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# α-Rhamnosidase activity in the marine isolate *Novosphingobium* sp. PP1Y and its use in the bioconversion of flavonoids

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ABSTRACT

Crude protein extracts of *Novosphingobium* sp. PP1Y, a microorganism isolated from polluted marine waters in Pozzuoli (Italy), were analyzed for the presence of glycosidase activities. Particular attention was devoted to a α-L-rhamnosidase activity able to hydrolyze several flavonoids of interest for the pharmaceutical and food industries. This activity had an alkaline pH optimum and a moderate tolerance to the presence of organic solvents, appealing features for its possible biotechnological uses. An increase of the α-L-rhamnosidase activity in PP1Y crude extracts was induced by adding naringin to the growth medium, suggesting the possibility to use material from *Citrus* industrial waste to induce the glycosidase activity expressed by strain PP1Y and produce simultaneously high-added-value molecules from the hydrolysis of their flavonoids. In order to investigate on the enzymatic mechanism of PP1Y α-L-rhamnosidase activity, hydrolysis products of PNP-α-L-rhamnopyranoside were analyzed by $^{1}$H-NMR experiments. The kinetic behaviour clearly indicated an inverting mechanism of hydrolysis for this novel enzymatic activity.


## 1. Introduction

Biocatalysis represents nowadays a versatile and valuable tool for industrial biotechnologies. The use of enzymes as biocatalysts can have significant benefits compared to conventional chemical technologies, for achieving high reaction selectivity, higher reaction rate, improved product purity, and a significant decrease in chemical waste production. A wide variety of chemical substances is already produced in industrial processes through the use of enzymes [1,2].

Within this framework, α-L-rhamnosidases [E. C. 3.2.1.40] have attracted great attention in the last decade due to their application as biocatalysts in a variety of food, pharmaceutical and chemical industrial processes [3].

α-L-Rhamnosidases (from now on indicated as α-RHAs) are a class of glycosyl hydrolases that specifically cleave terminal α-L-rhamnose from a large number of natural products, which include flavonoids, some terpenyl glycosides [4,5] and many other natural glycosides containing a terminal rhamnose, such as glycopeptides antibiotics and glycolipids [6]. These enzymes have recently been the focus of an increasing scientific interest. As an example, the use of α-RHAs to improve the bioavailability, and thus the biological activity, of flavonoids beneficial for human health as direct drugs or as nutritional supplements, has been reported [3].

Moreover, L-rhamnose has a central role in the organic synthesis as a chiral intermediate of pharmaceutically important compounds. Its production using the enzymatic activity of α-RHAs in hydrolysis reactions of glycosylated compounds that might be recovered from waste material of food processing industry (e.g. citrus peel), represents an interesting and useful biotechnological application these glycosidases [7].

Fungi are the main source of α-RHAs. However, these enzymes have been isolated also from animal tissues, such as the liver of both the marine gastropod *Turbo cornutus* [8] and pig [9], and from plants such as *Rhamnus daurica* [10] and *Fagopyrum esculentum* [11].

Bacteria represent a yet unexplored reservoir of α-RHAs, which might show novel interesting properties. An analysis of several α-RHAs isolated from various microbial sources reveals, for example, that one of the main differences found between fungal and bacterial enzymes is their optimal pH, with the fungal enzymes showing more acidic pH optima compared to bacterial counterparts, for which neutral and alkaline optimal pH values have generally been reported. This characteristic suggests different potential applications for fungal and bacterial enzymes, making bacterial α-RHAs

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suitable in biotechnological processes requiring good activity in more basic solutions such as the production of L-rhamnose by hydrolysis of naringin or hesperidin, compounds whose solubility strongly increases at higher pH values [3,12].

Despite the very limited number of bacterial α-RHAs that has been described so far, data in literature suggest that this enzymatic activity is widely distributed over a diverse range of ecological niches. α-RHAs have been detected and characterized in the human intestinal bacterium *Bacteroides* JY-6 [13], in cold-adapted *Pseudoalteromonas* species and *Ralstonia pickettii* isolated in the sea water of sub Antarctic environment [14], also in soil bacteria such as *Bacillus* sp. GL1 [15,16], *Sphingomonas paucimobilis* FP2001 [17] and *Sphingomonas* sp. R1 [18] and finally in wine strains of *Oenococcus oeni* [19].

α-RHAs from *Lactobacillus* species were identified and investigated for their potential biotechnological use to de-rhamnosylate flavonoids present in frequently consumed food commodities [20].

Moreover, α-RHAs genes have been cloned and expressed from thermophilic bacteria such as *Clostridium stercorarium* [21] and from the bacterium PRI-1686, which is a member of the phylum of Thermomicrobia [22].

Recently, the crystal structure of *Streptomyces avermitilis* α-L-rhamnosidase (SaCBM67) was reported [23]; its structure presented a novel catalytic carbohydrate-binding module and resulted different from the only two structures of α-L-rhamnosidases (GH78) previously reported: the BsRhaB isolated from *Bacillus* sp. GL1 [24] and the putative α-L-rhamnosidase BT1001 from *Bacteroides thetaiotaomicron* VPI-5482 [25].

It goes without saying that the biotechnological potential of bacterial α-RHAs, whose structural, functional and molecular biology aspects have not been sufficiently investigated, is strictly related to the acquisition of new information on the enzymatic systems isolated from new bacterial sources.

Recently, *Novosphingobium* sp. PP1Y, an organic solvent-resistant, biofilm-forming marine microorganism, was isolated from the surface waters of a docking bay in the harbour of Pozzuoli (Naples, Italy), an area heavily polluted with hydrocarbons [26]. The analysis of its genome, confirmed the presence of several genomic features of interest for the biotechnological potential of this microorganism, and more specifically for its exploitation as a source of enzymes active on carbohydrates. Strain PP1Y shows in fact a unique abundance among Sphingomonadales of genes encoding for glycosyl hydrolases (53 orfs) [27], which are distributed among 27 different families. This prompted our interest in investigating the presence of α-RHA activities in the crude protein extract of strain PP1Y.

In this work, cell extracts obtained from *Novosphingobium* sp. PP1Y cells grown in minimal medium were tested for the presence of glycosidase activities; in particular α-RHA and β-glucosidase activities were detected and partially characterized.

This α-RHA activity was successfully used in bioconversion studies of flavonoidic compounds, thus suggesting its potential use as an eco-friendly tool to modulate the biological and pharmacological properties of these molecules. It was further demonstrated by $^{1}$H-NMR experiments that α-RHA activity from *Novosphingobium* sp. PP1Y functioned with an inverting mechanism, in agreement with the enzymatic mechanism already proposed for other α-RHAs previously described.

## 2. Materials and methods

### 2.1. General

*Novosphingobium* sp. PP1Y was isolated as previously reported [26]. All reagents were purchased from Sigma-Aldrich and used without any further purification. Silica gel, reverse-phase silica gel C-18 and TLC silica gel plates were from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Compounds on TLC plates were visualized under UV light or charring with α-naphthol reagent.

TLC solvent systems used were (A) EtOAc:MeOH:$H_2O$, 70:20:10 and (B) EtOAc:AcOH:2-Propanol:HCOOH:$H_2O$, 25:10:5:1:15.

$^{1}$H-, $^{13}$C- and 2D-NMR spectra were acquired by the NMR Service of the Istituto di Chimica Biomolecolare of the National Research Council of Italy (C.N.R.-Pozzuoli, Naples, Italy) and recorded on a Bruker DRX-600 spectrometer, equipped with a TCI CryoProbe™, fitted with a gradient along the *Z*-axis, and on other Bruker instruments with fields at 400 and/or 300 MHz. Samples for NMR analysis were dissolved in the appropriate solvent; spectra in $D_2O$ were referenced to internal sodium 3-(trimethyl-silyl)-(2,2,3,3-$^{2}H_4$) propionate (Aldrich, Milwaukee, WI); for other solvents downfield shift of the signal of the solvent was used as internal standard.

In the bioconversion studies followed by $^{1}$H-NMR experiments, reported in paragraph 2.8 and 2.9, the selected signals integral values of products and reagents resulted affected by an error in integration (<5%) which depended upon instrument optimization.

Protein concentration was routinely estimated using the Bio-Rad Protein System [28]; bovine serum albumin was used as standard.

### 2.2. Bacterial growth

*Novosphingobium* sp. PP1Y was routinely grown in minimal medium. Potassium phosphate minimal medium (PPMM) contained 20 mM potassium phosphate pH 6.9, 1 g/L $NH_4Cl$ and 100 mM NaCl. After autoclaving, 5 mL of a trace element solution was added to each litre of cooled PPMM. The trace element solution contained in a 0.92% solution of HCl: 30.1 g/L $MgSO_4$, 4.75 g/L $FeSO_4 \times 7H_2O$, 5.4 g/L MgO, 1.0 g/L $CaCO_3$, 0.72 g/L $ZnSO_4 \times 7H_2O$, 0.56 g/L $MnSO_4 \times H_2O$, 0.125 g/L $CuSO_4 \times 5\ H_2O$, 0.14 g/L $CoSO_4 \times 7\ H_2O$, 0.03 g/L $H_3BO_3$, 0.004 g/L $NiCl_2 \times 6\ H_2O$, 0.006 g/L $Na_2MoO_4 \times 2H_2O$. When using PPMM as growth medium, 0.4% (w/v) of glutamic acid, prepared in deionized water and sterilized by filtration with a 0.22 μm Millipore membrane, was used as unique carbon and energy source and added to the autoclaved media. Cultures were incubated at 30 °C with orbital shaking (220 rpm).

Bacterial growth was monitored by measuring the optical density at 600 nm ($OD_{600}$). A pre-inoculum in rich medium was prepared by transferring 50 μL from a glycerol stock stored at −80 °C to a 50 mL Falcon tube containing 12.5 mL of sterile LB medium. LB was prepared according to Sambrook et al. [29]. The pre-inoculum was allowed to grow at 30 °C for at least 14 h at 220 rpm and then used to inoculate 1 L of a preparative growth in PPMM medium at an initial cell concentration of 0.01–0.02 $OD_{600}$/mL. Cells were usually harvested after 24 h at a final optical density of ca. 1 $OD_{600}$/mL. It is worthy to note that, when following the growth for more than 24 h, the formation of flocks was observed, thus hampering a correct estimation of the turbidity of the cell suspension.

### 2.3. Preparation of Novosphingobium sp. PP1Y crude protein extract

Cell cultures were harvested by centrifugation at 5,524 × *g* for 30 min at 4 °C. Cell pellets were suspended in 25 mM MOPS at pH 7.0 and disrupted by sonication (30′ total, 30 s ON and 30 s OFF) in an ice-water bath. Cell suspension was centrifuged at 17,418 × *g* at 4 °C for 60 min. The soluble fraction obtained after centrifugation was filtered on a 0.45 mm Millipore membrane, divided in aliquots and stored at −80 °C. When used for the screening of glycosidase

**Table 1**
Evaluation of different substrates bioconversion by using glycosidase activities in the crude extract of *Novosphingobium* sp. PP1Y cells.

| Substrates[a] | Crude extract of PP1Y cells grown in PPMM medium | | | |
|---|---|---|---|---|
| | Hydrolysis[b] | | Transglycosylation[c] | |
| | 3 h | 24 h | 3 h | 24 h |
| PNP-α-D-Glc*p* | + | + | – | +/– |
| **PNP-β-D-Glc*p*** | + | ++ | + | ++ |
| PNP-α-D-Gal*p* | +/– | + | – | +/– |
| **PNP-β-D-Gal*p*** | + | ++ | + | ++ |
| PNP-α-D-Man*p* | – | – | – | – |
| PNP-β-D-Man*p* | – | – | – | – |
| PNP-α-d-Xyl*p* | – | – | – | – |
| **PNP-β-D-Xyl*p*** | + | ++ | +/– | + |
| PNP-α-L-Fuc*p* | – | – | – | – |
| **PNP-β-D-Fuc*p*** | + | ++ | + | ++ |
| PNP α-D-NAG*p* | – | – | – | – |
| PNP β-D-NAG*p* | +/– | + | +/– | +/– |
| **PNP-α-L-Rha*p*** | ++ | +++ | – | +/– |
| PNP-α-L-Ara*f* | – | – | – | – |

*Note*: –/+: percentage of products below 10%; +: 10–30% of products; ++: 30–70% of products; +++: percentage of products higher than 70%.

[a] Ara*f*, arabinofuranose; Fuc*p*, fucopyranose; Gal*p*, galactopyranose; Glc*p*, glucopyranose; Man*p*, mannopyranose; NAG*p*, *N*-acetylglucosaminopyranose; Rha*p*, Rhamnopyranose; Xyl*p*, xylopyranose.

[b] Hydrolysis products were analyzed by comparing Rf values with appropriate standards in TLC solvent system A.

[c] Due to self-transglycosylation reactions, products showed positive UV absorbance and lower Rf than the corresponding aryl substrates in the selected TLC solvent system A.

activities (Section 2.4), the soluble fraction was dialyzed against 20 mM potassium phosphate at pH 7, and then divided in aliquots and stored at −80 °C. PP1Y crude extracts were assayed for their total protein content.

Without any different indication, all experiments reported in the following sections, were performed using a crude extract obtained from a 2 L culture of PP1Y cells grown in PPMM medium and with a protein concentration of 3.72 mg total protein/mL. Cells were recovered after 24 h at an optical density of 1.02 $OD_{600}$/mL.

### 2.4. Glycosidase activities screening

PP1Y crude protein extract, prepared as described in the previous paragraph, was tested for the presence of glycosidase activities by using several *p*NP-α- and *p*NP-β-substrates (Table 1) at a 20 mM concentration in 0.6 mL of 50 mM Na-phosphate buffer pH 7, at 35 °C and under magnetic stirring. All reactions were carried out using 4.42 mg of total protein/mmol of reagent. Reactions were monitored over time (0–24 h) by TLC analysis (system solvent A).

Moreover, hydrolysis reactions with maltose, lactose, cellobiose, sucrose, raffinose, laminaripentaose, laminarin, curdlan, β-glucan from barley, xylan from birch wood, pullulan, amylopectin and starch as substrates were carried out using crude extracts of PP1Y cells grown in PPMM. Each substrate (10 mg) was suspended in 1 mL of 50 mM Na-phosphate buffer pH 7, containing 41.6 μg of total protein/mL of reaction mixture. Reactions were performed at 35 °C. Hydrolysis products were monitored by TLC analysis (system solvents A and B).

Flavonoidic substrates such as naringin, diosmin, rutin, hesperidin, neohesperidin dihydrochalcone, quericitrin, were tested. To this purpose, a 2 mM solution of each compound prepared in a final volume of 1 mL of 50 mM Na-phosphate buffer pH 7 was incubated at 35 °C in the presence of 100 μL of PP1Y crude extract obtained from cells grown in PPMM (3.72 mg of total protein/mL) for 24 h. Reactions were checked over time by TLC analysis (solvent system A).

Rutinose hydrolysis was also investigated. In this case, 0.5 mL of a solution containing 6 mM rutinose, 0.25 mL of PP1Y crude extract in 50 mM of Tris/HCl buffer at pH 8.5 was incubated at 35 °C under magnetic stirring and monitored by TLC analysis (solvent system A) for 24 h.

In all experiments, TLC standard solutions of pure reagents and products were used for comparison.

### 2.5. α-L-rhamnosidase activity assay

α-RHA activity was determined using *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NPR) as substrate. The assay was performed in 0.5 mL of 50 mM potassium acetate buffer pH 5.5, containing a variable amount of PP1Y crude extract and *p*NPR at a final concentration of 0.28 mM. The assay mixture was incubated for 30 min at room temperature; afterwards, 0.5 mL of a 1 M sodium carbonate solution were added and the sample absorbance was recorded spectrophotometrically at 405 nm ($\varepsilon_{405}$ = 0.0182 $\mu M^{-1} cm^{-1}$). One milliunit of enzymatic activity was defined as the amount of enzyme that releases 1 nmol of *p*-nitrophenol per min.

### 2.6. Induction of α-L-rhamnosidase activity

To evaluate the possible influence of naringin on the expression of the intracellular α-rhamnosidase activity of the strain PP1Y, we compared the specific activity in cell extracts obtained from growths in PPMM to which increasing concentrations of naringin were added. To this purpose, four 250 mL-Erlenmeyer flasks were prepared, each containing 250 mL of PPMM medium and 0.4% glutamic acid as unique carbon and energy source. Variable amounts of naringin were added to autoclaved media, in order to have final concentrations of 0.1–0.2–0.3 mM. The four flasks were inoculated with an overnight culture of PP1Y in LB, as described in paragraph 2.2. The initial cell concentrations in the flasks were comparable and in the range 0.02–0.04 $OD_{600}$/mL.

### 2.7. Influence of pH and organic solvents on α-L-rhamnosidase and β-glucosidase activities

In experiments of pH monitoring, 2 mM solutions of naringin were prepared in HCl/KCl, Na-acetate, Na-phosphate, Tris/HCl, Na-carbonate buffers, in order to cover the pH range between 2.2 and 10.9 (Table 2). 100 μL of PP1Y crude extract were added to 0.5 mL of each solution and incubated at 35 °C under magnetic stirring. Reactions were checked every 20 min by TLC analyses (solvent system A) and naringin consumption was evaluated by using solutions of pure naringin, narigenin, rhamnose and glucose as chromatographic standards. Blank reactions at different pHs were performed to evaluate the chemical degradation of the flavanone glycoside, occurring preferentially in strongly basic conditions.

Similarly, solutions of 2 mM naringin in 50 mM Tris/HCl buffer pH 8.5 containing 1, 10 and 50% v/v of DMSO or $CH_3CN$ (Table 2) were placed under constant agitation at 35 °C in the presence of 200 μL of PP1Y crude enzymatic extract (0.74 mg total protein)/mL of reaction. As previously reported, reactions were monitored by TLC analysis (solvent system A).

### 2.8. Stereochemical course of α-L-rhamnosidase activity in hydrolysis reactions

To the purpose, 800 μL of a 6 mM *p*NPR solution prepared in 50 mM Tris/HCl buffer pH 7 was freeze-dried, re-suspended three times in $D_2O$ and re-freeze-dried to exchange the $^1H$ atoms involved in labile linkages for $^2H$ ones. A similar procedure of proton isotope exchange was applied to 200 μL of PP1Y crude extract. Just prior to the $^1H$-NMR experiment, the previously exchanged *p*NPR

**Table 2**
The influence of pH and organic solvents presence on naringin hydrolysis by α-RHA activity in PP1Y crude protein extract.

| pH values | Naringin hydrolysis | | | |
|---|---|---|---|---|
| | Time (min) | | | |
| | 60 | 120 | 140 | 180 |
| 2.2 | – | – | – | – |
| 3.6 | – | – | – | – |
| 5.4 | – | – | – | – |
| 5.9 | – | – | – | –/+ |
| 6.1 | – | –/+ | –/+ | + |
| 7.2 | –/+ | + | ++ | ++ |
| 8.1 | – | ++ | ++ | ++ |
| 8.3 | + | ++ | +++ | +++ |
| **8.5** | + | +++ | +++ | +++ |
| 8.8 | + | ++ | +++ | +++ |
| 9.1[a] | – | – | + | + |
| 9.9[a] | – | – | + | + |
| 10.9[a] | – | – | + | + |
| % of organic solvent | | | | |
| DMSO 1% | + | ++ | ++ | +++ |
| **DMSO 10%** | + | + | ++ | +++ |
| DMSO 50% | – | – | – | – |
| $CH_3CN$ 1% | – | – | – | –/+ |
| $CH_3CN$ 10% | – | – | – | – |
| $CH_3CN$ 50% | – | – | – | – |

*Note*: – no hydrolysis; –/+: percentage of hydrolysis below 10%; +: 10–30% of hydrolysis; ++: 30–70% of hydrolysis; +++: percentage of hydrolysis higher than 70%.

[a] In these conditions a partial chemical degradation of naringin was observed which was suggested by the presence on TLC, along with the disappearance of the substrate, of a product more polar than naringin and the absence of rhamnose and glucose spots, thus confirming the absence of an enzymatic hydrolysis.

solution was dissolved in 0.75 mL of $D_2O$, equilibrated at 35 °C in a NMR tube inside the spectrometer probe, and an initial spectrum of solution without enzyme was recorded ($T_0$). The aliquot of freeze-dried crude extract was suspended in 50 μL of $D_2O$ and added to the solution. Starting from that moment ($T_0$ with enzyme), the stereochemical course of hydrolysis was followed by recording $^1$H-NMR spectra at close intervals during the incubation time (from 0 to 60 min).

A 6 mM solution of free rhamnose dissolved in 50 mM Tris/HCl buffer pH 7 was treated and monitored by $^1$H-NMR experiments as previously reported.

### 2.9. Bioconversion studies followed by $^1$H-NMR analyses

*p*NPR, naringin, rutin and neohesperidin dihydrochalcone bioconversions were performed and compared by $^1$H-NMR spectroscopic analyses.

In particular, 11.4 μmol of each substrate were dissolved in 1.9 mL of Na-phosphate buffer (50 mM, pH 8.5 or pH 7) containing 1 mL of PP1Y crude extract. Reactions were incubated at 35 °C, under constant magnetic stirring for 3 h. Aliquots of 380 μL were withdrawn from each reaction at different time intervals from 0 to 173 min. Enzymatic reactions were stopped by boiling at 100 °C for 2 min and samples were freeze-dried. Collected aliquots were dissolved in MeOD or $D_2O$, as for *p*NPR, and analyzed by $^1$H-NMR experiments. These reactions were also monitored by TLC analysis (system solvent A). These reactions were also monitored by TLC analysis (system solvent A).

### 2.10. Prunin production

The reaction was performed using 2 mL of PP1Y crude extract which were previously lyophilized and then resuspended in a 1 mL solution of Na-phosphate buffer (50 mM, pH 8.5) containing 10% of DMSO. 72.5 mg of naringin (0.125 mmol) were added and the sample was incubated at 35 °C, under magnetic stirring. Reaction was stopped after 72 h by heating the sample at 100 °C for 2 min and prunin was isolated by silica gel purification with EtOAc:MeOH 9:1 (v/v) as mobile phase. 1D and 2D NMR analyses confirmed its structure.

## 3. Results and Discussion

### 3.1. Glycosidase activities screening

Glycosidase activities in crude protein extract of *Novosphingobium* sp. PP1Y cells collected after a 24 h growth in PPMM medium (Section 2.2) were preliminary investigated for their hydrolytic and transglycosylation potentials, as described in Section 2.4. Reaction products were analyzed over time by TLC analysis using solvent system A Section 2.1, and the results were reported in Table 1. Data indicated that α-L-rhamnosidase and β-glycosyl hydrolases activities resulted the most abundant ones. After 3 h, 30–70% of PNP-α-L-Rha*p* and 10–30% of PNP-β-D-Glc*p*, PNP-β-D-Gal*p*, and PNP-β-D-Xyl*p* were hydrolyzed. Hydrolysis reactions were allowed to proceed for 24 h. At this time, an almost complete conversion of PNP-α-L-Rha*p* was observed.

Transglycosylation reactions resulted more interesting by using PNP-β-glycosides as substrates, suggesting a better attitude of β-glycosyl hydrolases to transfer monosaccharidic units in self-condensation reactions than the α-L-rhamnosidase activity; aryl-β-oligosaccharides were present in the reaction media up to 24 h.

Polysaccharides and oligosaccharides were also tested as possible substrates for the enzymatic activities present in PP1Y crude extracts. Products formation was monitored by TLC analysis (system solvents A and B, Section 2.1), using pure compounds as chromatographic standards. After 24 h of reaction, curdlan and laminarin were only slightly consumed whereas laminaripentaose resulted the best hydrolyzed oligosaccharide, resulting in the major production of glucose; cellobiose, instead, was not a good substrate (data not shown).

α-glucans like pullulan, amylopectin, starch and maltose were partially or almost totally hydrolyzed to glucose, maltose or larger oligosaccharides, suggesting the possible presence of α-glucanase activities (data not shown).

These preliminary data confirmed the biotechnological potential of PP1Y strain as a source of a wide array of enzymatic activities that can be used for the modification of carbohydrates and glycoconjugates.

### 3.2. Hydrolytic behaviour of PP1Y crude extract enzymes on flavonoids: description, pH dependence and tolerance to organic solvents

The simultaneous presence in PP1Y crude extract of α-RHA and β-glucosidase activities prompted us to investigate the possibility to hydrolyze selected flavonoidic compounds, having in their chemical structure both α-rhamnose and β-glucose units, such as naringin, diosmin, rutin, hesperidin, neohesperidin dihydrochalcone and quericitrin (Fig. 1). It is worth to note that chemical modification of these compounds, which are endowed with therapeutic potential [12] is important to modulate their biological activity.

In the reaction conditions reported in Section 2.4, after 3 h of incubation, complete conversion of naringin, rutin and neohesperidin dihydrochalcone was detected by TLC analysis (system solvent A, Fig. 2): spots of rhamnose, glucose and the corresponding aglycone were observed for each reaction.

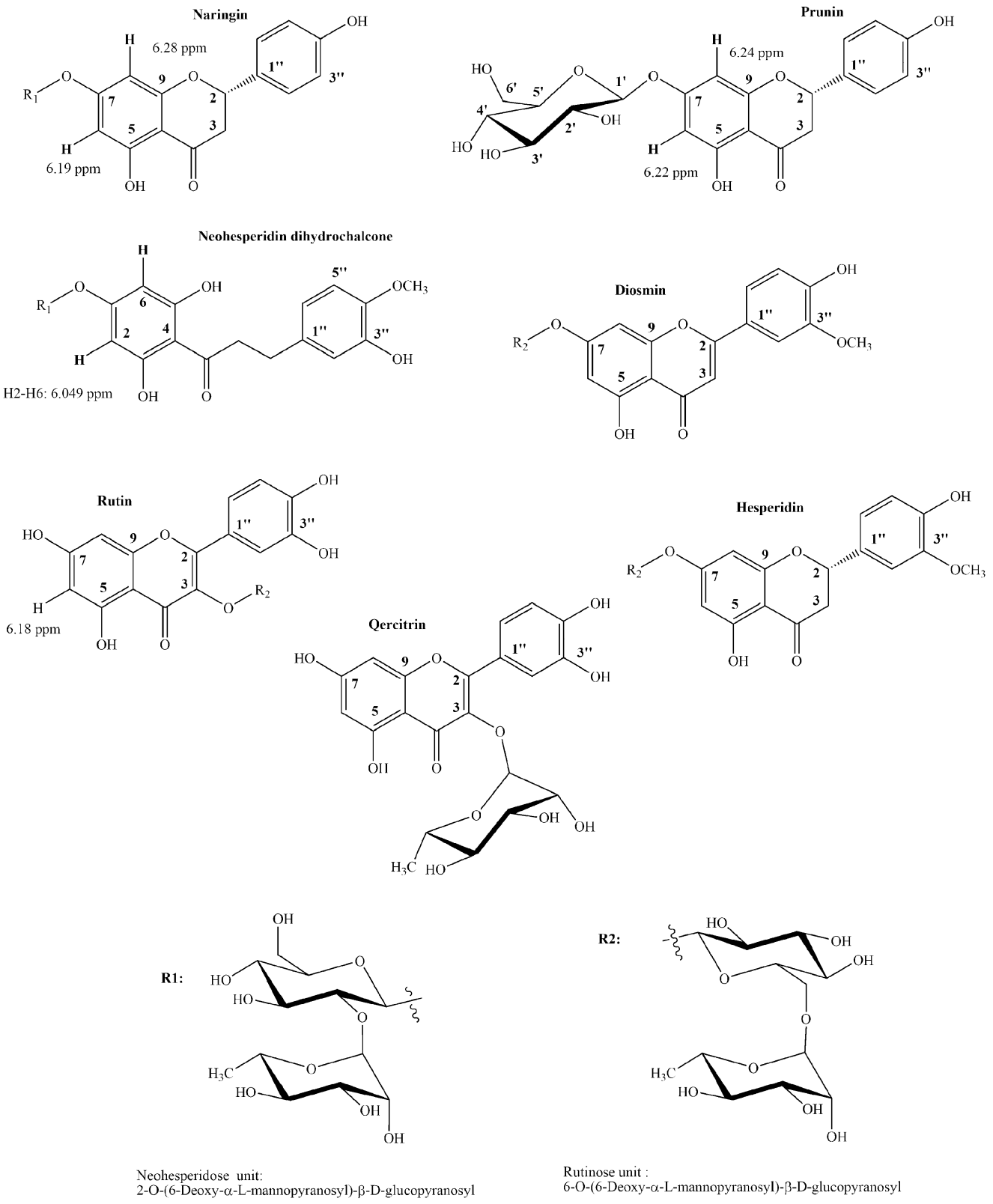


**Fig. 1.** Chemical structure of flavonoidic compounds used as substrates. Chemical shift values of signals that are integrated in bioconversion reactions are reported.

On the other hand, diosmin, hesperidin and quericitrin were not hydrolyzed; after 24 h as observed by TLC investigations (system solvent A) rhamnose and glucose spots, at Rf 0.56 and 0.3 respectively, were absent on TLC plates of diosmin and hesperidin reactions. Quericitrin was so poorly consumed (yield below 3%) that its hydrolysis was considered negligible; indeed, a very scarce presence of quercetin and rhamnose, at Rf 0.88 and 0.56 respectively, was observed.

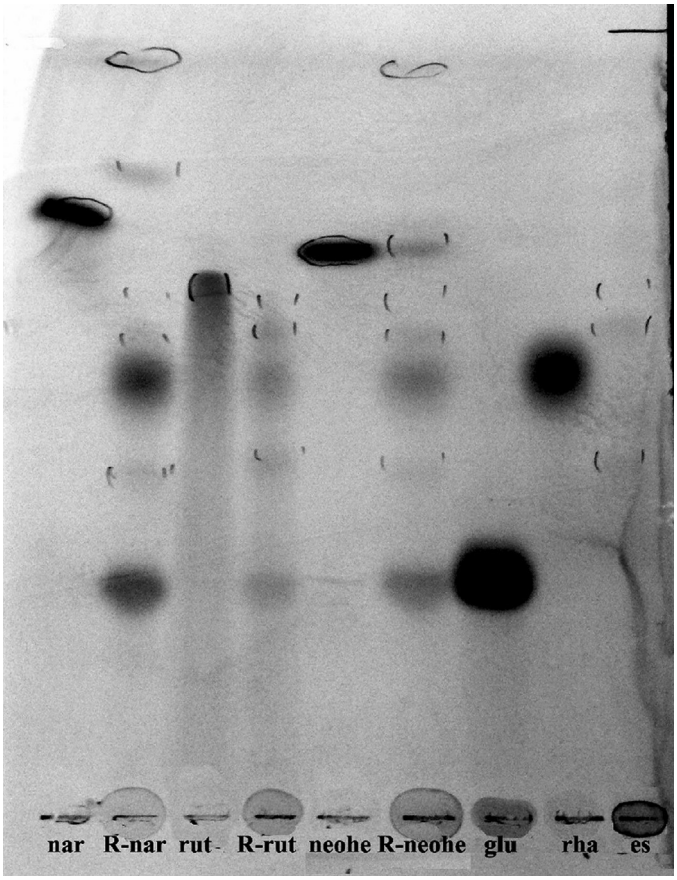


**Fig. 2.** Flavonoids hydrolysis followed by TLC (system solvent A). TLC standard solutions at a concentration of 2 mM were used. Nar: naringin, R-nar: naringin reaction; rut: rutin, R-rut: rutin reaction; neohe: nehoesperidin dihydrochalcone; R-neohe: nehoesperidin dihydrochalcone reaction; glu: glucose, rha: rhamnose; es: enzymatic solution.

These data gave some indication on the substrate preference of the α-RHA activity expressed by strain PP1Y under our experimental conditions.

Diosmin and hesperidin (Fig. 1), which contained the rutinose disaccharide (α-L-Rhamnopyranosyl-(1 → 6)-β-D-glucopyranose) linked to the phenolic portion of the molecules, were not hydrolyzed. Rutin, whose rutinoside portion was linked to the quercetin 3-enol position, was instead converted. Under similar reaction conditions, flavonoids with the neohesperidose disaccharide 2-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranose) linked to the phenolic portion (naringin and neohesperidin dihydrochalcone) were completely transformed.

These data suggested that the α-RHA activity was able to hydrolyze both α,1-2 and α,1-6 interglycosidic linkages. Most of the α-RHAs reported in literature are mainly active on α-1, 2 glycosidic linkages, to a smaller extent on α-1,6 linkages, and even less on other glycosidic bonds [30]. Interestingly, the enzymatic activity expressed by strain PP1Y was specific for α,1-2 interglycosidic bonds when the disaccharide unit of rutinose was linked to the phenolic hydroxyl groups. In fact, diosmin and hesperidin, which shared the presence of a α,1-6 interglycosidic bond in the disaccharide units linked to a phenolic site, were not hydrolyzed. The α,1-6 interglycosidic bond was instead hydrolyzed when the rutinose unit was linked to 3-enol position of rutin. This hydrolytic behaviour might be a consequence of a different steric effect deriving from the attachment of the disaccharidic units to different sites of the aglycons [3].

Free rutinose disaccharide was also examined as a possible substrate, resulting in a very low yield of products (data not shown), thus suggesting the importance of the aromatic portion of the flavonoidic moiety in the recognition mechanism of the enzymes involved.

Moreover, quercitrin, the quercetin 3-α-L-rhamnoside, in which L-rhamnose is directly linked to the aglycon, was not hydrolyzed. It should be noted that a marked preference for the of PNP-α-L-Rha*p* (from now on indicated as *p*NPR) in comparison to quercitrin, robonin or rutin has been reported also for other characterized α-RHAs [3].

The solubility of flavonoids (typically polyphenols) is pH-dependent and generally increases in alkaline media, which are often not compatible with the enzymatic activities used in bioprocesses. Using naringin as the most water-soluble available flavonoid (0.5 g/L at 20 °C) [31], the influence of pH and organic solvents on the hydrolytic behaviour of the enzymes present in PP1Y crude extracts was investigated.

Results of these experiments were reported in Table 2. A higher yield of naringin hydrolysis products was obtained at alkaline pH values ranging from 7.2 to 8.8 (Na-phosphate and Tris/HCl buffers) and an optimum pH value of 8.5 was determined. In this case, after 3 h of reaction, substrate spots were absent on TLC plates, thus suggesting a substrate complete depletion.

Within a reaction time of 3 h, TLC analyses of reaction systems in the pH range 8.2–8.8 showed the presence of a product with an Rf value higher than that of naringin. Free glucose and the narigenin aglycone were almost absent.

These data suggested the occurrence at these basic pH values of a marked decrease of the β-glucosidase activity, which should be responsible for the production of free glucose and narigenin aglycone, and the permanence of the α-RHA activity. This hypothesis was confirmed by the concomitant accumulation of a compound which was identified as prunin (Fig. 1), the de-rhamnosylated product of naringin, a molecule of biotechnological importance endowed with anti-inflammatory and antiviral activity against DNA/RNA viruses [32,33].

It is worth to note that bacterial α-RHAs having a pH optimum value at 8–8.5 have not been often reported in literature [30].

Prunin structure (Fig. 1) was confirmed by a 2D NMR spectroscopic investigation. The presence of the β-glucose residue was established by the value of the anomeric position signal in $^{13}$C NMR spectrum (in MeOD) at 101.25 ppm which resulted correlated in HSQC spectrum to the diastereoisomeric anomeric protons H-1′ at 5.01 ($J$ = 6.90 Hz), and 4.99 ($J$ = 6.90 Hz) ppm. The following signals (in ppm): C-2′ 74.66 (H-2′: 3.48), C-3′ 77.82 (H-3′: 3.47), C-4′ 71.16 (H-4′: 3.41), C-5′ 78.28 (H-5′: 3.42), C-6′ 62.34 (H-6′-H-6″: 3.90, 3.72) confirmed the glucose structure of prunin saccharidic portion. Aglycone structure was confirmed by the $^{13}$C spectrum signals at 80.69 ppm (C-2), 44.19 ppm (C-3), 166.98 and 167.05 ppm (C-7, the glycosylation site), 129.12 ppm (C-2″-C6″), 116.33 ppm (C-3″-C5″) and in $^{1}$H-NMR spectrum by the proton signals at 7.35 ppm (H-2″-H6″) and 6.85 ppm (H-3″-H5″). The other signals in the spectra were in agreement with data previously reported [34]. The increase of the α-RHA activity present in PP1Y protein extracts observed at alkaline pH values was also confirmed by the *p*NPR bioconversion study followed by NMR analysis reported in Section 3.3.

The pH variation is not the unique strategy that has been used to overcome the poor hydrosolubility of flavonoids; the use of water-miscible organic co-solvents, in fact, has also been suggested [31]. However, it should be noted that this strategy has several drawbacks, such as toxicity problems derived from the use of an organic solvent in a bioprocess, and the possible decrease in the enzymatic activities employed [35].

In this study, the influence of the presence of organic solvents on the hydrolysis of naringin was evaluated by TLC analysis of

reaction mixtures containing from 1 to 50% v/v DMSO or $CH_3CN$ (Table 2). After 90 min, in the presence of 1% and 10% of DMSO, naringin was almost totally consumed and traces of prunin were detected, suggesting a tolerance of the α-RHA activity to the presence of organic solvents along with a detrimental effect of DMSO on the β-glucosidase activity, as free glucose was almost absent in the reaction mixtures.

Finally, in the presence of either 50% DMSO or any concentration of $CH_3CN$, de-rhamnosylation and de-glucosylation reactions did not proceed even after 10 h.

Using the apparent best experimental conditions reported in Table 2 (pH value of 8.5 and 10% DMSO), and starting from a 125 mM naringin solution (Section 2.10), prunin was produced with a yield of 32.1%, corresponding to a final concentration of 0.02 M and an amount of 17.4 mg of recovered product.

Furthermore, free L-rhamnose was produced as a secondary product at a concentration of 6 g/L. The yield of products obtained in this experiment was comparable to that reported for a recombinant α-RHA from *Clostridium stercorarium* used for the hydrolysis of citrus peel waste naringin [7] and to other similar processes [30].

### 3.3. Bioconversion experiments followed by NMR

To confirm and better detail the observations on the substrate preference of the α-RHA activity in *Novosphingobium* sp. PP1Y crude protein extracts described in the previous paragraph, bioconversion reactions of *p*NPR, naringin, rutin and neohesperidin dihydrochalcone were performed using PP1Y cell extracts (Section 2.9), and monitored over time by $^1$H-NMR.

Based on diagnostic signals of reagents and products for each reaction, substrate conversions were calculated by measuring the percentage ratio between the integrals of diagnostic signals of the products and the sum of the integration values of selected signals of reagents and final products. Results were reported in Fig. 3.

The highest yield of bioconversion in a shortest time was obtained with rutin. After 20 min 76.2% of the substrate was consumed. At the same time, 30–35% of *p*NPR, 26% of naringin, and 7% of neohesperidin dihydrochalcone were transformed.

After 173 min, naringin and neohesperidin dihydrochalcone reached bioconversions yields (95.6 and 86%, respectively) similar to rutin (89.4%). Under these conditions, 3.1–3.3 g/L of flavonoids were converted to products and, although the flavonoidic substrates were not totally hydrolyzed to aglycone, free L-rhamnose was released into the reaction media at a concentration of about 0.77–0.86 g/L.

It should be added that *p*NPR bioconversion was performed not only at pH 8.5 but also at pH 7, looking for a possible pH influence on PP1Y α-RHA activity (see Section 3.2). In the experiment in which naringin was used as a substrate, the reaction course was evaluated by comparing in $^1$H-NMR spectra the integral values of two aromatic protons singlets (Fig. 1) H6–H8 in ring B at 6.19 ppm and 6.28 ppm, which were attributed a value of 2, to the integration of the corresponding protons of the final product narigenin (aglycon) at 5.78 and 5.8 ppm. $^1$H-NMR spectra in MeOD of naringin, narigenin and prunin (H6–H8 signals at 6.22–6.24, respectively) of pure solutions confirmed the identity of narigenin integrated signals.

When rutin was used as substrate, aromatic proton signal H6 of the reagent at 6.18 ppm was selected as reference for the integration (Fig. 1). As for the reaction products, an aromatic signal at 6.13 ppm, which increased over time, was selected for evaluating rutin bioconversion.

After 10 min of reaction, the anomeric proton of β-glucose in rutin at 5.07 ppm (d, $J$ = 7.61 Hz) almost disappeared (72% of conversion) and an anomeric proton signal at 5.035 ppm (d, $J$ = 8.07 Hz), possibly corresponding to the β-anomeric proton of quercetin 3-β-glucoside, appeared; anomeric signals of free glucose were absent.

On the other hand, in $^1$H-NMR spectra at 173 min, anomeric protons region was more crowded with signals due to the presence of free glucose (α-proton at 5.13 ppm $J$ = 3.91 Hz; β-proton at 4.50 ppm, $J$ = 7.8 Hz) and a signal at 5.023 ppm of free rhamnose α-proton (with the β-anomeric proton overlapped by the solvent residual water signal).

These data suggested a fast hydrolysis of the interglycosidic linkage inside the rutinose unit (α-L-Rha-(1 → 6)-β-D-Glu) with the production of free rhamnose at the beginning of the reaction, as confirmed by the presence of a further methyl signal at 1.27 ppm after 10 min of reaction, corresponding to protons at position 6 of free rhamnose, and the subsequent release of flavonol quercetin and glucose.

The Neohesperidin dihydrochalcone bioconversion was evaluated by $^1$H-NMR using the aromatic signals at 6.049 ppm, corresponding to two overlapped aromatic protons H2 and H6 (Fig. 1) linked to the saccharidic portion, as the reagent reference signals, with a integral value of 1, and a signal at 6.077 ppm, belonging to the reaction product.

An evaluation of the anomeric signals in the range 4.45:5.2 ppm of $^1$H-NMR experiment showed, after 173 min of reaction, the presence of the α-proton signal of linked rhamnose at 5.28 ppm and the anomeric signal of β-glucose at 5.065 ppm (d, $J$ = 7.63 Hz) which belonged to the substrate, the α- and β-anomeric protons signals of free glucose at 5.13 ppm (d, $J$ = 3.81 Hz) and 4.50 ppm (d, $J$ = 7.63 Hz) respectively. Moreover, an intense signal at 5.03 ppm of the α-anomeric proton of free rhamnose, a signal at 4.95 ppm (d, $J$ = 7.31 Hz) almost as intense as the previous signal, which was partially covered by residual $H_2O$ signal in MeOD, corresponding to β-glucose H1 signal of neohesperidin dihydrochalcone without rhamnose were also observed. On the contrary, the free rhamnose β-anomeric signal was totally overlapped by residual $H_2O$ signal in MeOD.

The high intensity of the α-anomeric proton of free rhamnose and the scarce presence of anomeric signals of free glucose, led us to suppose that the main reaction product was the de-rhamnosylated neohesperidin dihydrochalcone.

In experiments using *p*NPR as substrate, the integral value of aromatic proton signal at 7.23 ppm was set to 1 and the signal of *p*-nitrophenol at 6.69 ppm produced by the hydrolysis of glycosidic linkage was selected for the assessment of *p*NPR bioconversion. This bioconversion was performed at two pH values; as reported in Fig. 2, after 60 min, the conversion of *p*NPR was of about 61% at pH 7, whereas at higher pH it reached 97%, thus confirming the trend of the experiments in which naringin was used as substrate described in Section 3.2.

### 3.4. Induction of α-RHA activity in PP1Y cell extracts

α-RHA activity can be specifically induced by several flavonoids [36,37] among which naringin is of particular interest because this compound can be abundantly recovered in citrus solid wastes. These wastes are quite interesting because they can be used either as an energetic source for growing microorganisms, due to their content in carbon and other nutrient components, and/or as a specific inducer for the biosynthesis of glycosidases due to the presence of flavonoids. It goes without saying that the use of citrus waste as starting material in bioconversion processes is an advantage for industrial companies because, among others, it decreases the expenses for waste disposal. The possible role of naringin as an inducer of the α-RHA activity [37] in PP1Y protein extracts was investigated using PP1Y cells grown in PPMM medium supplemented with naringin at different initial concentrations up to 0.3 mM, as described in detail in Section 2.6.

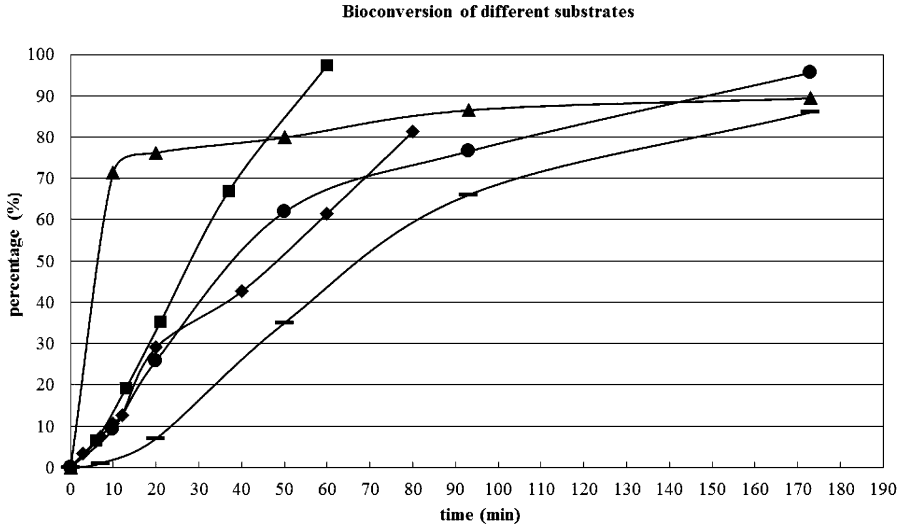


**Fig. 3.** Bioconversions of naringin ●, rutin ▲, neohesperidin dihydrochalcone —, *p*-nitrophenyl-α-L-rhamnopyranoside at pH 7 ♦ and 8.5 ■.

The effect of naringin could not be evaluated at concentrations higher than 0.3 mM due to its poor solubility under the experimental conditions used.

The effect of naringin could not be investigated at concentration values higher than 0.3 mM due to its poor solubility under the experimental conditions used. Results were reported in Fig. 4 and confirmed that naringin acts as an inducer leading, at a concentration of 0.3 mM, to a maximum 5-fold increase of the α-RHA activity (15.2 mU/mg) detected in the crude extract of PP1Y cells. These data encourage a future biotechnological use of PP1Y strain and of its α-RHA activity for the use of agro-industry vegetable residues.

### *3.5. Stereochemical course of α-RHA activity in hydrolysis reactions*

To shed light on the type of enzymatic mechanism of PP1Y α-RHA activity, hydrolysis products of *p*NPR were analyzed by recording $^{1}$H-NMR spectra over time. The integration of the anomeric proton signals of reagent and products was investigated; the analysis was performed taking into account that the anomeric proton of free rhamnose shows peaks at 5.06 ppm (α-anomeric form) and 4.83 ppm (β-anomeric form). Results were reported in Fig. 5, where the reaction time-dependent changes in amplitude of H-1 signals from substrate, α-1(S) (5.68 ppm), and products, α-1(P), and β-1(P), were plotted as % of total anomeric (H-1) signals. At $t_0$, the only α-anomeric proton signal present was that of *p*NPR at 5.68 ppm (α-1 (S)). During the first minutes of reaction a signal at 4.83 ppm, corresponding to the β-L-Rha*p* anomeric proton (β-1 (P)), appeared only slightly earlier than the appearance of a further signal at 5.06 ppm, corresponding to the α-L-Rha*p* anomeric proton (α-1 (P)).

These signals increased in intensity over time, while the anomeric signal of *p*NPR decreased concomitantly, as it is evident from Fig. 5. NMR analysis did not gave evidence for the onset of mutarotation equilibration during the first minutes of reaction.

Later in the incubation, mutarotation of the initially released β-L-Rha*p* increased the ratio between the intensities of the α- and β-anomer protons signals. After 7 min, at a conversion percentage of 7.5%, the relative intensities of the α- and β-anomer resonances (calculated from peaks integration) were in a ratio of about 1:3 (25% of the α- and 75% for the β-anomer). After 20 min, at a conversion percentage of 29%, the α-/β-anomer ratio was of about 1:2 (32% of the α- and 68% for the β-anomer). After 60 min, at a conversion percentage of 61.5%, the α/β ratio value increased to about 1:1. This ratio was kept constant also after 80 min, when the integration values showed that the yield of *p*NPR consumption was of

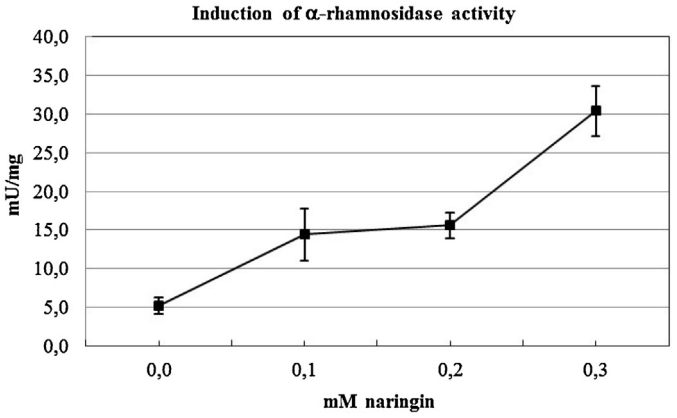


**Fig. 4.** α-RHA specific activity in the cell extract of strain PP1Y is expressed as mU/mg of total proteins (*Y*-axis). Specific activity is reported as a function of the concentration of naringin initially added to the growth medium.

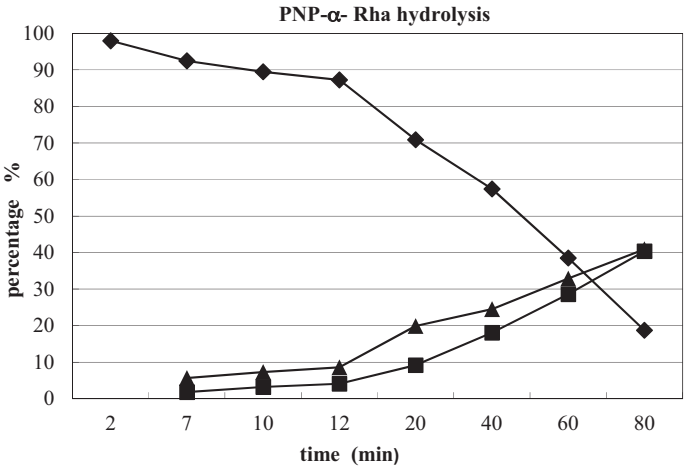


**Fig. 5.** % of total anomeric signals in *p*-nitrophenyl-α-L-rhamnopyranoside hydrolysis reaction: signals from substrate, α-1(S) ♦, and products, α-1 (P) ■, and β-1 (P) ▲, are plotted as % of total anomeric (H-1) signals.

about 86%, and was maintained up to 18 h, thus representing the equilibrium value of the ratio between both anomeric forms of L-rhamnopyranose. A solution of free rhamnose used in the same experimental conditions and monitored by $^1$H-NMR reached the mutarotational equilibrium after 1–2 min and the concentrations ratio of α- and β-rhamnose was 1:1.

The kinetic behaviour suggested by these experiments for PP1Y α-RHA activity was an inverting mechanism of hydrolysis in which β-rhamnose was formed from the α-rhamnose via a single displacement mechanism and was then spontaneously converted by mutarotation to the α-anomeric form.

## 4. Conclusions

Bacterial glycosyl hydrolases are the focus of an increasing number of researches because of their key role in fundamental biological processes and their biotechnological applications. Here, the attention was focused on a novel α-RHA activity from *Novosphingobium* sp. PP1Y, which resulted interesting for its alkaline pH optimum and moderate tolerance to organic solvents.

This α-RHA was used, even without any further purification of PP1Y crude protein extract, for the bioconversion of flavonoids useful for the food and pharmaceutical industries. However, a future detailed biochemical characterization of this enzyme is now crucial for a better understanding of its effective biotechnological potential.

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