PRODUCTION OF ENANTIOMERICALLY PURE D-PHENYLGLYCINE USING *Pseudomonas aeruginosa* 10145 AS BIOCATALYST

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Abstract - Different bacterial strains were screened to detect nitrilase and/or nitrile hidratase/amidase activities towards benzonitrile, to be used as biocatalyst to produce enantiomerically pure non-proteinogenic amino acids using amino nitriles as starting material. The best biocatalyst found was *Pseudomonas aeruginosa* 10145, which showed high enzyme activities. Whole cells were used as catalyst for the transformation of 2-phenyl-2-amino-acetonitrile for the corresponding D-phenylglycine. The percentage conversion was followed by chiral HPLC. After 1 hour reaction 18% of 2-phenyl-2-amino-acetonitrile was converted into D-phenylglycine with an enantiomeric excess of over 95%. When an inducer was added to the media, an increase in nitrile hydrolyzing activities was detected, hence leading to total conversion of (R)-2-phenyl-2-amino-acetonitrile to the corresponding amino acid in 30 min reaction. The isolated yield of the target product was 50% and its characterization was performed by polarimetry, chiral HPLC, IR-FT spectroscopy and GC-MS.
Keywords: Nitrilase, Amidase; Nitrile hydratase; Amino acids; Kinetic resolution.

INTRODUCTION

The continuous search for microorganisms capable of carrying out chemical transformations has intensified in recent years. Reduction (Medina, Stefani and Brandelli, 2006; Lacerda *et al*, 2006a; Lacerda *et al*, 2006b; Ribeiro *et al*, 2006), Hydrolysis (Wang *et al*, 2006); Oxidation (Rozenbaum *et al*, 2006; Durand *et al*, 2006; Toniazzo *et al*, 2006; Toniazzo *et al*, 2005; Santos *et al*, 2004; Santos *et al*, 2003) and other chemical transformations (Xie and West, 2006; Paggy, Rodriguez and Fay, 2005) have been successfully undertaken by several groups.

Chiral synthesis, particularly devoted to the production of enantiomerically pure non-proteinogenic amino acids, has received special attention in the pharmaceutical and chemical industries (Ager *et al.*, 2001). In this sense, some amino acids and derivatives have shown pharmacological activities (LeTiran, Stables and Kohn, 2001) and can be incorporated into the backbone of peptide or peptide mimetic compounds to be used as protease inhibitors (Kondekar, Kandula, and Kumar, 2004; Matsumoto *et al*, 2000; Markoff, Falgout and Chang, 1997).

Amino nitrile compounds have been used as starting material to obtain amino acids, submitting the nitrile group to enzymatic hydrolysis, Scheme 1, (Alonso, Oestreicher and Antunes, 2007; Chaplin *et al*, 2004). Many bacteria, fungi and vegetal species have been reported as nitrile hydrolyzing catalysts (Kaul *et al.*, 2004; Effenberger and Oβwald, 2001; Webster, Ramsden, Hughes, 2001; Dadd *et al,* 2001,

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Hoyle, Bunch and Knowles, 1998; Layh, and Willetts, 1998; Battistel, Bernardi, and Maestri, 1997; Gradley and Knowles, 1994; Macadam, and Knowles, 1985). These cell activities are attributed to the presence of nitrilases. Nitrilase (E.C. 3.5.5.1) is an important enzyme of the hydrolase family class, in which catalysis is attributed to a triad formed by glutamic acid, lysine and a nucleophilic cysteine (Brenner, 2002). These enzymes hydrolyze the nitrile group to the corresponding acid and ammonia (Kobayashi, and Shimizu, 1994). Nitriles may also follow a sequential bi-enzymatic pathway leading to the same acid. This enzymatic route involves the sequential action of nitrile hydratase (E.C. 4.2.1.84) and amidase (E.C. 3.5.14). The hydrolytic conversion of nitriles catalyzed by nitrile hydratase/amidase system occurs in two well established steps. Initially the nitrile group is converted to the corresponding amide by nitrile hydratase, in most cases without distinction between the two isomers of a racemic substrate. Then the amide group is hydrolyzed to the corresponding acid and ammonia, by the action of amidase. Normally, these enzymes discriminate between the two amide isomers produced by the hydration of the nitrile (Wang and Lin, 2001). Nitrile hydratase activity comprises either one non-heme iron (III) or a non-corrinoid cobalt (III) (Mascharak, 2002), and amidase activity is attributed to the presence of a nucleophilic serine.
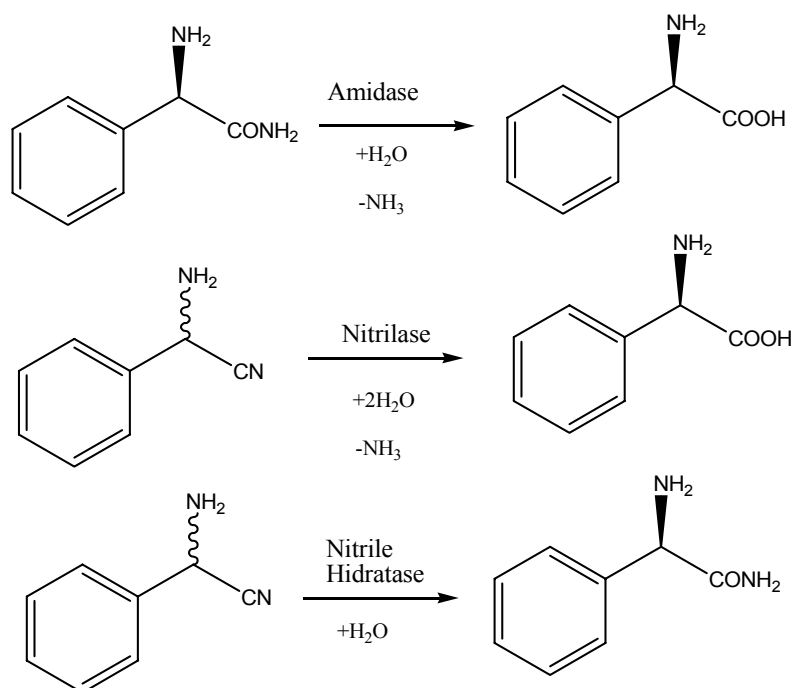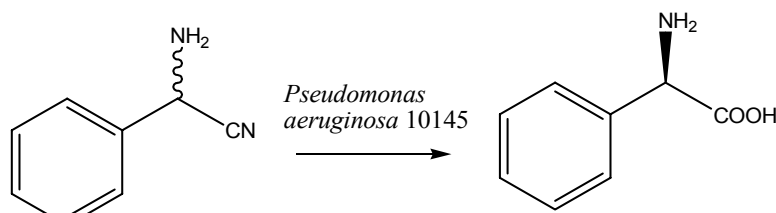

Scheme 1

The use of whole cells submitted or not to non-proliferative conditions, has been extensively studied for producing a wide range of compounds. *Bradyrhizobium* strains were used to obtain indole-3-acetic acid from the corresponding nitrile in phosphate buffer (Vega-Hernández, Léon-Barrios, Pérez-Galdona, 2002). Dinitrile, aliphatic and cyclic nitriles were transformed using free and immobilized cells of a thermophilic *Bacillus* sp. (Graham *et al.*, 2000). Racemic phenylaminoacetonitrile, ibuprofen nitrile and methylbutyronitriles were hydrolyzed to the corresponding acids, with high enantiomeric excess, using resting cells of different *Rhodococcus* strains (Effenberger, and Harper, 1998; Kaul *et al.*, 2004).

In the present work, our main goal was to isolate new bacteria strains with enantioselective nitrile hydrolyzing activities against aromatic nitriles and arylacetonitriles. The most promising biocatalyst selected (*Pseudomonas aeruginosa* 10145) was incubated with racemic 2-phenyl-2-amino-acetonitrile, to produce enantiomerically pure D-phenylglycine, in short reaction times (Scheme 2).

Scheme 2

MATERIALS AND METHODS

Chemicals

Racemic 2-phenyl-2-amino-acetonitrile, racemic and enantiomerically pure phenylglycine and benzoic acid were acquired from Aldrich. Benzonitrile (technical grade) was acquired from Reagen. Other chemicals (of analytical grade) used for the preparation of culture media were purchased from Difco and BD.

Bacteria Strains

Four bacterial strains (*Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescens* and *Bacillus subtilis*) were kindly supplied by INCQS – FIOCRUZ – Rio de Janeiro – Brazil.

Cell Resuspension

The lyophilized cultures were resuspended with saline solution under aseptical conditions, with sterile NaCl 0,85% (w/v). These cell suspensions were inoculated in solid Luria-Bertani (LB) media.

Cell Maintenance

Cells were maintained in 20% glycerol and stocked at -70°C. Cells stocks were transferred to solid Luria-Bertani (LB) media (Tryptone 1.0%; yeast extract 0.5%; NaCl 1.0% and agar 2.0%) and incubated at 30°C for 24 h.

Biomass Production

Colonies from solid media were collected and incubated under orbital agitation, in 250 mL conical flasks containing 50 mL of liquid LB sterile media at 30°C, 140 rpm for 24 h. Cell growth was monitored via turbity at 570 nm.

Nitrile Hydrolysing Activity Screening

The four strains were grown in LB media as previously described. The cells were harvested by centrifugation at 4°C and 4000 rpm for 15 minutes. The biomass was resuspended with 100 mM phosphate buffer pH 7.0 and transferred to 125 mL conical flasks containing a final volume of 25 mL of the same phosphate buffer. The biomass concentration in each flask was 500 mg (wet weight) of cells per 25 mL of phosphate buffer.

To evaluate nitrile hydrolyzing activities, benzonitrile was used as a model substrate. The cells were incubated with 1.0 mM of benzonitrile, under orbital shaking (140 rpm) at 30°C. Samples were taken periodically and the cells were harvested by centrifugation. The biotransformation was evaluated by RP-HPLC, using a mobile phase containing methanol and water (70:30), flow rate at 0.8 mL/min and UV detector set at 247 nm.

Activity Induction Assays

P. aeruginosa cells were grown in LB media for 24 hours in the presence of benzonitrile 0.5% (v/v); 1.5% (v/v) and 2.5% (v/v), respectively. The addition of the inducer at the beginning of the assay led to inhibition of cell growth. To avoid this deleterious effect of benzonitrile, cells were grown in the same media for 24 hours until stationary growth phase was achieved, afterwards benzonitrile was added to the media in three different concentrations [0.025; 0.05 and 0.1% (v/v)]. Cells were incubated for 24 hours in the presence of the inducer.

Production of Enantiomerically Pure D-Phenylglycine

The biomass obtained with and without induction was harvested by centrifugation at 4°C, 4000 rpm for 15 minutes. The biomass was suspended in 25 mL

100 mM phosphate buffer pH 7.0 to a final concentration of 500 mg (wet weight) of cells per total volume of media. 2-phenyl-2-amino-acetonitrile was added to the flasks to a final concentration of 1.0 mM. and cells were incubated as previously described. An 8-fold increase of the biomass production was also performed. In this case all the previously specified numbers were proportionally increased. Samples were periodically withdrawn and the cells harvested by centrifugation. The supernatant of each sample was then analyzed by chiral-HPLC.

Characterization of Target Product

Analytical HPLC was carried out in a Shimadzu LC-10AS chromatograph. Activity screening assays were performed on a 250 mm x 4.6 mm Spherisorb ODS-2 C_{18} column, using methanol:water (70:30) as mobile phase (0.8 mL/min), the UV detector was set at 247 nm. The analysis of 2-phenyl-2-amino-acetonitrile biotransformation was performed on a 250 mm x 4.6 mm Nucleosil Chiral-1 (Macherey Nagel) column, using $CuSO_4$ 1.0 mM as mobile phase (1.0 mL/min) and the UV detector was set at 240 nm. Polarimetric analysis were conducted in a Perkin-Elmer 243B digital polarimeter, infrared spectra in a Perkin-Elmer 467 FTIR spectrometer. After silanization, the reaction product was analyzed by GCMS. To assure the identity of the reaction product an authentic sample of D-phenylglycine was also silanized and analyzed. These analyses were performed in a HP 5890 series II gas chromatograph coupled to a HP 5972 mass spectrometer, and the two products showed the same retention time and mass spectra, m/z 294, 178 (100%), 73 40%). Polarimetry showed $[\alpha]^{25}_D = -158,8$. FTIR analysis revealed the following bands of axial deformation: 3439 cm^{-1} (N-H bond of α-NH$_3^+$); 2985 and 2934 cm^{-1} (C-H bond of α-C atom); 1612 cm^{-1} (C=O bond of COOH); 1395 cm^{-1} (C-N bond); and 729 and 693 cm^{-1}, bands of angular deformation of aromatic ring C-C bonds.

RESULTS AND DISCUSSION

It was found that *Pseudomonas aeruginosa* 10145 strain was the most efficient microorganism tested for nitrile hydrolyzing activities, with a model aromatic nitrile used to detect such activities (benzonitrile). This strain converted benzonitrile to benzoic acid, with maximum yield (70% of conversion) in the shortest reaction time, approximately 5 hours, when compared with the other strains used. The three other strains studied were also able to convert benzonitrile to the benzoic acid, although substantially lower in efficiency than *P. aeruginosa* (Table 1), indicating lower nitrile hydrolyzing activities. Longer incubation times were investigated with all microorganisms, but a decrease of conversion was observed. Since this conversion decrease was parallel to an increase in turbidity, indicating cell growth, these results suggest that the acid released to the media was used as carbon and nitrogen sources together with the residual nitrile to support cell growth.

No trace of the intermediate amide benzamide was detected in these experiments, hence suggesting that the reaction may go through an enzymatic pathway (nitrilase) or by the bi-enzyme pathway constituted by nitrile hidratase/amidase activities. In the latter case, absence of the amide is explained considering a much higher amidase activity than the nitrile hydratase activity.

In view of these results *P. aeruginosa* was chosen as the most promising strain to be used in enantiomerically enriched amino acids synthesis due to its high nitrile converting activities, within shortest reaction times.

To perform the synthesis of phenylglycine, *P. aeruginosa* cells were grown in the absence and in the presence of benzonitrile [0.5; 1.5 and 2.5% (v/v)]. When benzonitrile was added to the culture media as enzymatic inductor, at the beginning of the incubation period, the turbidity of the media did not change, suggesting that benzonitrile toxicity had led to the decrease of cell viability. According to this observation, cells were grown in the absence of the inducer using LB liquid media as growth broth. The biomass then obtained, was able to convert 2-phenyl-2-amino-acetonitrile to the corresponding D-phenylglycine, with 18% of maximum yield in 1 hour of incubation and approximately 95% ee (Figure 1). As the time of incubation became longer, the non converted substrate as well as the amino acid decreased their concentrations in the media, with a concomitant turbidity increase. These results suggest that this non-proliferant condition forced the cells to use both 2-phenyl-2-amino-acetonitrile and the amino acid released to the media, as sole nitrogen and carbon source to maintain cell viability.

Table 1: Screening of Nitrile Converting Microorganisms by the conversion of Benzonitrile (1mM) to Benzoic Acid – Isolated Yields

Time	1 h		3 h		5 h		6 h	
Strain	Yield (%)	Substrate (%)*	Yield (%)	Substrate (%)*	Yield (%)	Substrate (%)*	Yield (%)	Substrate (%)*
Bacillus subtilis	15	86	20	80	20	70	35	64
Pseudomonas aeruginosa	25	38	48	35	70	25	70	18
Pseudomas putida	11	50	11	50	15	45	15	33
Pseudomas fluorescens	12	45	14	40	15	45	20	40

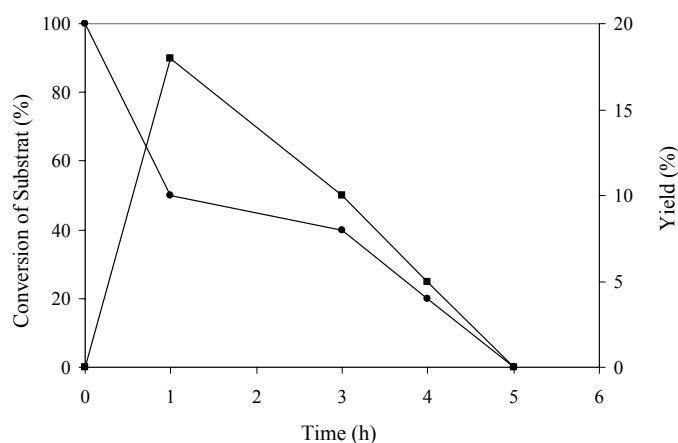*Percentage of substrate remaining in reaction media.

Figure 1: Biotransformation of racemic 2-Phenyl-2-amino-acetonitrile 1mM to D-Phenylglycine with *P. aeruginosa* as biocatalyst. (■) D-Phenylglycine and (●) 2-Phenyl-2-amino-acetonitrile.

In order to increase the conversion rates in shorter reaction times, induction assays were performed. Cells were grown in LB media, until stationary growth phase (24 hours), and afterwards different amounts of benzonitrile [0.025; 0.05 and 0.1% (v/v)] were added to the culture media. After exposure to the different inducer concentrations a diauxie growth pattern was observed (Figure 2), corresponding to an increase in the biomass of 24, 35 and 46% as compared to the control with 0.025; 0.05 and 0.1% of inducer, respectively. These results suggest that with higher cell density the presence of benzonitrile is well tolerated by the culture, decreasing the effects of inducer toxicity. The cells induced with 0.025% of benzonitrile showed maximum conversion of the D-isomer of 2-phenyl-2-amino-acetonitrile, yielding complete conversion (50%) of D-phenylglycine within 30 minutes of reaction. When the inducer concentration was raised, conversion decreased in the same time interval (30 minutes), with maximum conversion of 30% and 20% when 0.05 and 0.1% of benzonitrile was used, respectively. The decrease in the amino acid production, with the increase of the inducer concentration added to the media in the stationary growth phase, can be attributed to the toxic effects observed in the induction assays already described, once the lower concentration of benzonitrile led to the higher conversion rates. Target product characterization was achieved with the material resulting from the scaled up biomass production.

It is important to emphasize that there is no competitive chemical process for the above described to obtain enantioenriched D- or L-phenylglycine and its derivatives. The only competitive protocols would be based on resolution of *N*-acetyl-DL-phenylglycine, via acylases (Dunes *et al.*, 2005) or dynamic kinetic resolution of DL-phenylhydantoin (Arcuri *et al.*, 2000; Arcuri *et al.*, 2002; Arcuri *et al.*, 2004).
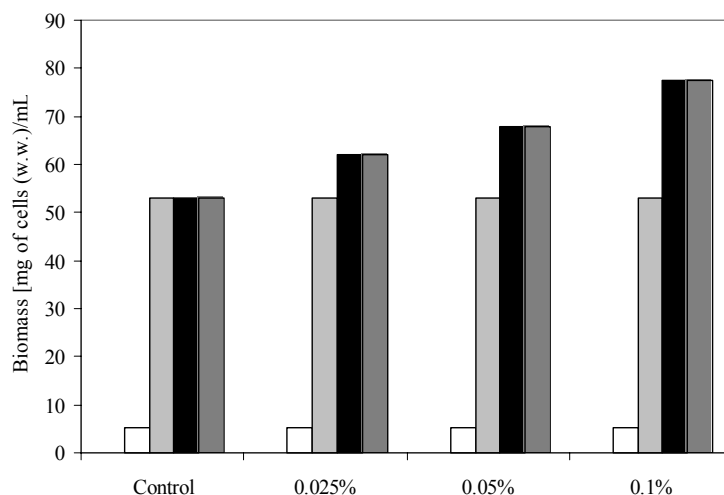
Figure 2: Effect of different inducer concentrations on *P. aeruginosa* growth profile.
(White bar-12 h; Gray bar-24 h; Black bar-36 h and White striped bar-48 h).

CONCLUSIONS

All the wild type strains screened in the present work were able to convert the aromatic nitrile substrate (benzonitrile) into the corresponding benzoic acid. These results indicate that these bacteria strains constitutively express nitrile hydrolyzing activities as being a new source of biocatalyst for nitrile conversion. The absence of an amide in RP-HPLC chromatograms do not discard the occurrence of the bi-enzimatic route (nitrile hydratase/amidase) if the amidase activity is higher than that of the corresponding hydratase. The most efficient nitrile converting biocatalyst was *Pseudomonas aeruginosa* strain, both with benzonitrile and 2-phenyl-2-amino-acetonitrile as substrate. These results indicate that this new nitrile converting microorganism expresses nitrile hydrolyzing activities that evidenced being active against both aromatic nitriles and phenylamino-acetonitriles. The presence of an inducer in the culture media led to cell toxicity, but when the same inducer was added to the stationary phase of growth, the enzyme activities increased considerably, leading to higher conversion rates in shorter times while keeping the enantiomeric excess.

Although *Rodococcus rhodochrous* is the most commonly used nitrile converting microorganism [17-19] and *Pantoea endophytica* and *Pantoea* sp. have been reported as good biocatalyst for the production of amino acids from nitrile substrate (Hensel, Lutz-Wahl and Fischer, 2002), this *Pseudomonas aeruginosa* strain emerges as a new promising biocatalyst for the conversion into amino acids of nitrile-containing compounds, since short reaction times were enough to achieve high conversion. The improvement of this nitrile hydrolyzing enzymes expression can be useful in the production of enantiomerically pure amino acids.

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