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56 Abstract

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ORIGINAL ARTICLE

Marine Microorganisms for Biocatalysis: Selective Hydrolysis of Nitriles with a Salt-Resistant Strain of *Meyerozyma guilliermondii*

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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 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 displayed high activity on aromatic substrates, but also arylaliphatic and aliphatic substrates were accepted. Salt-resistant *M. guilliermondii* LM2 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.

Keywords Marine yeast · Nitrilase · *Meyerozyma guilliermondii* · Seawater · Biocatalysis

Introduction

Important traits for the development of biotechnological processes are the ability, exhibited by some microorganisms, to tolerate some of the most 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 bio-prospecting 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, thermostability, barophilicity, and cold adaptivity (Trincone 2011). Exploitation of extremophile microorganisms from marine environment can represent a reliable alternative to mesophilic systems. 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

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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 3.5.1.14). 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 production 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.

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 Resource 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 $MgSO_4$, 1 g/L K_2HPO_4, 0.1 g/L $CaCl_2$, 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 $g_{dry\ weight}$/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 SpeedDigester 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 μm glass microfiber GF/A filter; Whatman). The filters were washed with three volumes of de-ionized 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 $g_{dry\ weight}$/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 cell 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; 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, 6.3 min; 2-(cyanophenyl)acetic acid, 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-phenylbutyronitrile, 7.7 min; (*S*)-2-phenylbutyronitrile, 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 cyanoacetic acid, 13.7 min.

Lyophilized Cell Preparation

After cultivation and induction, cells were harvested by centrifugation (5000 rpm, 10 min), washed once with 0.9% NaCl, and suspended in 10 mL of either de-ionized 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 re-hydrated in the biotransformation buffer for 15 min at 28 °C under orbital shaking at 150 rpm before use.

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 concentration 0.02–0.06%). Only *Meyerozyma 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 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 $g_{dry\ weight}$/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. 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 strategy 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,

Table 1 Growth of marine yeasts on solid YNB medium containing different nitriles as sole nitrogen source (0.06–0.12%)

	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

Nd not detectable growth

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/$mg_{dry\ cells}$) when induced by 12.5 mM cyclohexanecarbonitrile (CECN) (Fig. 1).

To optimize the nitrilase expression, an appropriate ratio between the amount of cells and inducer was established. Two concentrations of cells (2 $g_{dry\ weight}$/L and 7 $g_{dry\ weight}$/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 (6–9 mM) 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/$mg_{dry\ cells}$).

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/$mg_{dry\ cells}$ 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/L K_2HPO_4, 0.1 g/L $CaCl_2$, 0.1 M 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 for details). At the end of this process, the biomass showed 0.06 U/$mg_{dry\ cells}$ 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. In this case, more than 50% reduction in specific nitrilase activity (0.024 U/$mg_{dry\ cells}$) 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}

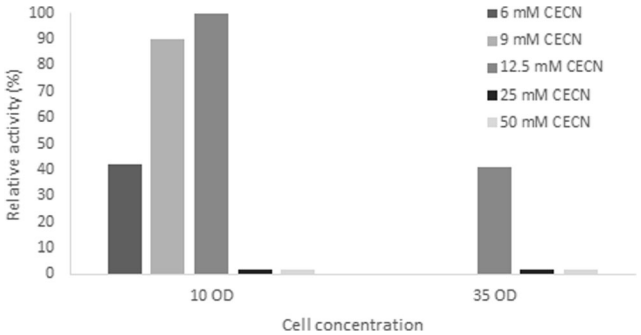
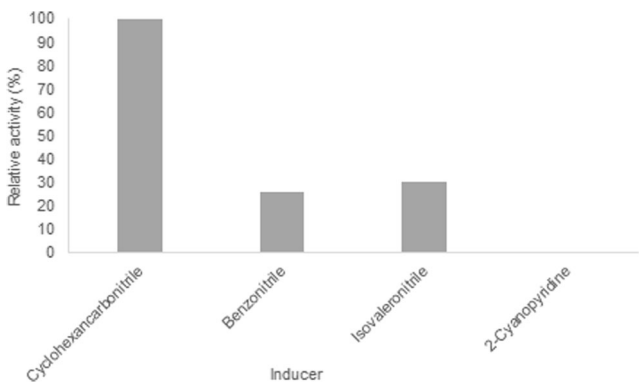


Fig. 2 Effect of inducer (cyclohexanecarbonitrile)/cells concentration ratio on nitrilase specific activity on 10 mM benzonitrile



Q2 **Fig. 1** Induction of nitrilase by different compounds (12.5 mM each), calculated as specific activity on 10 mM benzonitrile

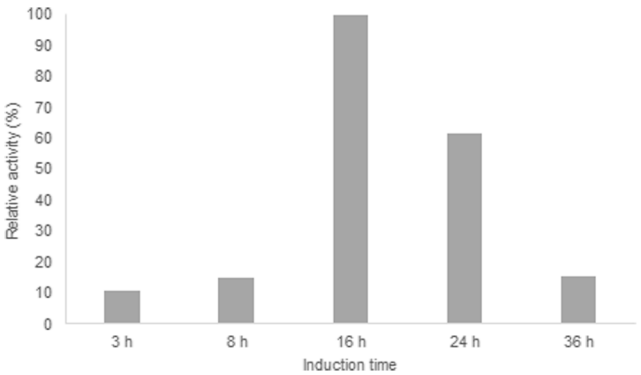
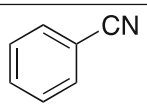
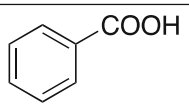
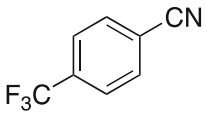
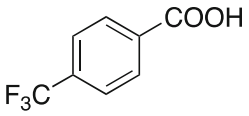
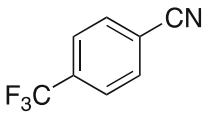
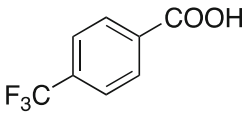
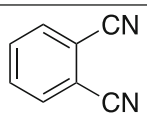
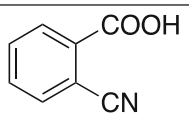
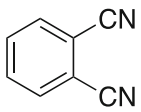
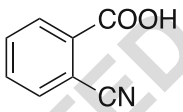
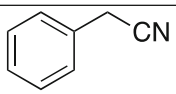
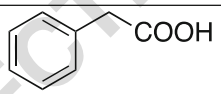
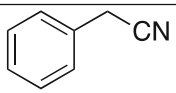
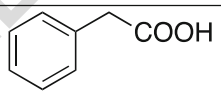
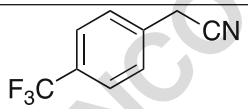
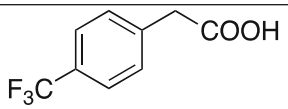
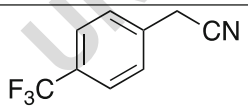
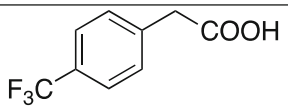
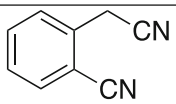
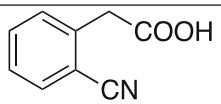
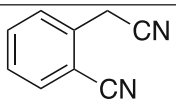
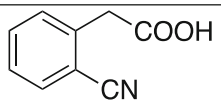


Fig. 3 Induction time of nitrilase specific activity (assessed on benzonitrile) by 2 $g_{dry\ weight}$/L of cells and 12.5 mM CECN

t2.1 **Table 2** Hydrolysis of different achiral aromatic nitriles with whole cells of *M. guillermondii* LM2. 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

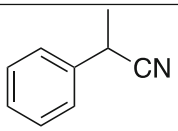
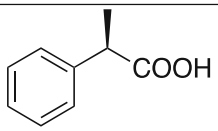
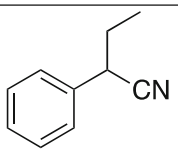
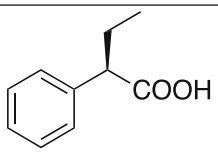
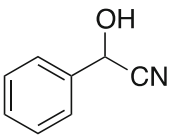
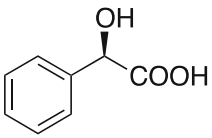
Entry	Substrate	Product	Yield (%)	Time (h)
1			>95	2
2			84	2
3			95	3
4			< 3	2
5			10	24
6			90	2
7			>95	3
8			56	2
9			90	5
10			12	2
11			70	24

$_{cells}$). 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. guillermondii* were tested for biotransformation of different aromatic mono- and di-nitriles (Table 2).

t3.1 **Table 3** Hydrolysis of different racemic aromatic nitriles with whole cells of *M. guillermondii* LM2. 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

Benzonitrile and 4-(trifluoromethyl)benzonitrile were converted rapidly and quantitatively by whole cells of *M. guillermondii* LM2 (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 of the 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. guillermondii* 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 2 $mg_{dry\ weight}$/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 $-C\equiv N$ hydrolysis is mostly due to true nitrilase(s).

Whole cells of *M. guillermondii* LM2 were also used for hydrolyzing racemic nitriles (Table 3); *M. guillermondii* LM2 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). (*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.

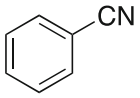
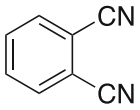
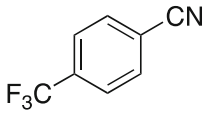
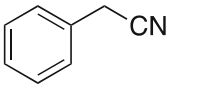
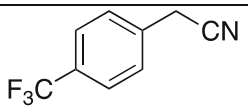
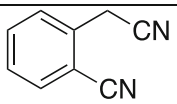
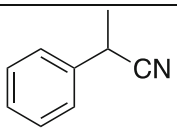
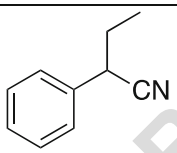
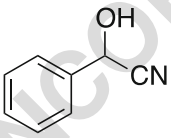
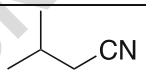
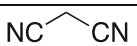
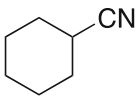
Finally, the biotransformation was also carried out with aliphatic substrates: isovaleronitrile was converted into the corresponding acid with good conversion (92% after 24 h), whereas malononitrile gave 2-cyanoacetic acid with low rates and yield (10% after 24 h). Table 4 summarizes the activity of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

The comparison of the relative activity shows that nitrilase of *M. guillermondii* 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

t4.1 **Table 4** Relative activity of resting cells of *M. guillermondii* toward different nitriles

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

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.

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/$mg_{dry\ weight}$). Moreover,

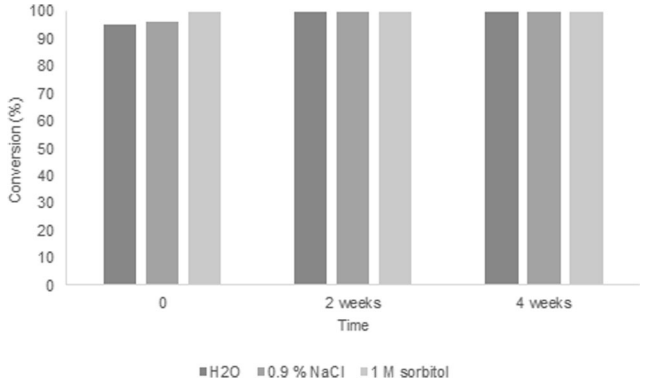


Fig. 4 Stability of lyophilized cells using different lyophilizing solvents. Conversions of 10 mM benzonitrile after 3 h are reported

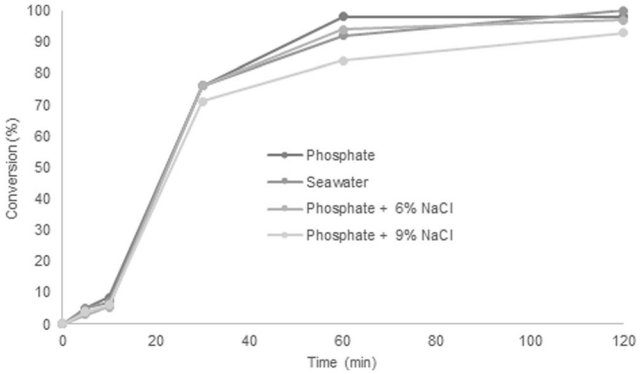


Fig. 6 Conversion of 10 mM benzonitrile in media with different salinity by *M. guillermondii* grown in seawater-based medium

similar time-courses were observed when the biotransformation was carried out in seawater and in media with increasing concentrations of NaCl (Fig. 6).

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. guillermondii* LM2, achieving the complete conversion into (*R*)-mandelic acid after 8 h.

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/L dry weight 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 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; Kaplan et 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, optimized for growth and nitrilase activity, converted aromatic substrates with high yields, being also able to transform arylacetonitriles and aliphatic nitriles. This feature was allowed for the conversion of chiral α-substituted benzyl nitriles, such as 2-phenylpropanenitrile, 2-phenylbutanenitrile, and mandelonitrile; in all the cases, a strong enantiopreference for hydrolysis of the *R*-enantiomer, as often encountered with nitrilases. The biotransformation of racemic

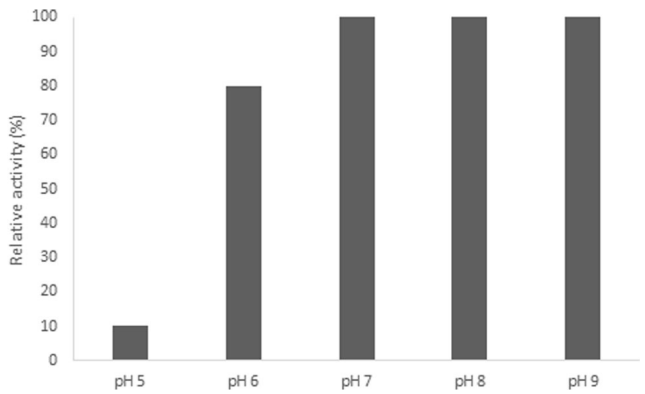


Fig. 5 Activity of lyophilized cells on 10 mM benzonitrile at different pHs. pH 7 is considered as 100%

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 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 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 toward isovaleronitrile and mandelonitrile was lower than what observed with *M. guilliermondii* CGMCC12935 grown on a synthetic medium (Zhang et al. 2017).

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.

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 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%).

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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References

Anderson NG (2012) Solvent selection. In: Anderson NG (ed) Practical processes & development-a guide for organic chemists, 2nd edn. Academic Press, Oxford, pp 121–168

Banerjee A, Sharma R, Banerjee UC (2002) The nitrile-degrading enzymes: current status and future prospects. Appl Microbiol Biotechnol 60:33–44

Brewis EA, Van Der Walt JP, Prior BA (1995) The utilization of aromatic, cyclic and heterocyclic nitriles by yeasts. Syst Appl Microbiol 18: 338–342

Burgaud G, Hue NTM, Arzur D, Coton M, Perrier-Cornet JM, Jebbar M, Barbier G (2015) Effects of hydrostatic pressure on yeasts isolated from deep-sea hydrothermal vents. Res Microbiol 166:700–709

Chi Z, Zhang T, Liu G, Li J, Wang X (2009) Production, characterization and gene cloning of the extracellular enzymes from the marine-derived yeasts and their potential applications. Biotechnol Adv 27: 236–225

de Oliveira JR, Mizuno CM, Seleghim MH, Javaroti DC, Rezende MO, Landgraf MD, Sette LD, Porto AL (2013) Biotransformation of phenylacetonitrile to 2- hydroxyphenylacetic acid by marine fungi. Mar Biotechnol 15:97–103

De Vitis V, Guidi B, Contente ML Granato T, Conti P, Molinari F, Crotti E, Mapelli F, Borin S Daffonchio D, Romano D (2015) Marine microorganisms as source of stereoselective esterases and ketoreductases: kinetic resolution of a prostaglandin intermediate. Mar Biotechnol 17:144–152

Debabov VG, Yanenko AS (2011) Biocatalytic hydrolysis of nitriles. Rev J Chem 1:385–402

Dias JCT, Rezende RP, Rosa CA, Lachance M, Linardi VR (2000) Enzymatic degradation of nitriles by a *Candida guilliermondii* UFMG-Y65. Can J Microbiol 46:525–531

Domínguez de María P (2013) On the use of seawater as reaction media for large-scale applications in biorefineries. ChemCatChem 5:1643–1648

Gong J, Lu Z, Li H, Shi J, Zhou Z, Xu Z (2012) Nitrilases in nitrile biocatalysis: recent progress and forthcoming research. Microb Cell Factories 11:142

He Y, Xu J, Su J, Zhou L (2010) Bioproduction of glycolic acid from glycolonitrile with a new bacterial isolate of *Alcaligenes* sp. ECU0401. Appl Biochem Biotechnol 160:1428–1440

Hoyle AJ, Bunch AW, Knowles CJ (1998) The nitrilases of *Rhodococcus rhodochrous* NCIMB 11216. Enzym Microb Technol. https://doi.org/10.1016/S0141-0229(98)00076-3

Kaplan O, Vejvoda V, Charvátová-Pinvejcová A, Martínková L (2006) Hyperinduction of nitrilases in filamentous fungi. J Ind Microbiol Biotechnol 33:891–896

Margesin R, Schinner F (2001) Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles 5:73–83

Martínková L, Křen V (2010) Biotransformations with nitrilases. Curr Opin Chem Biol. https://doi.org/10.1016/j.cbpa.2009.11.018

Martínková L, Vejvoda V, Kaplan O, Kubáč D, Malandra A, Cantarella M, Bezouška K, Křen V (2009) Fungal nitrilases as biocatalysts: recent developments. Biotechnol Adv 27:661–670

Meth-Cohn O, Wang M (1997) Rationalisation of the regioselective hydrolysis of aliphatic dinitriles with *Rhodococcus rhodochrous* AJ270. Chem Commun 0:1041–1042

Mukherjee C, Zhu D, Biehl ER, Hua L (2006) Exploring the synthetic applicability of a cyanobacterium nitrilase as catalyst for nitrile hydrolysis. Eur J Org Chem. https://doi.org/10.1002/ejoc.200600699

O'Reilly C, Turner PD (2003) The nitrilase family of CN hydrolysing enzymes – a comparative study. J Appl Microbiol 95:1161–1174

Petrícková A, Sosedov O, Baum S, Stolz A, Martínková L (2012) Influence of point mutations near the active site on the catalytic properties of fungal arylacetonitrilases from *Aspergillus niger* and

Neurospora crassa. J Mol Catal B Enzym. https://doi.org/10.1016/j.molcatb.2012.01.005

Rédou V, Navarri M, Meslet-Cadière L, Barbier G, Burgaud G (2015) Species richness and adaptation of marine fungi from deep-subseafloor sediments. Appl Environ Microbiol 81:3571–3583

Rezende RP, Dias JCT, Rosa CA, Carazza F, Linardi VR (1999) Utilization of nitriles by yeasts isolated from a Brazilian gold mine. J Gen Appl Microbiol 45:185–192

Rustler S, Chmura A, Sheldon RA, Stolz A (2008) Characterisation of the substrate specificity of the nitrile hydrolyzing system of the acidotolerant black yeast *Exophiala oligosperma* R1. Stud Mycol 61:165–174

Serra I, Guidi B, Burgaud G, Contente ML, Ferraboschi P, Pinto A, Compagno C, Molinari F, Romano D (2016) Seawater-based biocatalytic strategy: stereoselective reductions of ketones with marine yeasts. ChemCatChem. https://doi.org/10.1002/cctc.201600947

Spizzo P, Basso A, Ebert C, Gardossi L, Ferrario V, Romano D, Molinari F (2007) Resolution of (*R*,*S*)-flurbiprofen catalysed by dry mycelia in organic solvent. Tetrahedron 63:11005–11010

Thuku RN, Brady D, Benedik MJ, Sewell BT (2009) Microbial nitrilases: versatile, spiral forming, industrial enzymes. J Appl Microbiol 106:703–727

Trincone A (2011) Marine biocatalysts: enzymatic features and applications. Mar Drugs 9:478–499

Vejvoda V, Kaplan O, Klozová J, Masák J, Cejková A, Jirku V, Stloukal R, Martínková L (2006) Mild hydrolysis of nitriles by *Fusarium solani* strain O1. Folia Microbiol 51:251–256

Yamamoto K, Oishi K, Fujimatsu I, Komatsu K (1991) Production of *R*-(−)-Mandelic acid from Mandelonitrile by *Alcaligenes faecalis* ATCC 8750. Appl Environ Microbiol 57:3028–3032

Zaky AS, Tucker GA, Daw ZY, Du C (2014) Marine yeast isolation and industrial application. FEMS Yeast Res 14:813–825

Zambelli P, Serra I, Fernandez-Arrojo L, Plou FJ, Tamborini L, Conti P, Contente ML, Molinari F, Romano D (2015) Sweet-and-salty biocatalysis: fructooligosaccharides production using *Cladosporium cladosporioides* in seawater. Process Biochem 50:1086–1090

Zhang XH, Liu ZQ, Xue YP, Xu M, Zheng YG (2016) Nitrilase-catalyzed conversion of (*R,S*)-mandelonitrile by immobilized recombinant *Escherichia coli* cells harboring nitrilase. Biotechnol Appl Biochem 63:479–448

Zhang Q, Gong J, Dong T, Liu T, Li H, Dou W, Lu Z, Shi J, Xu Z (2017) Nitrile-hydrolyzing enzyme from *Meyerozyma guilliermondii* and its potential in biosynthesis of 3-hydroxypropionic acid. Bioprocess Biosyst Eng. https://doi.org/10.1007/s00449-017-1754-6

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