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Optimized Production of Hydroxamic Acid Derivatives with Antioxidant and Anticholinergic Potential by Immobilized *Pseudomonas aeruginosa* Cells

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Abstract
In this study were investigated, the synthesis, acetylcholinesterase inhibition and antioxidant activity of a series of hydroxamic acid derivatives (HAD), with different chemical group characteristics, such as aliphatic (acetohydroxamic acid and butyryl hydroxamic acid), aromatic (benzohydroxamic acid and phenylalanine hydroxamic acid) and amino acid (glycine hydroxamic acid and alanine hydroxamic acid). It was observed that these HAD compounds present very promising activity as acetylcholinesterase (AChE) inhibitors and as antioxidants. The aliphatic HAD demonstrated to have a higher inhibitory activity of AChE than amino acid or aromatic HAD. As for the antioxidant activity, a high antioxidant potential was found for all the compounds with EC_{50} values ranging from 0.19 µM to 1.65 µM. Aiming these applications, a biocatalysis approach was used to obtain these HADs with optimal reactional conditions. In this study, reverse micelles with immobilized *Pseudomonas aeruginosa* intact cells containing amidase were used as a biocatalyst to catalyze the acyltransferase reaction of the corresponding substrate amide and hydroxylamine to obtain various HAD and this was achieved for the first time with yields of approximately 100%.

1. Introduction

Hydroxamic acids, with the general formula of RC (O) NHOH, can chelate metal ions and are known to have multiple biological activities [1]. Several hydroxamic acid derivatives (HAD) are enzymes inhibitors and therefore have clinical potential as tumour inhibitors [2, 3] and as antibiotics [4], they can also be used for the treatment of several

diseases, such as Alzheimer disease (AD) [5], AIDS [6] and cardiac dysfunction diseases [7]. These compounds can also present an antioxidant potential [8-10] and may also have applications in other areas, such as the food industry and in wastewater treatment [11, 12].

In contrast to these numerous reports of the pharmacological potential of HAD, there are no reports on the acetylcholinesterase (AChE) inhibition by this type of compounds. Acetylcholinesterase (AChE, E. C. 3.1.1.7.) is an enzyme localized at the cholinergic synapses that terminate the neurotransmission signal by catalyzing the hydrolysis of the acetylcholine, the neurotransmitter in the cholinergic synapses [13, 14]. The reversible inhibition of this enzyme is the major target for the pharmacotherapy of AD symptoms and other forms of dementia [15, 16]. The most common form of dementia, AD pathogenesis, is complex and there is evidence that oxidative damage is associated with the disease, therefore antioxidant therapies have also been successfully applied in the prevention and treatment of AD [17, 18]. Consequently, there are efforts in the development of potential drug candidates for the treatment of AD in search for multi-target compounds that can act both as antioxidants and as reversible AChE inhibitors [19, 20]. In this regard, there is a great need for developing relatively simple, economical and commercially feasible processes for the synthesis of HAD having these properties and with acceptable yield. A number of traditional approaches are available for HAD preparation by organic synthesis, but some are tedious, time-consuming and costly as well. Bacterial amidases (acrylamide amidohydrolase; EC 3.5.1.4.) can be used for the production of various HAD by catalyzing transamidation reactions using a wide range of amides as substrates and hydroxylamine as acyl group acceptor [21-23]. Amidases have significant potential due to their broad substrate specificity and there are numerous reports on the immobilization of microbial cells with amidase activity providing several advantages for its various applications [21, 24-26].

It was previously carried out the kinetic analysis of the production of HAD by immobilized *Pseudomonas aeruginosa* cells in reverse micelles and it was observed that the immobilization increases not only stability but also the overall reaction rate relatively to the free form, which is advantageous [21, 25]. But it was also seen that the substrate nature present a very important role, the use of some amides as substrates to obtain HAD, other than short chain aliphatic, leads to much more difficult reactions [21], and since that there are several factors that can influence the reactions in reverse micelles, expanding upon previous work in this paper, the main goal was to optimize the reactional conditions for the synthesis of various HAD such as aliphatic (acetohydroxamic acid and butyryl hydroxamic acid), aromatic (benzohydroxamic acid and phenylalanine hydroxamic acid) and amino acid (glycine hydroxamic acid and alanine hydroxamic acid).

Therefore, this study demonstrates first, the above stated HAD as promising anticholinergic compounds and antioxidants and, second, the successfully consolidated conditions for bioprocessing by immobilized *Pseudomonas aeruginosa* cells.

2. Materials and Methods

2.1. Chemicals

All chemicals used were of analytical grade or HPLC grade as per requirement. Acetylcholinesterase (AChE) (E. C.3.1.1.7) type VI-S from electric eel 411 U/mg protein, 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), acetylthiocholine iodide (AChI), Thiazolyl Blue Tetrazolium Blue (MTT), 2,2-diphenyl-1-picrylhydrazil (DPPH), heptane, octanol, benzamide was purchased from Merck (Darmstadt, Germany). Tetradecyltrimethylammonium bromide (TTAB), L-alaninamide (L-AlaNH2), phenylalaninamide (PheNH2), benzohydroxamic acid (BenzHA), phenylalanine hydroxamic acid (PheHA) and butyrylhydroxamic acid (ButyHA) were obtained from Sigma-Aldrich (Madrid, Spain). Acetamide, acetohydroxamic acid (AcetHA) and iron chloride were purchased from Fluka (Madrid, Spain). Hydroxylamine was obtained from Panreac (Barcelona, Spain). Glycinamide was obtained from Bachem (Bubendorf, Switzerland). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was obtained from Fisher Scientific (Leicestershire, United Kingdom). Glycine hydroxamic acid (GlyHA) and alanine hydroxamic acid (AlaHA) were obtained from Key Organics (Cornwall, United Kingdom). Human Cell lines culture media DMEM (Dubbleco's Modified Eagle Medium), penicillin/streptomycin, L-glutamine, and heat inactivated FBS (Fetal Bovine Serum) were obtained from Lonza, VWR International.

2.2. AChE Inhibition by HADs

The AChE enzymatic activity was measured using an adaptation of the Ellman's colorimetric method [27]. Briefly: 325 µl of 50 mM HEPES buffer (pH 8), 100 µl of the HAD solution at different concentrations in water, 25 µl of AChE (0.1 U/mL) solution in 50 mM HEPES buffer pH 8 were incubated for 15 min. Subsequently, 75 µl of 79 µM AChI (0.023 mg/mL) and 475 µl of 3 mM DTNB in 50 mM HEPES buffer (pH 8) containing 0.05M NaCl and 0.021M MgCl2, were added to initiate the reaction. The initial rate of the enzymatic reaction was quantified by measuring the absorbance at 405 nm during 5 min. A control reaction was carried out using water instead of the HAD solution and this was considered 100% activity. The percentage of AChE inhibition by each concentration of HAD was calculated by using: IE (%)= 100-100 × (v_sample/v_control) where IE is the percent inhibition of AChE, v_sample is the initial rate of enzymatic reaction in the presence of HAD solution and v_control is the initial rate of the control AChE reaction in the absence of HAD solution. The tests were carried out in triplicate.

2.3. Antioxidant Activity of HADs

The antioxidant activity of the HADs was determined

using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) method [28] slightly modified. To a 2.5 ml solution of DPPH (0.002% (w/v) in methanol), 25 µl of HADs solutions were added at various concentrations in water. The mixture was incubated for 30 min at room temperature and the absorbance was measured at 517 nm against a corresponding blank. Radical scavenging activity (RSA) for DPPH free radical of each HAD concentration, was calculated using the expression: AA (%)=100-100×(A_sample/A_DPPH) where AA is the antioxidant activity, A_sample is the absorbance of the DPPH solution with HAD and A_DPPH is the absorbance of the DPPH solution. RSA was expressed as the concentration of the HAD that scavenges 50% of DPPH free radicals (EC_{50}). The assays were carried out in triplicate.

2.4. Cytotoxicity of HADs

HeLa (ATCC® CCL-2, human cervix adenocarcinoma epithelial cell line), Hep G2 (ATCC® HB-8065, human hepatocellular carcinoma cell line) and MCF7 (ATCC® HTB-22, human mammary gland adenocarcinoma epithelial cell line) were used for cytotoxicity studies using the MTT viability test, according to Mosmann method [29]. Briefly: the cells were seeded in 96 well plates at the density of 5 $x10^3$ cells per well in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mM L-glutamine and incubated at 37°C in an atmosphere with 5% CO_2 for 48 hours to assure attachment and 80% confluency. The media was aspirated and replaced with HAD solutions in DMEM medium at various concentrations. A control assay was performed by adding DMEM medium to the wells. Each concentration and the controls were replicated eight times. The plates were incubated for 24 h at the same conditions. After incubation, the HAD solutions were removed and replaced by 0.05 mg MTT per well in DMEM medium. The plates were incubated for 2 to 4 h and the MTT solution was removed and replaced with DMSO to mix the formazan crystals until dissolved. The absorbance at 570 nm was registered. For each concentration of HAD the percentage of viability was evaluated considering 100% viability in the control assay.

2.5. Optimization of HADs Synthesis by Biocatalysis in Reverse Micellar System

Acyltransferase activity of amidase (E. C. 3.5.1.4.) from *P. aeruginosa* L10 strain was used for the various HAD synthesis using as substrates, aliphatic, amino acid or aromatic amides, namely acetamide, butyramide, glycinamide, alaninamide, phenylalaninamide and benzamide. *P. aeruginosa* strain L10 was grown overnight and the cells were harvested and stored as previously described [30]. Whole cells from *P. aeruginosa* strain L10 were encapsulated in a reverse micellar system of surfactant TTAB in heptane: octanol (80:20) at various reactional conditions.

A sequence of experiments was designed using a Central Composite Design (CCD) [31]. Firstly, it was studied the influence in HAD production of substrate concentration, hydroxylamine concentration, and W_0 = [H_2O] / [surfactant] (Table 1). The various substrates have differences in the solubility which influenced the choice of substrate concentrations to be used in the CCD analysis. Subsequently using the optimal conditions of these factors it was studied the effect in HAD production of surfactant concentration, pH and buffer molarity (Table 2).

For each experiment, the initial velocity and yield of HAD synthesis were determined as described in [21]. Statistical analysis was performed to the experimental data through a second order model relating all the factors, three-dimensional surface plots were drawn to show the effects of the parameters on response and the significance of the model was evaluated using Design Expert software. A set of experiments using the optimum values of the parameters determined by the model were also conducted in order to validate the predicted optimum response of the model.

HPLC-DAD analysis was additionally carried in order to identify and quantify the produced HADs in reaction mixtures. The HPLC analysis was carried in JASCO HPLC system consisting of a model PU-2089 Plus pump, a model CO-2065 Plus oven and a Model MD-2018 Diode-Array Detector. The chromatographic column used was a Macharey-Nagel C18 reverse-phase column Model NUCLEOSIL 100-5 C18 AB at 40ºC and using as eluent acetonitrile: water (30%:70%) for the separation. The concentration of HADs in the reaction mixtures was determined by injecting 25 µL of the reaction mixture to the column using a Model Rheodyne injector. Standards of the different concentrations of the HADs were additionally injected in order to establish calibration curves for each standard. The area of hydroxamic acid derivative retention time peak was determined using the ChromNav software.

Table 1. *Parameters (factors) and levels studied on the first step of central composite design for HADs production, (Subst) used for HADs produntion were: Acetamide, Butyramide, Glycinamide, Alaninamide, Phenylalaninamide and Benzamide. Hydroxylamine ($HyNH_2$) was used as the second substrate. Surfactant concentration was 200 mM TTAB.*

	AcetHA					**ButyHA GlyHA AlaHA PheHA**					**BenzHA**				
Levels / Factor	**-2**	**-1**	**0**	**+1**	**+2**	**-2**	**-1**	**0**	**+1**	**+2**	**-2**	**-1**	**0**	**+1**	**+2**
Subst (mM)	10	20	30	40	50	2.5	7.5	12.5	17.5	22.5	1	2	3	4	5
$HyNH_2$ (mM)	10	25	40	55	70	2.5	10	17.5	25	32.5	2.5	10	17.5	25	32.5
W_0	3	5	7	9	11	5	6.5	8	9.5	11	5	6.5	8	9.5	11

Table 2. *Parameters (factors) and levels studied on the second step of central composite design for analysis of production of HADs.*

	AcetHA					**GlyHA AlaHA**					**ButyHA PheHA BenzHA**				
Levels / Factor	**-2**	**-1**	**0**	**+1**	**+2**	**-2**	**-1**	**0**	**+1**	**+2**	**-2**	**-1**	**0**	**+1**	**+2**
[TTAB] (mM)	50	100	150	200	250	150	180	210	240	270	110	150	180	230	270
HEPES pH (mM)						6.5	7	7.5	8	8.5					
[HEPES] (mM)						10	60	110	160	210					

HADs: AcetHA- Acetohydroxamic acid; ButyHA- Butyhydroxamic acid; GlyHA- Glycine Hydroxamic acid; AlaHA- Alanine Hydroxamic acid; PheHA- Phenylalanine Hydroxamic acid; BenzHA- Benzohydroxamic acid.

3. Results and Discussion

3.1. Biological Activities Studies

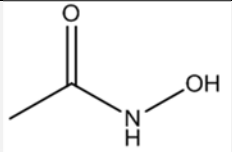
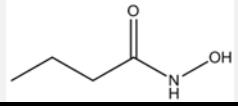
The biological activities of HADs were evaluated, the HADs were assayed both as AChE inhibitors and antioxidants. The capacity of HADs compounds to inhibit AChE was studied and it was seen (Table 3) that the percentage of enzyme AChE inhibition (IE%) was approximately 50% for aliphatic HADs (AcetHA and ButyHA) and aromatic benzohydroxamic acid (BenzHA), using 0.6 mM of these compounds. Amino acid HADs, GlyHA, AlaHA and also PheHA were seen to be poorer AChE inhibitors with IE% lower than 30%. With the lowest IE% value obtained for the PheHA with 14.81%.

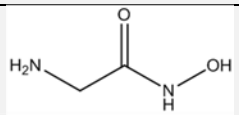
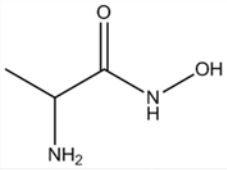
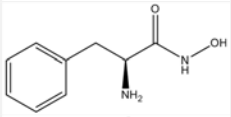
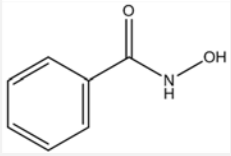
Several other compounds, some of which based on natural products, demonstrated AChE inhibition and are referenced as having therapeutic use, such as alkaloids like galantamine (Table 3), nonalkaloids compounds, plant extracts, fractions and essential oils [32-34]. As mentioned, hydroxamic acids are claimed to be efficient enzyme inhibitors [1, 2], due to their metal chelating properties, however, to the best of author's knowledge, this is the first report of AChE inhibition using these compounds.

Oxidative stress is also an important process in the search for treatment for several diseases including AD [17, 18]. The antioxidant activity of HADs was additionally determined by the DPPH radical assay. The DPPH test aims to measure the capacity of the compounds to scavenge free radicals in solution and the EC_{50} represents the compound concentration providing 50% of antioxidant activity. Table 3 shows the antioxidant activity of the several HADs. These compounds were found to be effective scavengers against DPPH radical and the higher the concentration of the compounds the higher the capacity to scavenge free radicals. The investigated HADs demonstrated remarkable radical scavenging activity and the higher antioxidant potential (lower EC_{50} value) was found for smaller chain compounds such as aliphatic HAD, AcetHA, and amino acid HAD, GlyHA, and for PheHA with EC50 values of 0.26 μM, 0.19 μM and 0.25 μM, respectively. The EC_{50} values obtained were much lower in comparison with butylated hydroxyanisole (BHA, E 320), a known standard frequently used in pharmaceutical and food industry, with referenced EC_{50} of 50 μM [8, 35]. Other studies have reported on the capacity of other hydroxamic acids to exhibit antioxidant activities, however, EC50 values between 36 μM and 0.06 mM were reported [8-10].

Therefore, all the HADs studied show to be promising agents that could be used to protect cell damage due to oxidative stress often associated with a range of disorders such as cancer and chronic neurodegenerative diseases [18, 36, 37]. These HADs also showed to have potential as AChE inhibitors. As mentioned AChE is an enzyme used in the development of drugs for the treatment of several diseases, among which AD [15, 16], on the other side, neurodegenerative diseases can be correlated with oxidative stress [18]. So the development of drugs with both targets is a research going on due to the multifactorial nature of this disease and the inefficiency and secondary effects associated with the drugs in current clinical use [19, 38].

Table 3. *Percentage of inhibition of AChE activity (IE%) and antioxidant activity (EC_{50} values) of hydroxamic acid derivatives (HADs) acetohydroxamic acid (AcetHA), butyrylhydroxamic acid (ButyHA), glycine hydroxamic acid (GlyHA), alanine hydroxamic acid (AlaHA), phenylalanine hydroxamic acid (PheHA) and benzohydroxamic acid (BenzHA) in comparison with butylated hydroxyanisole (BHA).*

		AChE inhibition		**DPPH**
HADs	**Structure**	**[Conc] (mM)**	**IE (%)**	**EC_{50} (μM)**
AcetHA		0.6	50	0.26 ± 0.06
ButyHA		0.6	41.18 ± 1.00	0.42 ± 0.03

		AChE inhibition		DPPH
HADs	**Structure**	**[Conc] (mM)**	**IE (%)**	**EC_{50} (μM)**
GlyHA		0.6	29.51 ± 0.19	0.19 ± 0.07
AlaHA		0.6	23.12 ± 0.74	1.65 ± 0.40
PheHA		0.6	14.81 ± 2.22	0.25 ± 0.05
BenzHA		0.6	50	0.49 ± 0.12
BHA [17]		-	-	50
Galantamine [32]		$0.7x10^{-3}$	50	-

Another important aspect regarding the safety of HADs compounds was to understand the toxic potential of these compounds and therefore three different cell lines, HeLa, HepG2 and MCF-7 cells, were used to screen for the toxicity of the HAD at various concentrations.

It was seen that the cell lines reacted differently to the same compound in a dose-dependent manner, HepG2 cells appeared to be slightly less susceptible to HADs as compared to the other cell lines. The evaluation was conducted in accordance with the Protocol of the American Cancer Institute (NCI), which recommends that the compound concentration inhibiting the growth of 50% of the cells IC_{50} value lower than $\leq$ 4 $\mu g.mL^{-1}$ should be considered significantly toxic [39]. However, our results showed that the HADs used in this study decreased 50% of cancer cell lines viability for concentrations higher than 100 μg/mL, therefore the HADs used in this study were not considered toxic. The *in vitro* assays using mammalian cell cultures avoid the excessive use of laboratory animals which is expensive, time-consuming and involves ethical problems [40]. As cell culture systems can be more sensitive and more reproducible than tests involving intact animals [41] it was reasonable to assume that the studied HADs were considered safe in addition to the previously mentioned potential.

3.2. Optimized HADs Biosynthesis

As mentioned above the HADs showed promising biological activity as antioxidant and AChE inhibitors, and therefore further studies were carried out in order to set the optimal reactional conditions for the HADs synthesis.

As biocatalyst for HAD synthesis, whole cells from *P. aeruginosa* strain L10 containing amidase (E.C. 3.5.1.4.) were used encapsulated in a reverse micellar system of surfactant TTAB in heptane: octanol (80:20). This reactional system used hydroxylamine as acyl group acceptor and several amides were used as substrates, acetamide, butyramide, glycinamide, alaninamide phenylalaninamide or benzamide, to synthesize the HADs AcetHA, ButyHA, GlyHA, AlaHA, PheHA, and BenzHA, respectively.

The reactional conditions for the synthesis of the different HAD studied were optimized according to a Response Surface Methodology. Several authors have used this technique for optimization of bioprocesses [42-44]. By using a central composite design (CCD) methodology, the effect of the variation of the reaction medium composition in the HADs production was investigated. In a first stage substrate amide concentration, hydroxylamine concentration, and micellar water content (W_0) varied accordingly to Table 1 in Materials and Methods section.

Table 4 summarizes the optimum reactional conditions predicted by the model for obtaining the highest values of the yield of HADs synthesis. High correlation coefficients were obtained in the statistical analysis applied to the data from the set of experiments performed, which varied from 0.853 to 0.950 indicating that the data were close to the predicted values, supporting the model.

By analyzing the results obtained in Table 4, it can be seen that in order to obtain higher yields of HADs synthesis, the micellar water content W_0 should be high for aliphatic HADs. A W_0 of 11 was obtained for AcetHA which is in accordance with previous results [25]. As for ButyHA synthesis, a W_0 of 8 was predicted, a W_0 lower than 11 indicates the decrease of the micelles volume [45, 46] which could increase the proximity of the butyramide substrate, which is known to have a lower affinity with the biocatalyst [21], contributing to enhance the biocatalyst performance. This could also explain the use of lower W_0 of 5 for reactions producing amino acid HAD (GlyHA and AlaHA) and BenzHA. As for reactions producing PheHA,

the obtained W_0 10 was high, possibly because the substrate phenylalaninamide is a bulky molecule [21]. As seen by others, the control of the water content using reverse micelles can result in a great improvement of enzyme activity [46-48]

Table 4. *Model predicted conditions of substrates concentration, hydroxylamine ($HyNH_2$) concentration and W_0 for obtaining the best response of yield of corresponding HADs production. Substrates (Subst) used from top to bottom: acetamide, butyramide, glycinamide, alaninamide, phenylalaninamide, and benzamide.*

HADs	[Subst] mM	[$HyNH_2$] (mM)	W_0	Yield (%)
AcetHA	10	40	11	84.70
ButyHA	2.5	17.5	8	89.45
GlyHA	2.5	2.5	5	100
AlaHA	2.5	2.5	5	100
PheHA	2.5	2.5	10	22.34
BenzHA	2.5	32.5	5	56.80

HADs: AcetHA- Acetohydroxamic acid; ButyHA- Butyhydroxamic acid; GlyHA- Glycine Hydroxamic acid; AlaHA- Alanine Hydroxamic acid; PheHA-Phenylalanine Hydroxamic acid; BenzHA- Benzohydroxamic acid.

For amino acid HAD, GlyHA, AlaHA and PheHA, synthesis, small concentrations of substrates and hydroxylamine, 2.5 mM were used for obtaining optimum yield, which is an economic advantage. For all these HADs the ratio between the substrate and co-substrate hydroxylamine concentration was of 1:1. This finding can possibly be explained by the presence of an NH_2 group in the acyl group of the substrates, as can it be seen in the compounds structures of Table 3. According to the Ping Pong Bi Bi system of the reaction [49], the presence of such an NH_2 group could facilitate the transfer of the acyl-enzyme complex to the second substrate hydroxylamine in order to form the HAD. Oppositely, for the production of aliphatic derived, such as AcetHA and ButyHA, and also for BenzHA, an increase in nucleophile concentration (hydroxylamine) relative to the acyl donor substrate is required for obtaining higher yields. This former result was additionally seen in aqueous solutions for reactions using amidase from *Rhodococcus* strain [50].

In this study, the predicted parameters conditions allowed to reach aliphatic and amino acid HADs yields of synthesis higher than 80%. However, lower yields were achieved for aromatic HADs such as PheHA and BenzHA, 22.34 and 56.80%, respectively. Nevertheless, it was clear that the predicted reactional conditions significantly improved the yield of HADs synthesis, especially for the production of ButyHA and BenzHA, as much as 4-6 fold increase, and for PheHA a 3 fold increase, relatively to previous work [21].

Based on these results other reactional conditions, such as surfactant TTAB concentration, HEPES buffer pH, and molarity were further optimized using response surface methodology by analysis at five levels as mentioned in Materials and Methods section Table 2. The optimum conditions determined in Table 4 were maintained.

The model allowed drawing of response surface curves for a yield of HADs production in different reactional conditions, as the represented in Figure 1 for AlaHA production at pH 8.5. The plots of the response surface curves for all HADs produced demonstrated that it was possible to achieve the maximum yield (100%) and to predict the reaction system conditions for obtaining total substrate conversion. The set conditions are resumed in Table 5 for each of the produced HADs.

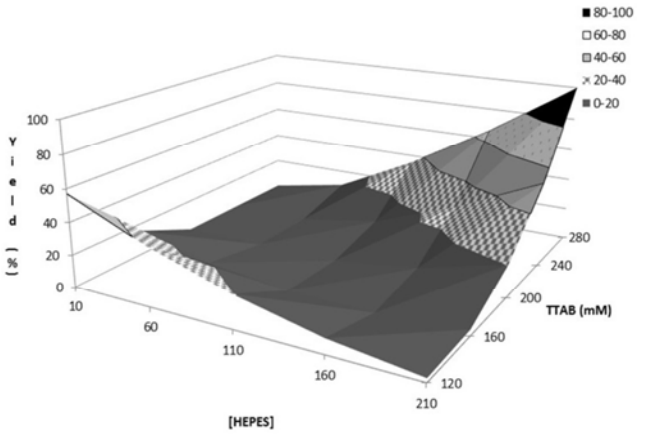


Figure 1. *Response surface plot of central composite design for optimization of the yield of alanine hydroxamic acid (AlaHA) synthesis using P. aeruginosa strain L10 cells in reverse micelles in heptane: octanol (80.20) at pH 8.5 using as substrate 2.5 mM alaninamide and 2.5 mM hydroxylamine. Figures show the interaction between TTAB surfactant concentration and HEPES buffer molarity.*

It was noticed that at the optimum pH, high buffer molarity and high surfactant concentration should be used in the reactional conditions in order to obtain maximum HADs production, as can be seen also in Figure 1. In previously reported work it was shown that the increase in buffer molarity at high surfactant concentration decreased the micellar volume [51] and here confirmed to be fundamental for the efficiency of the reverse micellar system to achieve 100% yield for all the studied HADs synthesis.

3.3. Validation Studies

In order to evaluate if the conditions predicted by the model for the reaction parameters were able to achieve the yield of HADs synthesis, independent experiments were performed for each HADs under the optimal reactional estimated conditions. The obtained experimental results for the set conditions are shown in Table 5. Table 5 displays the results obtained for the quantification of HADs synthesis, both using a colorimetric assay and HADs HPLC-DAD analysis described in Materials and Methods section. The results obtained in both cases were similar. As it can be seen in Table 5 an average yield of HADs of (90 – 100%) was achieved under the reaction conditions which allowed to conclude that the reactional conditions established are well within the range predicted by the model.

4. Conclusions

In conclusion, the search for antioxidant and additionally

AChE inhibitors clearly appears as a research worth focusing on due to the multifactorial nature of some disorders such as AD, neurodegenerative diseases and cancer [17-19]. Only a few compounds are known to present this ability, the several HADs studied in this work were tested and showed not to be toxic, have a great potential both as antioxidant and as AChE inhibitors, rendering them also as promising lead structures in the search for multifunctional agents. To author's knowledge, this study is also the first report of the HADs as AChE inhibitors. This work also enabled the successful synthesis by biocatalysis, with high yields, of these HADs in the reverse micellar system of surfactant TTAB in heptane: octanol using as biocatalyst the intact cells of *P. aeruginosa* strain L10 containing enzyme amidase. The use of intact cells, immobilization, and reaction in organic solvents is a simple and rapid approach to overpass the limitation of the catalysis and an alternative to the chemical synthesis of these important compounds with the additional advantage to allow high yields of production of various HADs using the same biocatalyst.

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