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Production of hydroxamic acids by immobilized *Pseudomonas aeruginosa* cells: Kinetic analysis in reverse micelles

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ABSTRACT

Intact cells from *Pseudomonas aeruginosa* strain L10 containing amidase were used as biocatalysts both free and immobilized in a reverse micellar system. The apparent kinetic constants for the transamidation reaction in hydroxamic acids synthesis, were determined using substrates such as aliphatic, amino acid and aromatic amides and esters, in both media. In reverse micelles, K_m values decreased 2–7 fold relatively to the free biocatalyst using as substrates acetamide, acrylamide, propionamide and glycinamide ethyl ester. We have concluded that overall the affinity of the biocatalyst to each substrate increases when reactions are performed in the reversed micellar system as opposed to the buffer system. The immobilized biocatalyst in general, exhibits higher stability and faster rates of reactions at lower substrates concentration relatively to the free form, which is advantageous. Additionally, the immobilization revealed to be suitable for obtaining the highest yields of hydroxamic acids derivatives, in some cases higher than 80%.

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1. Introduction

Enzymes are used as biocatalysts in the synthesis of products mainly due to their versatility catalyzing a wide range of reactions with high reaction rates, regiospecificity and under milder conditions than their chemical counterparts [1]. Biocatalysis can be carried out using either whole cells or lysates containing the enzyme of interest, or using purified enzymes. However, enzyme purification is costly and time consuming and often enzymes are less stable in the purified form than in whole cells [2,3].

Biocatalytic processes are often carried out in aqueous environments. However, observations that biocatalysts are able to catalyze reactions in organic solvents encouraged an intense research and development of applications for enzymatic catalysis in nonaqueous environments. A wide range of reactions were carried out in organic media namely oxidation–reductions, hydroxylations, esterifications, hydrolysis and transamidations reactions [3–8].

Microemulsions are typical non-conventional media suitable to promote biocatalysis and are often used to obtain higher solubility of substrates or products, to lower the water content to promote reversal of hydrolytic reactions or to modify the specificity or the stability of the biocatalyst [1,4–7,9]. Reversed micelles are microemulsions in which a shell formed by surfactant molecules protects the biocatalyst against denaturation by the organic solvent. The biocatalyst is solubilized inside this shell in a water pool maintaining the catalytic activity and often can exhibit an increased stability [1,10,11].

Amidases (acylamide amidohydrolase E.C.3.5.1.4) from *Pseudomonas aeruginosa* [12], *Rhodococcus* sp. [13,14] and *Arthrobacter* sp. [15] have been used for the catalysis of amides or carboxylic acids hydrolysis reactions and of amides or esters acyltransferase reactions (transfer of R—CO groups to hydroxylamine) synthesizing hydroxamic acids derivatives. [3,8,9,16].

Synthesis of acetohydroxamic acid, using amidases from *P. aeruginosa* as biocatalysts, has been performed by our group in aqueous media [17] and in a reverse micellar system [3,8]. The success of the latter microenvironment, where yields of product synthesis higher than 90% were accomplished in the reverse micellar system of cationic surfactant tetradecyltrimethyl ammonium bromide (TTAB) in heptane/octanol (80/20%), encouraged the present study in which we investigated the biocatalysed synthesis reaction of several hydroxamic acid derivatives in this non-conventional medium. Furthermore we hereby use whole cells from *P. aeruginosa* L10, containing amidase as biocatalysts, since it is also well known from our previous work that there is no need for the enzyme purification steps prior to the synthesis reaction [3].

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Hydroxamic acids have received considerable attention and are considered very attractive compounds due to their high chelating potential with metal ions. Several hydroxamic acids have been used as drugs and have been reported as tumor inhibitors, anti-malarial agents, anti-HIV and recommended for treatment of ureaplasma infections and anemia [14,18,19]. Other hydroxamic acids have been used to eliminate metal ions in wastewater treatment and nuclear technology [20]. The application of these compounds in food industry, waste water treatment and in pharmaceutical industry as antimicrobial agents, antibiotic antagonists and tumor inhibitors was investigated by [21–24]. However chemical synthesis of these compounds is a complex procedure [25] and therefore the results obtained from this work show great potential for the simplified synthesis of these highly important products. This work aims to present the kinetic analysis of the acyltranferase reaction catalyzed by amidase in intact cells of *P. aeruginosa* strain L10, using a wide range of substrates (aliphatic, aromatic and amino acids amides and esters) for the synthesis of several hydroxamic acid derivatives, both free in aqueous media or immobilized in reverse micelles.

2. Experimental

2.1. Chemicals

Heptane, octanol, benzamide and propionamide were purchased from Merck (Darmstadt, Germany). Tetradecyltrimethyl ammonium bromide (TTAB), benzohydroxamic acid, L-alaninamide, phenylalaninamide, phenylalaninamide ethyl ester and glycine ethyl ester were obtained from Sigma–Aldrich (Madrid, Spain). Acetamide, acetohydroxamic acid and iron chloride were purchased from Fluka (Madrid, Spain). Hydroxylamine was obtained from Panreac (Barcelona, Spain). Glycinamide and leucinamide were obtained from Bachem (Bubendorf, Switzerland), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer from Fisher Scientific (Leicestershire, United Kingdom). All chemicals used were of analytical grade. Benzohydroxamate and butyrylhydroxamate were obtained from Sigma and glycine hydroxamate and alanine hydroxamate were obtained from Key Organics.

2.2. Pseudomonas aeruginosa strain L10 cell culture

P. aeruginosa L10 strain was used as the source of amidase (E.C. 3.5.1.4.) The strain was grown overnight as previously described [26,27] and the cells were harvested by centrifugation at 10,000 × *g* for 5 min. The pellet containing cells was washed twice with NaCl (8.5%, w/v) and stored at −20 °C.

2.3. Enzyme assays

2.3.1. Acyltransferase activity assay of immobilized P. aeruginosa cells in the reverse micellar system

The activity assays in the reverse micellar medium were performed using intact cells from *P. aeruginosa* L10 strain containing amidase and according to a methodology previously described [3], in a thermostatic water bath at 40 °C. Briefly, the acyltransferase activity was investigated in a 5 mL stirred reverse micellar system of 200 mM TTAB in heptane/octanol (80/20%, v/v) containing *P. aeruginosa* cells (0.050 g cells/mL in 10 mM HEPES buffer at pH 7.2), hydroxylamine solution (freshly neutralized to pH 7). The water content in the system, usually defined through the parameter $w_0 = [H_2O]/[\text{surfactant}]$, was controlled by adjusting the added volume of buffer 10 mM HEPES at pH 7.2 and established to a value of 10. The amide or ester substrate solution in 10 mM HEPES buffer at pH 7.2 was injected in order to initiate the reaction.

The rate of reaction was determined by following the increase in the concentration of the reaction product a, hydroxamic acid derivative, during the course of the reaction as described in [3]. Calibration curves with acetohydroxamate, benzohydroxamate, butyrylhydroxamate, glycine hydroxamate and alanine hydroxamate were established and values for the extinction coefficient (ε) were obtained of 0.105 mM^{-1} ($R^2 = 0.993$), 0.1210 mM^{-1} ($R^2 = 0.964$), 0.038 mM^{-1} ($R^2 = 0.993$), 0.0072 mM^{-1} ($R^2 = 0.965$), 0.0057 mM^{-1} ($R^2 = 0.972$), respectively. One enzyme unit (U) is defined as the amount of enzyme required to produce 1 μmol of hydroxamic acid derivative per min under these experimental conditions.

2.3.2. Acyltransferase activity assay of free P. aeruginosa cells in buffer solution

The acyltransferase activity was determined as described above but the reaction was carried out in 10 mM HEPES buffer pH 7.2 containing *P. aeruginosa* cells (0.050 g cells/mL in 10 mM HEPES buffer at pH 7.2) and hydroxylamine solution (freshly neutralized to pH 7). The amide or ester substrate solution in 10 mM HEPES buffer at pH 7.2 was injected in order to initiate the reaction. The rate of the reaction was determined as described previously [3] by following the increase in the concentration of the reaction product, hydroxamic acid derivative, during the course of the reaction. Calibration curves with acetohydroxamate, benzohydroxamate, butyrylhydroxamate, glycine hydroxamate and alanine hydroxamate were established and values for the extinction coefficient (ε) were obtained of 0.063 mM^{-1} ($R^2 = 0.993$), 0.0885 mM^{-1} ($R^2 = 0.989$), 0.0328 mM^{-1} ($R^2 = 0.988$), 0.002 mM^{-1} ($R^2 = 0.935$), 0.0017 mM^{-1} ($R^2 = 0.951$) respectively. One enzyme unit (U) is defined as the amount of enzyme required to produce 1 μmol of hydroxamic acid derivative per min under these experimental conditions.

2.4. Storage stability

Storage stability of the cells in the reverse micellar system was demonstrated by incubating the cell solution (0.012 g/mL in 10 mM HEPES buffer at pH 7.2) in 200 mM TTAB in heptane/octanol (80/20%, v/v) at w_0 of 10 at 25 °C under constant agitation during several hours. Sampling was performed at time intervals and activity was assayed as described in Section 2.3.1 by the injection of 30 mM hydroxylamine and 15 mM acetamide in order to initiate the reaction.

Storage stability of the cells in HEPES buffer was demonstrated by incubating the cell solution 0.012 g/mL in 10 mM HEPES buffer at pH 7.2 at 25 °C under constant agitation during several hours. Aliquots were taken from the mixture during incubation, 30 mM hydroxylamine plus 15 mM acetamide were added in order to initiate the reaction and activity was assayed as described in Section 2.3.2.

The first order rate inactivation constants, *k*, were determined by the slope of the semilogarithmic plot of residual activity versus storage time and used to calculate the half-life values, $t_{1/2}$ [28].

2.5. Kinetic studies

For the kinetic analysis the activity assays were performed using a constant *P. aeruginosa* cell concentration at different concentrations of each of the substrates in 10 mM HEPES buffer at pH 7.2. The hydroxylamine solution was used in a 2 fold concentration of the substrate concentration. The activity assays were run in duplicate both in the reverse micellar system and in the buffer solution according to the methodology described in Sections 2.3.1 and 2.3.2 respectively.

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