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D-Aminoacylase from a novel producer: *Stenotrophomonas maltophilia* ITV-0595

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A novel bacterial strain producing D-aminoacylase was isolated from organic waste and identified as *Stenotrophomonas maltophilia* ITV-0595. The isolation was performed using *N*-acetyl-D-phenylglycine (NAcDPG) as the sole source of C and N. The optimum pH for enzyme expression was 8 at 37°C. Using N-Ac-DPG concentrations from 0.5 up to 3% w/v, it was observed that at the 1% level, the microorganism showed acceptable responses in both enzyme activities and cell growth. From the different tested compounds N-acetyl-D-methionine (1%) was the best enzyme inducer (Sp. act. = 4.14 U mg^{-1} protein, Vol. act. = 0.17 U ml^{-1}) and the only one that increased cell growth.

Keywords: D-aminoacylase; *Stenotrophomonas maltophilia*; *Xanthomonas maltophilia*; *Alcaligenes* sp

Introduction

Aminoacylases comprise those enzymes that enantioselectively catalyze hydrolytic reactions of N-acyl groups in amino acid derivatives (Figure 1).

l-aminoacylases have industrial applications in the resolution of racemic mixtures for the production of l-amino acids, as l-methionine. The origin of the commercially available l-aminoacylase is, up to the present, *Sus scrofa* (Hog) kidney, and the microorganism *Aspergillus oryzae*.

In addition, d-aminoacylases could have the same applications as l-aminoacylases, producing amino acids with a d-configuration, such as d-valine, d-phenylglycine and d-*p*-hydroxyphenylglycine, which are used in the production of important pharmaceutical and agricultural compounds. However, there are no commercially available d-aminoacylases. Therefore these d-amino acids are produced by other methods.

The initial report of studies concerning d-aminoacylases from microorganisms was in the early fifties, when Kameda *et al* [6] demonstrated the occurrence of this intracellular enzyme in soil bacteria. From this time until 1980, species of *Pseudomonas* and *Streptomyces* [12] were reported as d-aminoacylase producers. Due to the lack of a strict stereospecificity of d-aminoacylase produced by species of these genera, further screening programs were required. At the end of the eighties, a new genus, *Alcaligenes*, was introduced as producer of this enzyme [8,13]. One of the groups involved in this contribution stated that the d-aminoacylase from *Alcaligenes* DA181 was superior to those previously studied, because it showed 'only negligible hydrolysis activity toward all the N-acetyl-l-amino acids tested' [13].

As far as we know, a d-aminoacylase producer from another genus has not been reported. In view of the limited number of microorganisms with this capability and the need to increase knowledge about d-aminoacylases, we have developed a research program in relation to d-aminoacylases from microorganisms [2,3]. We describe here the isolation of a novel d-aminoacylase-producing microorganism, the taxonomic study of the strain and characterization of its enzyme production.

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Materials and methods

Chemicals

All N-acetyl derivatives used in our studies, with the exception of phenylglycine which was a gift from Fersinsa-Gist Brocades (Ramos Arizpe, Coah, Mexico), were prepared from free amino acids, purchased from Sigma Chemical Co (St Louis, MO, USA), by procedures already described [2,4]. The identification of N-acetyl derivatives was performed determining their melting point and IR spectrum. Yeast extract, maltose and tryptone were supplied by Bioxon (Oaxaca, Oax, Mexico) and Bacto Agar by Difco (Detroit, MI, USA). Other chemicals, all of analytical grade were purchased from JT Baker (Mexico, DF) and used without further purification.

Isolation procedure

For the isolation of d-aminocylase-producing bacteria, 5.0 g of organic waste (soil taken from the bottom of a container of plant residues at a local market), were suspended in 100 ml of sterile distilled water by vigorous mixing. After filtration, 20 ml of this extract were inoculated into 100 ml of the following medium (medium X): 1% w/v N-acetyl-d-phenylglycine, 0.1% w/v KH_2PO_4, 0.1% w/v K_2HPO_4, 0.05% w/v $MgSO_4 \cdot 7H_2O$; pH = 7.0. It was then incubated for 4 days at 37°C. Dilutions were spread on agar plates (medium X plus 2% agar), and incubated at 37°C for 3 days. Microorganisms able to grow on this medium were purified and cultivated at the same temperature in medium X supplemented with 0.5% yeast extract (medium Y). The cells were collected by centrifugation, and the intracellular d-aminoacylase activity was measured after the cells were treated with toluene. A bacterial strain, named ITV-0595, was selected and deposited in the Instituto Technológico de Veracruz culture collection.

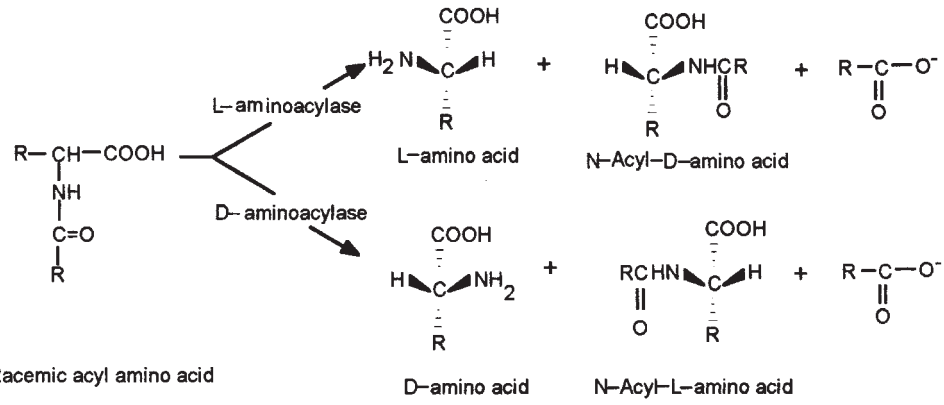

Figure 1 Enzymatic resolution of acyl amino acids.

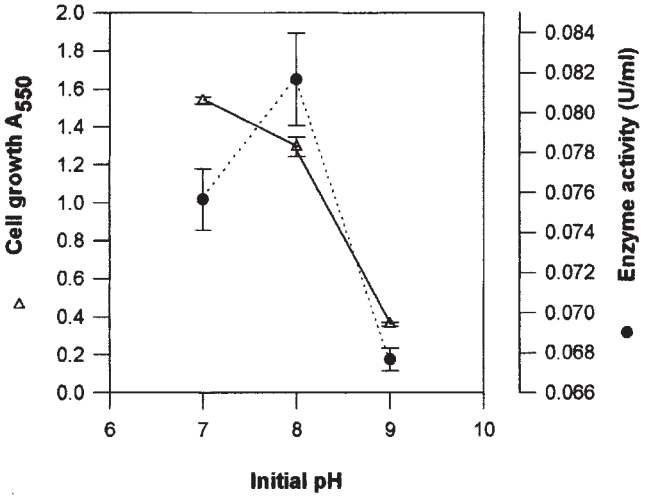

Figure 2 Effect of medium pH on the production of d-aminoacylase. Strain ITV-0595 (inoculum 1E8 cells ml^{-1}), was grown in medium Y, for 24 h, at 37°C, 250 rpm.

Taxonomic study of the strain

In order to obtain information that could make possible characterization and identification of the selected strain, we applied procedures described in the literature [1,5,15]. We developed part of this identification study with the assistance of a standarized micromethod, employing a panel or identification system for Gram-negative rods (non enteric and Enterobacteriaceae), with 25 biochemical tests and 19 antimicrobial susceptibility tests (Mics) (Dade-Baxter, USA). The results were obtained using an automated microplate reader (Microscan, Dade-Baxter, USA).

Analytical methods

D-Aminoacylase activity: Enzyme activity was measured using the ninhydrin method [10] to monitor d-phenylglycine released by hydrolysis of N-acetyl-d-phenylglycine. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of d-phenylglycine per min at 37°C.

Standard enzyme reaction: A reaction mixture, containing 250 μl 10 mM N-acetyl-d-phenylglycine, 250 μl Tris buffer (50 mM, pH 7.8), and appropriately diluted enzyme solution was incubated at 37°C for 20 min. The reaction was stopped by adding 1 ml 20% w/v trichloroacetic acid solution. The d-phenylglycine liberated was measured by the ninhydrin method [10].

Protein determination: Protein was determined by the method of Lowry *et al* [7] using bovine serum albumin as a standard.

Results

Characteristics of Stenotrophomonas maltophilia ITV-0595

This strain is an aerobic, Gram-negative, motile, rod-shaped bacterium. Colonies are smooth, glistening, mucoid and beige in the isolation medium, and yellow in XMSM (*Xanthomonas maltophilia* Selective Medium: 10 g L^{-1} maltose, 5 g L^{-1} tryptone, 4 ml 2% aq. sol. bromthymol blue, 15 g L^{-1} Bacto-Agar) [1], oxidase negative, catalase positive.

The following are results obtained with the assistance of an identification system for Gram-negative rods (Microscan-Dade-Baxter, USA). No production of acid was noted from glucose, sucrose, sorbose, raffinose, rhamnose, arabinose, inositol, adonitol or melibiose. The presence of lysine decarboxylase was observed but not that corresponding to arginine and ornithine. Tryptophan deaminase, urease and β-galactosidase were not produced. Neither H_2S nor indol was produced. The organism utilized citrate as a carbon source but not acetamide, tartrate and malonate. Esculin hydrolysis and Voges-Proskauer reaction were negative. Nitrate was reduced to nitrite, and growth was inhibited by cetrimide.

These characteristics plus a positive oxidase reaction, identify strain ITV-0595 as *Xanthomonas maltophilia* (*Stenotrophomonas maltophilia*), according to the data bank software linked to the automated microplate reader (Microscan).

Strain ITV-0595 or *Stenotrophomonas maltophilia* did produce acid from maltose, using XMSM, a characteristic observed in all the isolates of *S. maltophilia* [1].

Stenotrophomonas maltophilia has recently been classified. It was formerly known as *Pseudomonas maltophilia*

and then as *Xanthomonas maltophilia* [15]. *S. maltophilia* is not a plant pathogen. On the contrary, there is evidence of its antifungal activity in the rhizosphere of oilseed rape [1]. However *S. maltophilia* is also known as an opportunistic pathogen causing nosocomial infections [16].

Effect of medium pH on the production of D-aminoacylase

Even when the microorganism could be isolated using a pH of 7, it was necessary to ascertain the effect of the initial pH on the production of d-aminoacylase. Three different pH conditions (7, 8, 9) were used in medium Y. Experiments were performed in triplicate. The highest enzyme production was obtained with an initial pH of 8 (Figure 2).

Effect of N-acetyl-D-phenylglycine concentration on D-aminoacylase production

In order to determine the best expression of d-aminoacylase under specific conditions, we investigated the effect of different concentrations of this non-natural amino acid derivative, originally used for the microbial isolation. Concentrations of NAcDPG up to 1.5%, increased d-aminoacylase specific activity (Figure 3) which diminished at higher concentrations, although activity was present even in the absence of NAcDPG. NAcDPG (1%) was chosen for further studies, considering that at this level there is an acceptable expression of specific activity, cell growth and volumetric activity.

Effect of acetylated amino acids (D or D,L) and free amino acids on D-aminoacylase production and cell growth

N-acetyl-d-valine, N-acetyl-d-leucine, N-acetyl-d-methionine, N-acetyl-d- phenylglycine, N-acetyl-d,l-valine, N-acetyl-d,l-leucine, N-acetyl-d,l-phenylglycine, d-phenylglycine and d-methionine, were tested to ascertain their ability to induce d-aminoacylase. Table 1 shows the results of using 1% N-acetyl-d-(phenylglycine,-valine or-methionine) and 0.78% d-(methionine and phenylglycine). All these compounds with the exception of N-acetyl-d-leucine increased the induction of d-aminocylase from 1.48 to 6.34 times. In relation to cell growth, only N-acetyl-d-methionine did not inhibit it, and in fact was the only inducer.

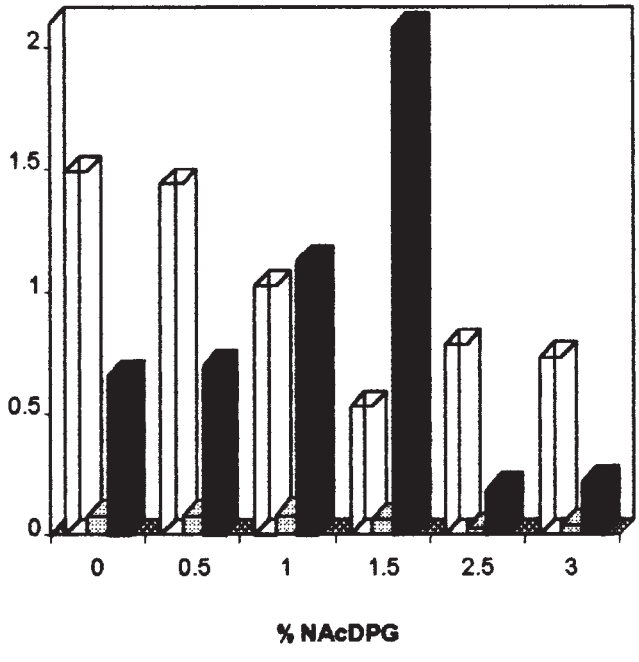

Figure 3 Effect of N-acetyl-d-phenylglycine concentration on d-aminoacylase production. □, Cell growth A_{550}; ▩, d-aminoacylase activity (U ml^{-1}); ■, specific activity (U mg^{-1} protein).

Table 1 Effect of inducers (1% N-Ac-d-AA and 0.78% d-AA) on the production of d-aminoacylase

Inducer	Cell growth (A550)	Specific activity (U mg^{-1} protein)
None	1.486	0.653
N-Ac-d-phenylglycine	1.022	1.124
N-Ac-d-valine	1.094	1.060
N-Ac-d-leucine	1.182	0.627
N-Ac-d-methionine	2.529	4.138
d-Methionine	0.097	0.958
d-Phenylglycine	0.078	1.122

Table 2 includes the responses of *S. maltophilia* ITV-0595, when 1% NAc-d,l-AA and 0.5% NAc-d-AA were used. In this case, d-aminoacylase activities (U ml^{-1}) induced by 0.5% N-Acetyl-d-(phenylglycine, -valine or -leucine) were about two-fold higher than activities induced by the corresponding 1% N-acetyl-d,l-amino acids. However, in the absence of an inducer, substantial enzyme activity was observed. Cell growth was inhibited with all the amino acid derivatives listed in Table 2, but racemic mixtures inhibited twice as much as the pure enantiomers. These results suggest that N-acetyl derivatives could play two roles, interfering with the relative enzyme induction and/or reducing the low availability as carbon sources observed in N-acetyl-d-amino acids.

Discussion

Stenotrophomonas maltophilia ITV-0595 is a new member of d-aminoacylase-producing microorganisms. Using 1% N-Ac-d-methionine, the best d-aminoacylase specific and volumetric activities (4.14 U mg^{-1} protein, 0.17 U ml^{-1} respectively) were observed. The same amino acid derivative was also the best inducer of cell growth. These characteristics favor using this substance in further studies as the

Table 2 Effect of inducers (1% N-Ac-d,l-AA and 0.5% N-Ac-d-AA) on the production of d-aminoacylase)

Inducer	Activity (U ml^{-1})	Cell growth (A550)
None	0.076	1.486
NAc-d,l-phenylglycine	0.045	0.568
NAc-d-phenylglycine	0.078	1.438
NAc-d,l-valine	0.033	0.828
NAc-d-valine	0.086	1.360
NAc-d,l-leucine	0.037	0.756
NAc-d-leucine	0.052	1.347

carbon source for cell growth and as the inducer for enzyme production.

As a possible explanation of the remarkable cell growth inhibition observed in the experiments with d-methionine or d-phenylglycine, and the absence of this behavior when N-Ac-d-methionine or other N-Ac-d-amino acids were used, it can be said that this microorganism as well as others with a d-aminoacylase expression, produces acetate and the d-amino acid as a result of hydrolytic activity. Acetate can be used as a carbon source and the d-amino acid could follow two paths, one of them being racemization, the other could be a deamination of the d-amino acid due to either a transaminase for d-amino acids or a d-amino acid oxidase, both routes favoring nitrogen and carbon source availability.

It seems that under the conditions used, *Stenotrophomonas maltophilia* ITV-0595 uses acetate as a carbon source for its growth, and the possible absence of enzymes that metabolize d-amino acids could explain their limited availability as carbon and/or nitrogen sources.

On the other hand, the relevant stimulation of cell growth and specific activity of N-Ac-d-methionine in *Stenotrophomonas maltophilia* ITV-0595 could be explained in terms of a high substrate specificity of this d-aminoacylase. This observation may be connected with the conditions of the rhizospheres, the usual habitat of this kind of bacteria, where several studies have suggested that methionine may play an important role [1].

In *Alcaligenes* spp, the most reported and studied d-aminoacylase-producing microorganisms, Moriguchi *et al* [9,11] measured a specific activity of 5.34 U mg^{-1} protein using 0.25% N-Ac-γ-methyl-d-leucine with *A. xylosoxydans* A-6, and a specific activity of 2.16 U mg^{-1} protein using 0.25% γ-methyl-d-leucine with *A. denitrificans* MI-4. Tsai *et al* [13,14] found that *A. denitrificans* DA181 with 0.5% N-acetyl-d-valine had a volumetric activity of 2.59 U ml^{-1} and *A. faecalis* DA1 an activity of 1.07 U ml^{-1}. In three of these studies [11,13,14], N-acetyl-d-methionine was used as the substrate for d-aminoacylase, and in the other [9] N-acetyl-d-leucine we used N-acetyl-d-phenylglycine.

A comparative analysis of cell growth between our results and those of others reported [14] for the same type of amino acids, shows an opposite behavior. In our case, 1% N-acetyl-d-methionine was the only one of the tested compounds that increased cell growth (1.7 times), whereas in *A. faecalis* DA1 this compound inhibited it [14]. All the remaining amino acids (N-ac-d-valine, N-Ac-d,l-leucine, N-Ac-d,l-valine and N-Ac-d,l-leucine) tested by Tsai *et al* and also by our group, stimulated cell growth in *A. faecalis* DA1 [14] but inhibited it in *S. maltophilia* ITV-0595. In *A. denitrificans* DA181, all the above amino acids, including N-Ac-d-methionine, stimulated cell growth.

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