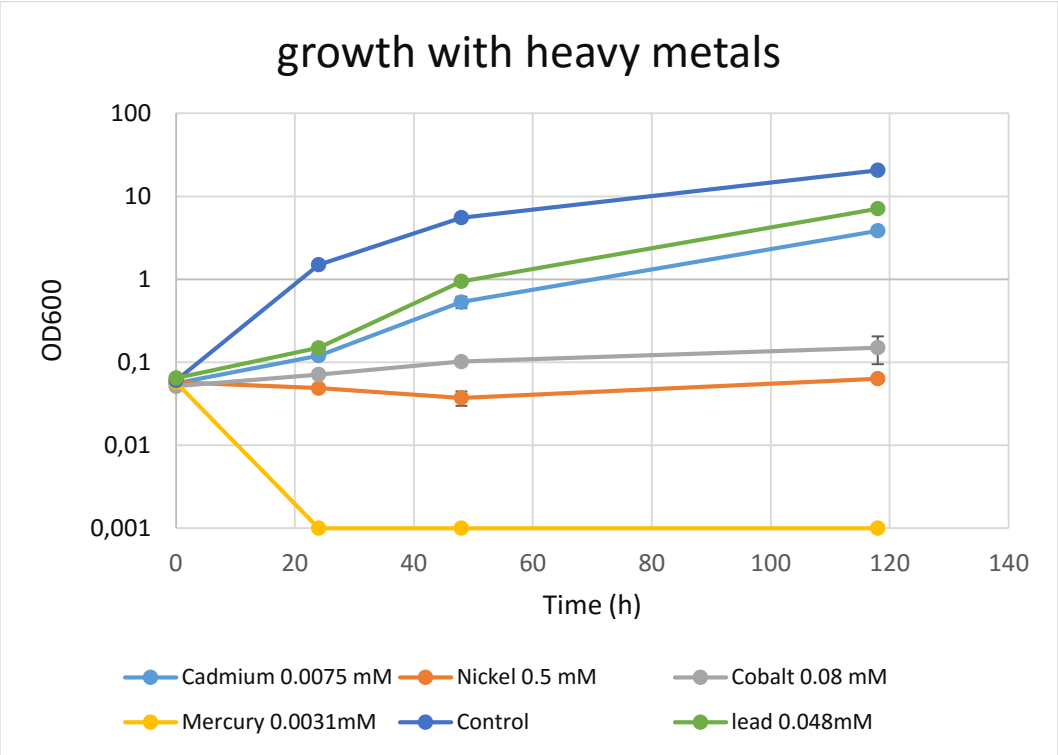


Figure 18: Growth in presence of heavy metals cations

4.3 Bio-reactor scaling-up

Preliminary experiments of scaling-up in 3 L batch bio-reactors were performed to understand the feasibility of large-scale bioremediation processes.

The growth conditions tested have been already described in §3, in the presence or absence of forced aeration. The results are reported in Figure 19 A and B.

Growth curves followed the same pattern observed for small batch experiments, being promising for further scaling-up. However, forced aeration seems to be a crucial requirement, since in its absence significant limitation of the growth was observed.

In accordance with limited biomass production, also nitrite removal and glucose consumption were slowed in absence of forced aeration

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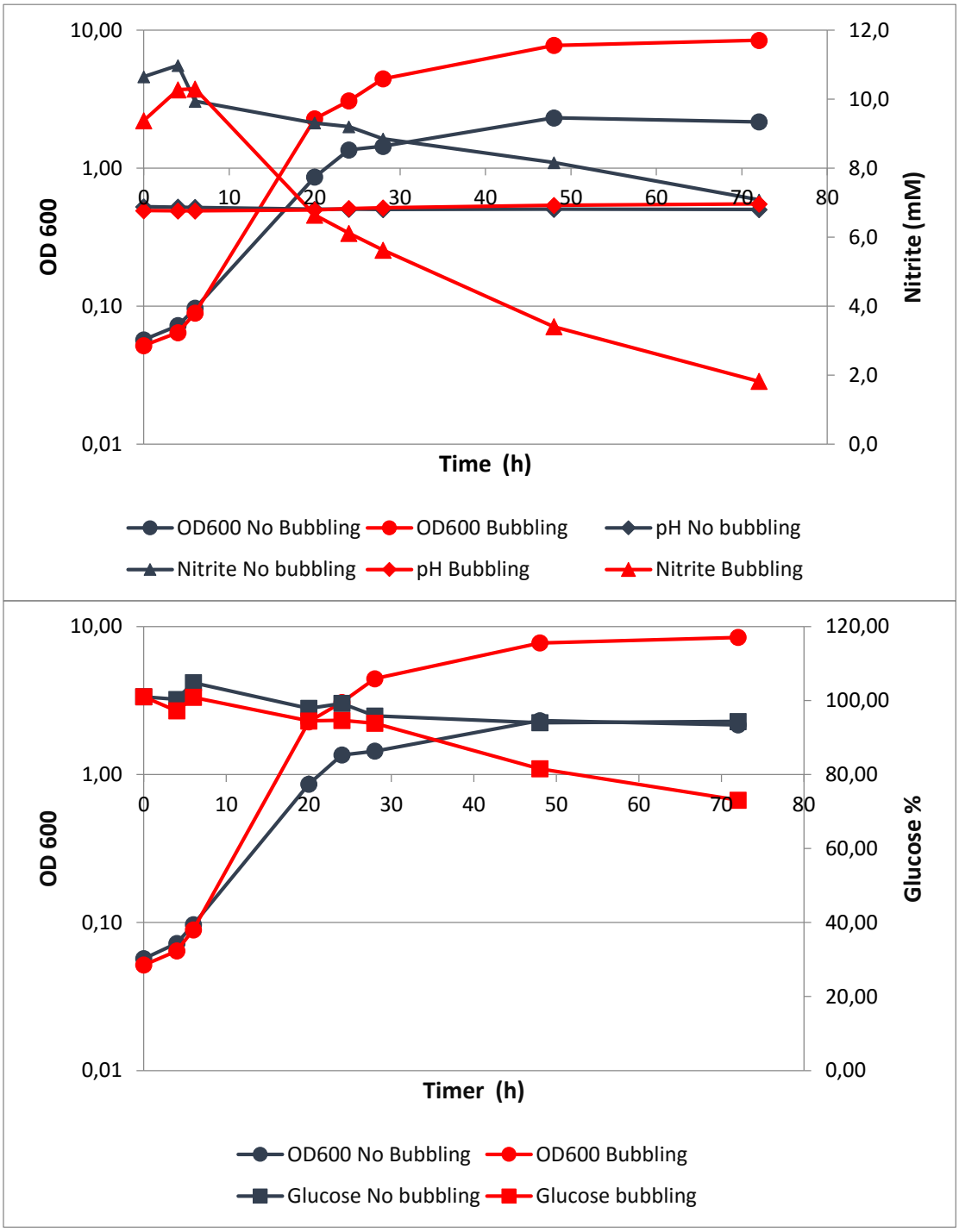

Figure 19: A) Growth, nitrite removal and pH measures in fermenter
B) glucose removal with and w/o aeration.

Chapter 5: Anabolic features of R. glutinis

5.1 Carotenoids production in different nitrogen sources

R. glutinis is known for its ability to accumulate lipids and carotenoids (Hernández-Almanza et al., 2014; Schneider et al., 2013). Carotenoid production by our strain was accordingly tested using mechanical destruction of cells and subsequent extraction of carotenoids with acetone (Aksu & Eren, 2007). Several nitrogen sources were tested.

Carotenoids are isoprenoids derivatives produced from mevalonate path (Figure 20).

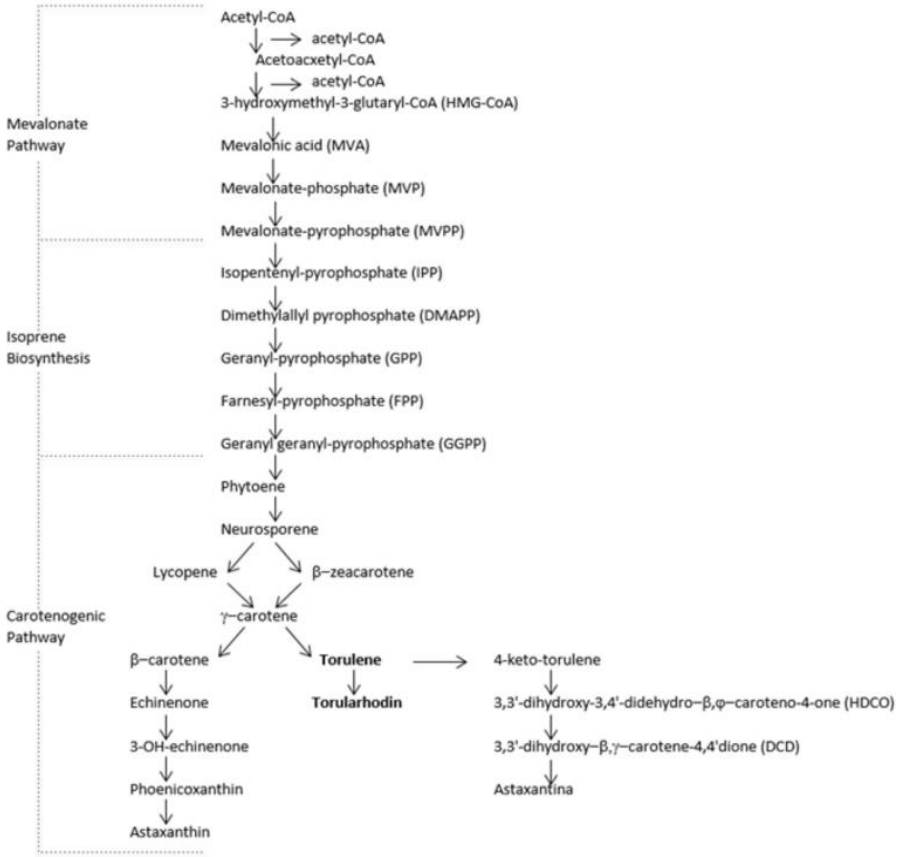

Figure 20: pathway of carotenoids biosynthesis

Analysis was made with HPLC at 24h to have reference values, which coincided with the end of exponential growth and after 72h because carotenoids are secondary metabolites, usually produced and accumulated during the stationary phase.

The results reported in Table 2 confirmed production of carotenoids by our strain, with values similar to what reported in others publications in different substrates and after longer times (Bhosale & Gadre, 2001; P. Buzzini; Pietro Buzzini & Martini, 2000).

total carotenoids mg/L	
$NH_4$24h	**3,4**
$NH_4$72h	**8,2**
$NO_2$24h	**1,1**
$NO_2$72h	**5,0**
$NO_3$24h	**1,1**
$NO_3$72h	**5,4**

Table 2: Total production of carotenoids in different nitrogen sources

Nitrite and nitrate as the nitrogen sources gave similar results of carotenoid productions, whereas ammonia led to a 2- or 3-fold increase. This finding is not surprising, provided that assimilation of nitrite and nitrate is rather costly for the cell. So, less reducing power is available for the synthesis of secondary metabolites such as carotenoids.

Carotenoid production is related to exposition (and resistance) at light and to the C/N ratio. In this experiment, the only difference was the nitrogen source and final growth values were similar, so the same C/N ratio was present. Accordingly, nitrogen in the medium.

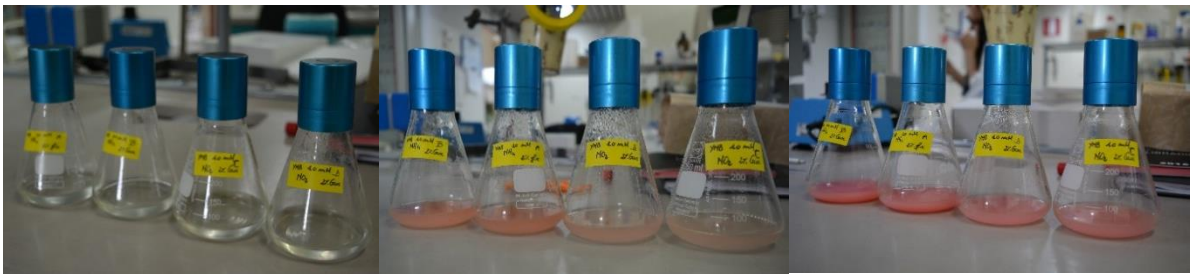

Figure 21: Cultures for carotenoids production at 0, 24, and 72 h

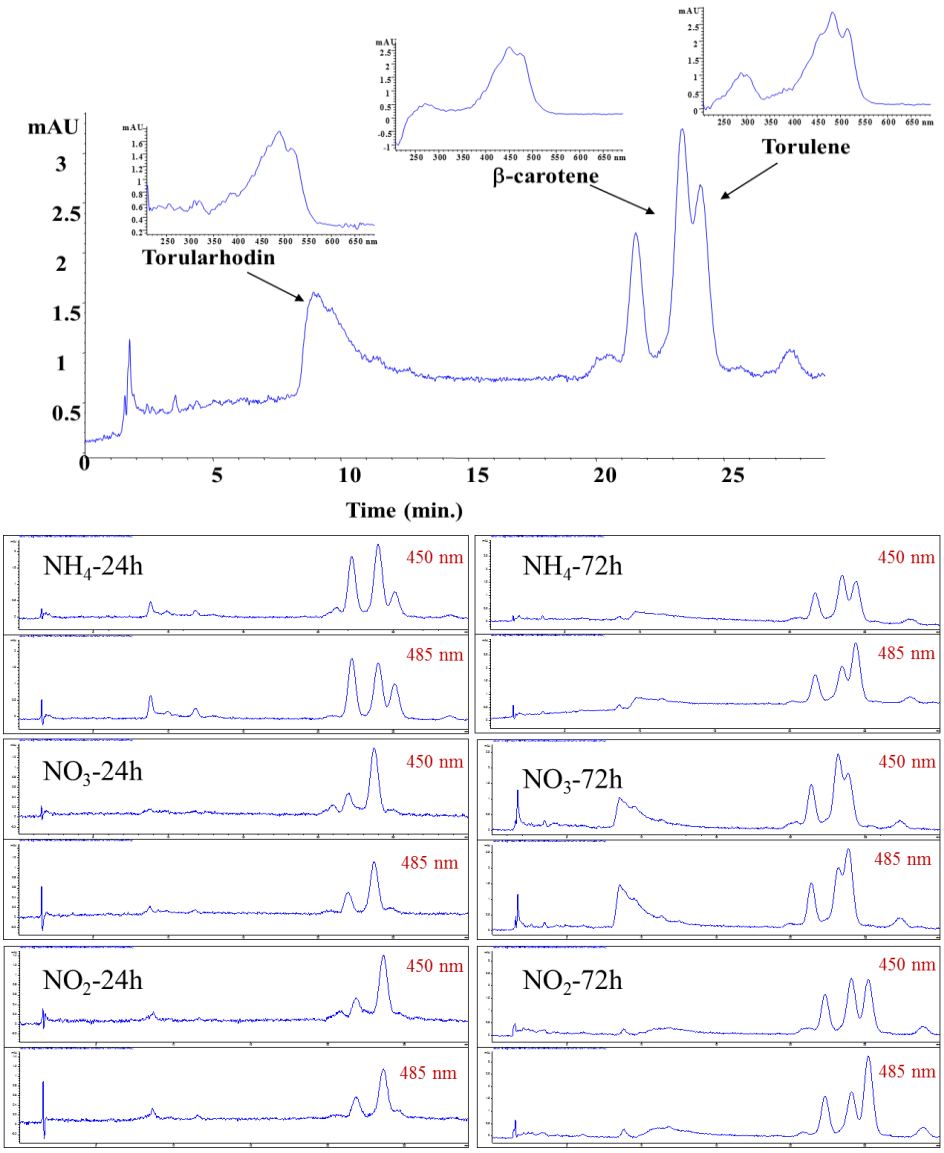

Figure 22: HPLC measures for carotenoid identification and quantification

HPLC-DAD was used to allow a secure identification of produced metabolites, and the data are reported in Figure 22.

Four peaks were identified, two with maximum absorption at 455 nm. One of them was recognized as β-carotene and the other (peak 2) was believed to be a carotene isomer because presented the same UV-spectrum. The other two peaks were identified as torulene and torularhodin with a maximum of absorbance at 485nm, in accordance to what has been reported for other *Rhodotorula* strains (Banzatto, Freita, & Mutton, 2013)

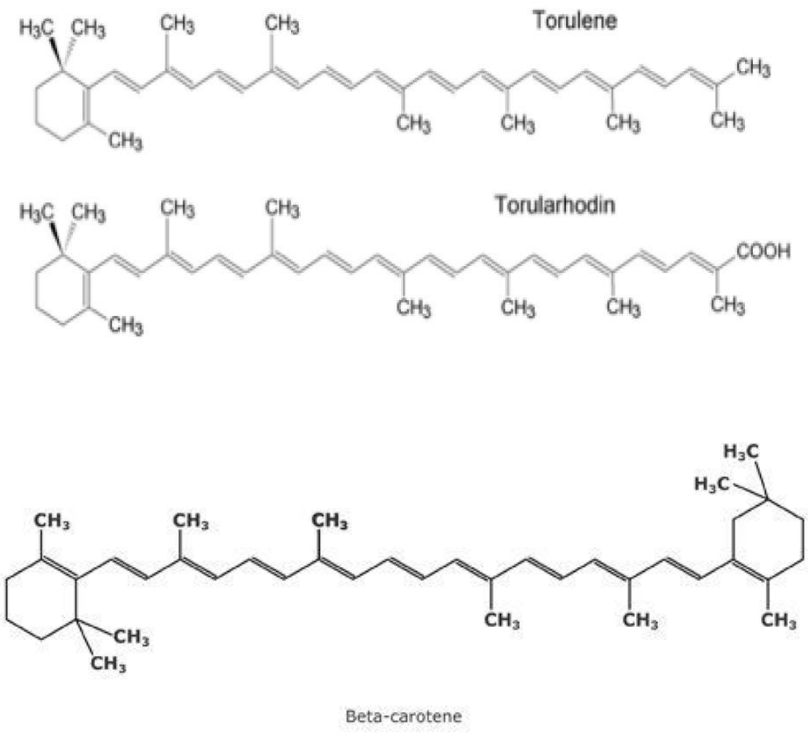

Figure 23: Relevant carotenoids produced by *R. glutinis*

Differences were not only related to total carotenoid production but also to the pattern of produced carotenoids (figures 22-24).

After 24h the speed of growth in ammonia certainly influenced carotenoid production which was higher but we had a similar relationship within each type of carotenoid produced. Particularly, beta-carotene was the main compound detected for all nitrogen sources.

In stationary phase the pattern changed in all samples, since torulene was the carotenoid most produced. In nitrate medium torularhodin reaches comparable production levels.

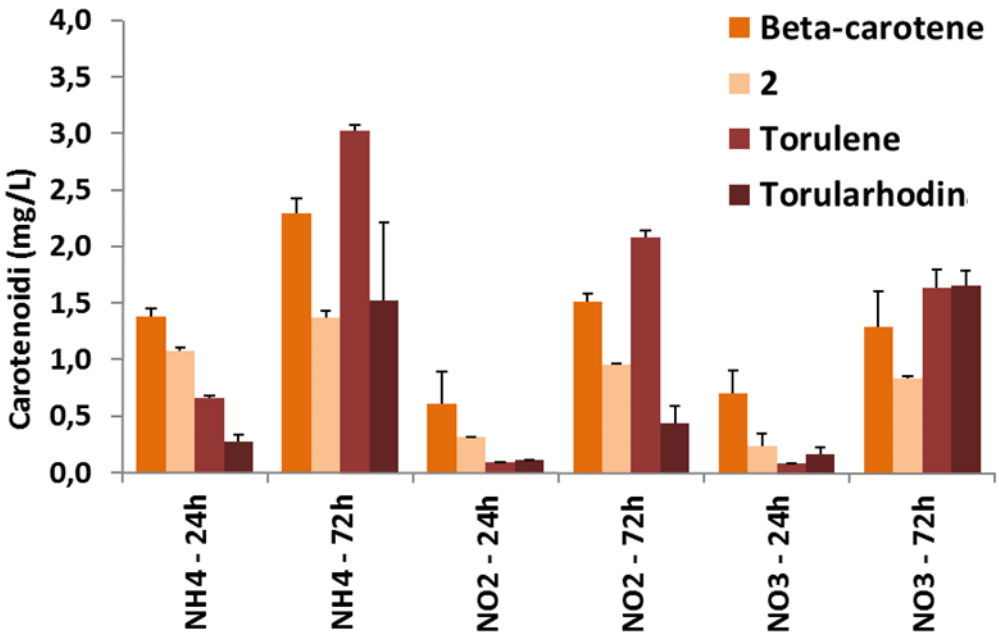

Figure 24: Different carotenoids produced by *Rhodotorula* DSBCA06 in different nitrogen sources

Chapter 6: Nitrogen metabolism by R. glutinis DSBCA06

R. glutinis proved to be capable of assimilating nitrate as the sole nitrogen source as yeasts studied by the team of ULL (Barnett et al., 1983).

A part of the PhD research project was performed at "Department of Biochemistry and Molecular Biology" of La Laguna University (ULL), under the supervision of Prof. J. M. Siverio, to gain insight in the molecular mechanism allowing *R. glutinis* DSBCA06 to assimilate different nitrogen sources.

6.1 Nitrate reductase activity

Nitrate reductase (NR) is the principal enzyme for nitrate assimilation, reducing nitrate with formation of nitrite.

Measuring nitrate reductase activity and its inhibition is the first step to study the metabolism of any nitrate-assimilating strain, and to understand regulatory systems of nitrogen metabolism.

This experiment evaluates kinetic of NR in *R. glutinis* DSBCA06 with systems used for yeast *H. polymorpha*, *H. anomala* (CECT 1 1 12) and *H. wingei* (CBS 2432) to evaluate nitrate reduction.

We used a culture of *R. glutinis* DSBCA06 with OD_{600} 3.0 A.U..

We added KNO_3 at final concentration 5mM after 30 minutes of starvation in YNB medium without nitrogen sources.

Culture samples were collected at regular times since KNO_3 addition. After three hours' ammonia was added to check enzymatic inhibition.

Enzymatic activity was verified by quantification of nitrite produced by cellular extraction after cell disruption with glass balls.

With this procedure the yield of enzymatic reaction with NADH and NADPH was evaluated, and the effect of protease inhibitor and dithiothreitol (DTT) to detect best conditions to measure NR activity.

Nitrate reductase activity was measured with Griess assay (to measure nitrite produced by enzyme) and Bradford method (to measure total protein content).

Figure 25 shows that the use of NADPH is crucial to perform the NR assays, provided that NADH is a non-substrate for the enzyme. Also DTT and protease inhibitors (not shown) significantly improve the detection limit of the assays.

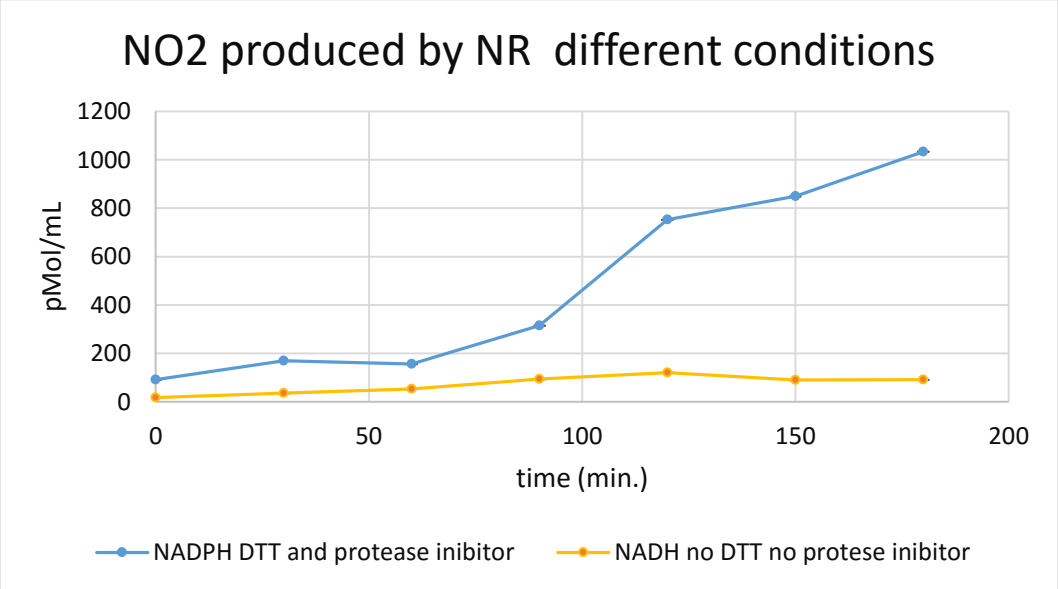

Figure 25: optimization of NR assay

In figure 26 the increment in NR activity, stimulated by nitrate in the medium, is evident in the first 180 min. As expected, the addition of ammonia, on the contrary, led to inhibition of NR.

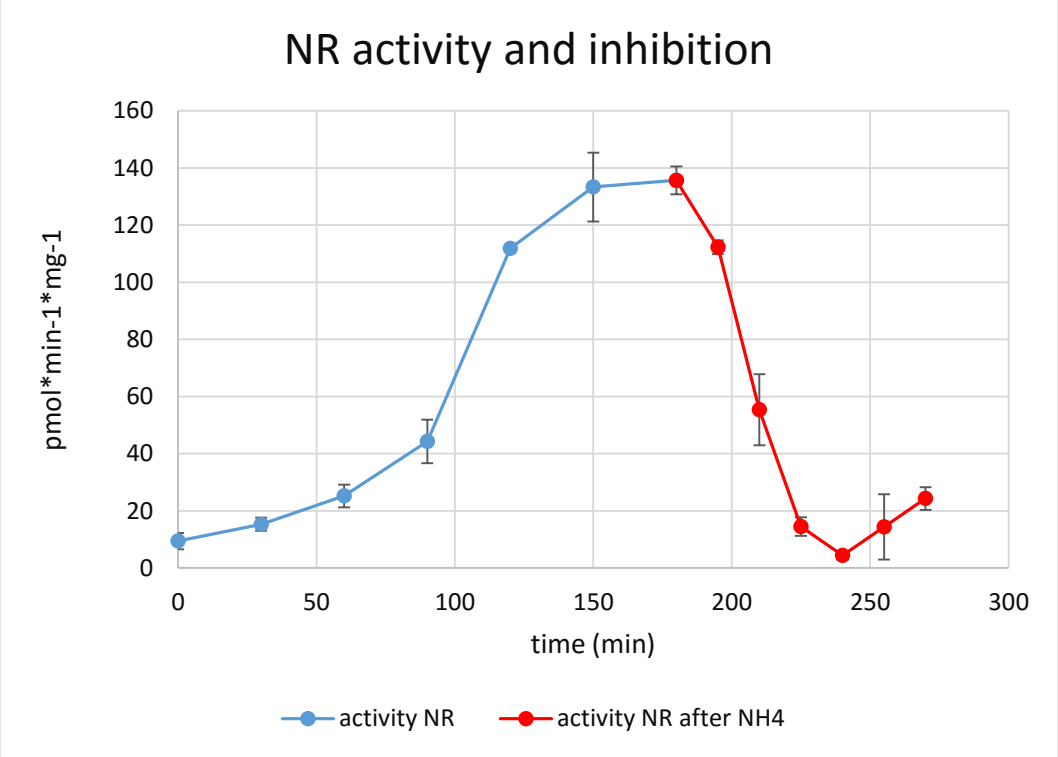

Figure 26: NR activity and its inhibition by ammonium

6.2 Nitrate assimilation

In these experiments *R. glutinis* was grown in 5 mM YNB, at initial OD_{600} of 1.0. In Figure 27 nitrate disappearance can be observed, and the nitrite was simultaneously released in medium. In Figure 28 nMol of nitrate removed per mg of cells.

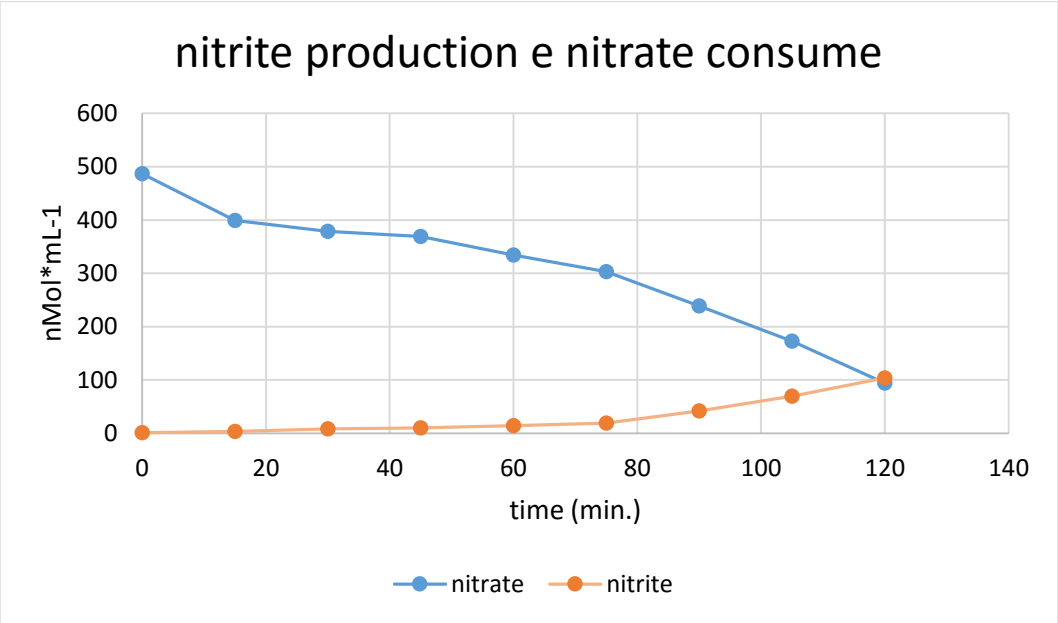

Figure 27: nitrate absorption and contemporary nitrite production

Nitrate uptake and assimilation is evident even within a short time, suggesting that *R. glutinis* excretes excess nitrite deriving from nitrate reduction, to neutralize toxic effect of this anion.

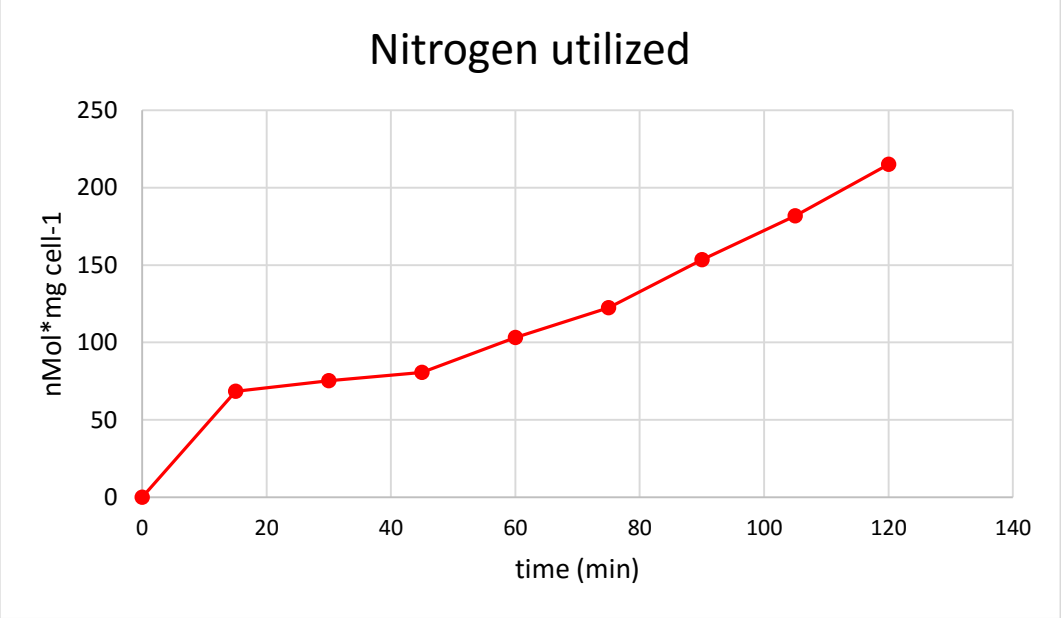

Figure 28: nitrogen assimilation by *R. glutinis*

6.3 Nitrite assimilation in short time

In this experiment starvation cultures of *R. glutinis* were supplemented with 5 mM nitrite. And nitrite uptake was measured in the first two hours (in this period no considerable variations in OD_{600} values are detectable). In Figure 27pmoles of nitrite assimilated by 1 mg of cells are reported.

Inspection of the Figures reveals that nitrite uptake takes place during lag phase, anticipating biomass increase.

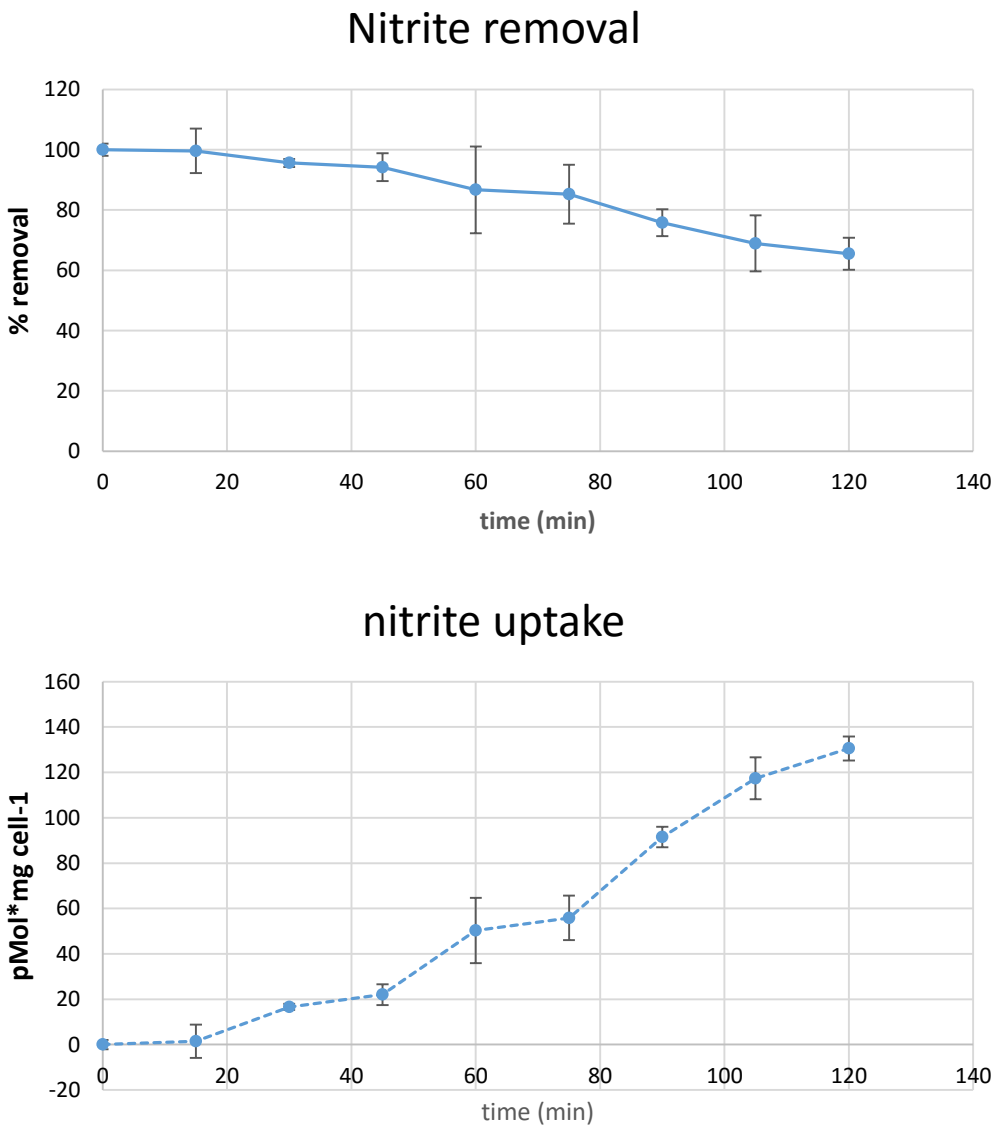

Figure 29: nitrite removal and uptake from *Rhodotorula glutinis* in short time

Conclusions

During this study a nitratophile yeast was isolated, and molecularly identified as *R. glutinis* by rRNA sequencing. The experiments carried out showed that this strain is an extremely adaptable nitrophile yeast, capable of growing in presence of nitrogen compounds in high concentrations.

The strain has been proven capable of growing using nitrate, nitrite and ammonia as the sole nitrogen sources, and of removing them from the medium. This makes it particularly suitable for future application in the field of bioremediation and wastewater treatment. Particularly, *R. glutinis* was able to tolerate high concentrations of these polluting nitrogen compounds, being promising for both *in situ* and *ex situ* applications.

We decided to further concentrate our efforts in the characterization of the ability of our strain to degrade nitrite, chemical species with documented high toxicity, among the studied ones. The strain in the presence of nitrite was able to use different pure and renewable carbon sources, including the quite inexpensive glucose and several agro-industrial wastes.

Particularly good results have been found employing vegetable oils as a sole carbon source for its growth, being a promising alternative for their disposal.

The optimal conditions of growth were established, in terms of pH and nutrient concentrations. The ability of the strain to tolerate some toxic heavy metals was evaluated, taking into account the presence of large contaminated areas in Sardinia. *R. glutinis* DSBCA06 was able to resist against noticeable concentrations of lead and cadmium.

The costs of bioremediation and bio-purification treatments are often knocked down by coupling with processes of secondary metabolites production. In this perspective, we analysed the ability of the strain to produce carotenoids from the culture media with high concentrations of nitrite, nitrate and ammonia. Moreover, we have assessed that the chemical form in which the nitrogen was found in soil is capable of influencing the relationship between the type of produced carotenoids. The strain was proven able to produce more carotenoids in the presence of ammonium. The carotenoid pattern was also identified by HPLC-DAD.

In the light of all these findings, we can consider *R. glutinis* DSBCA06 a strain with different features exploitable in the field of bioremediation and in the production of secondary metabolites with considerable economic value.

During the stage at La Laguna University, enzymatic pathway of nitrogen assimilation was partially characterized.

Several aspects of *R. glutinis* metabolism require anyway to be further investigated. Particularly, optimization of growth conditions using advanced statistical approach (i.e. response surface methodology) seems to be mandatory before large-scale applications. Besides, several other secondary metabolites (i.e. exopolysaccharides) could help to get a potential process economically affordable.

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