

# Characterization of a novel amine transaminase from *Halomonas elongata*

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## 1. Introduction

Classification of transaminases have recently been reviewed [1]. Amine transaminases are most well known for asymmetric chiral amine synthesis and some are also able to convert  $\omega$ -amino acids [2]. They do not require the presence of carboxylic moieties in the substrate to convert chiral amines. Transaminases are a family of enzymes with high potential in biotechnological applications. They can be very useful for the enantioselective production of a series of compounds with high value such as chiral amines and enantiopure amino alcohols which find use in many chemical fields; above all, for the synthesis of biologically active compounds (Scheme 1) [3,4]. The synthesis of enantiopure amines by enzymatically catalyzed reactions presents several advantages as an

alternative to traditional approaches such as mild reaction conditions, high stereoselectivity, fewer synthetic steps, potential total substrate conversion and no environmental issues unlike in the case of transition metal catalysts [5,6]. However to be recognized as economically viable, the catalyst must have a high space-time yield, which refers to the amount of product produced per quantity of catalyst per unit time [7].

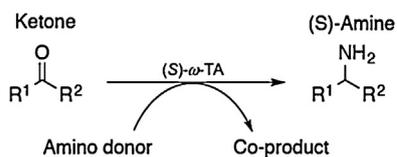
Within the class of  $\omega$ -transaminases, amine transaminases play a central role in the biocatalytic preparation of enantiopure amines. Some transaminases displayed high turnover rate, high stability, broad substrate specificity and no requirement for external cofactors [8,9]. Issues such as unfavourable equilibrium constant, inability to accept larger molecules, substrate and product inhibition and reduced stability at extreme pHs, temperature and organic solvents are often still challenging but by protein engineering several aspects can be addressed. For instance, the remarkable work by Savile et al. led to an engineered version of ATA-117, an (*R*)-selective variant of the  $\omega$ -transaminase from *Arthrobacter* sp. which was able to accept the very bulky substrate precursor of sitagliptin [10].

Recently, an alternative substrate to the use of an excess of the amino donor to shift the equilibrium of the reaction has been reported by O'Reilly [11]. Still, it is apparent that the pool of

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**Scheme 1.** Asymmetric synthesis by  $\omega$ -transaminase.

enzymes effectively suitable for these biotransformations is limited and there is scope to investigate new sources and novel enzymes [12].

As part of a larger project with the aim to identify new biocatalysts with particular characteristics such as high temperature resistance, organic solvent compatibility and uncommon substrate specificity, we focused our interest on enzymes from extremophiles. The adaptation process for extremophiles has affected the features of their enzymes providing them with improvements in respect to their mesophilic counterparts [13]. In particular, we have developed an interest in halophilic organisms and their enzymes as they often have a remarkable tolerance to organic solvents [14–16].

In this study we identified, cloned, and expressed a putative amine transaminase (HEWT) from the genome of the bacterium *Halomonas elongata*; this is a moderate halophile which evolved an organic-osmolyte strategy to overcome the high osmotic pressure of its natural environment [17]. *H. elongata* does not exhibit the extensive adaptation of the intracellular macromolecules present in archaea halophiles, which preferentially evolved to maintain their proper osmotic pressure by the accumulation of high cytoplasmic concentration of potassium chloride [18]. On the contrary, *H. elongata* preserves an appropriate cytoplasm osmotic pressure by accumulation and/or biosynthesis of organic solute.

Since *H. elongata* is a haloadapted bacterium, it allows for heterologous expression of its proteins inside a common mesophilic host like *Escherichia coli* hence avoiding less conventional expression systems required for true halophilic proteins [19,20]. *H. elongata* aminotransferase sequence presents an identity of 38% with the  $\omega$ -TA from *Vibrio fluvialis* and 55% with  $\omega$ -TA from *Chromobacterium violaceum*. The former is the most investigated (and commercially available)  $\omega$ -TA [21]. The latter was identified in a precedent study for its 38% sequence identity with the *V. fluvialis* enzyme and shares with it the substrates preference for aromatic amines [3]. Moreover, the  $\omega$ -TA from *C. violaceum* presents an uncommon selectivity for TAs, exploiting the amination on  $\alpha,\alpha'$ -dihydroxyketones which are not usually accepted as substrates [22,23].

## 2. Materials and methods

### 2.1. *spuC* gene and HEWT bioinformatic analysis

The gene named *spuC* (HELO\_1904) was identified inside the genome of the moderate halophilic bacterium *H. elongata* DSM 2581. The genome of this bacterium was obtained from DSMZ (Braunschweig, Germany). Conserved domain analysis was performed on the amino acid sequence of HEWT using the NCBI conserved domain database (CDD) (<http://www.ncbi.nlm.nih.gov/cdd/>). A specific hit was obtained with the OAT<sub>I</sub> like, acetyl ornithine aminotransferase family a member of the pyridoxal phosphate (PLP)-dependent aspartate aminotransferase superfamily (fold I). The amino acid sequence of HEWT was subsequently submitted to the web based server Phyre2 (Protein Homology/Analogy Recognition Engine) for protein structure prediction [24]. The sequence of the *H. elongata* aminotransferase showed a sequence identity of 55% with 100% of query cover for *C. violaceum* CV2025  $\omega$ -TA

and a sequence identity of 38% with 100% of query cover for *V. fluvialis* js17  $\omega$ -TA. From the sequence alignment was possible to identify the three residues considered to be responsible for the transaminase catalytic activity: D255 Aspartic acid: salt bridge/H-bond to N1 of pyridoxal 5'-phosphate, K284 Lysine: Schiff base with pyridoxal 5'-phosphate, R412 Arginine: salt bridge/H-bond with  $\alpha$ -carboxylate group of substrate [25–27].

### 2.2. Molecular biology tools, bacteria, enzymes and chemicals

Phusion™ DNA polymerase was purchased from Finnzymes (Espoo, Finland). PCR primers production and protein sequencing were performed by Eurofins MWG Operon (Ebersberg, Germany). Molecular weight marker HyperLadder™ I was purchased from Bionline (London, UK). Agarose Gel DNA Extraction Kit, High Pure PCR Product Purification Kit and PD-10Columns were purchased from Roche (Mannheim, Germany). Plasmid extraction kit GeneJET™ Plasmid Miniprep Kit was purchased from Thermo Fisher Scientific GmbH (Dreieich, Germany). T4 DNA Ligase, restriction enzymes *EcoRI* and *BamRI* and broad range protein marker P7702 (2–212 kDa) were purchased from New England Biolabs (Beverly, MA, USA). The pRSET B vector was purchased from Invitrogen (Carlsbad, CA, USA). *E. coli* XL10-Gold® Ultracompetent cells were purchased from Stratagene (San Diego, USA). *E. coli* BL21(DE3) competent cells were purchased from Novagen (Darmstadt, Germany). Ampicillin sodium salt was purchased from Molekula Ltd. (Gillingham, UK). Millex®HV 0.45  $\mu\text{m}$  PVDF filters were purchased from Millipore Ltd. (London, UK). His-tagged protein purification columns Ni-NTA Superflow Cartridges were purchased from QIAGEN GmbH (Hilden, Germany). Stain/DeStain-Xpress™ protein detection kit was provided by EnzoLife Technologies Ltd. EnzoLife Technologies Ltd. (Dublin, Ireland). All chemical reagents, unless stated otherwise, were purchased as analytical grade from Sigma–Aldrich Chemie GmbH (Munich, Germany).

### 2.3. Construction of the expression vector

The *spuC* gene was amplified by PCR from genomic DNA of *H. elongata* with Phusion DNA polymerase with the oligonucleotide primers FWD-*spuC* (5'-AAAGGATCCGATGCAAACCAAGACTATCAGG-3') and REW-*spuC* (5'-AAAGAATTCTCATGCCGTTGGCTCCTCTTGC-3'). The primers were designed to incorporate *BamHI* and *EcoRI* restriction sites, respectively (underlined). The manufacturer's protocol for Phusion DNA polymerase PCR in 50  $\mu\text{L}$  reaction was used. PCR was carried out with 1 U of enzyme in presence of *H. elongata* genome (2 ng/ $\mu\text{L}$ ) and the two primers (0.5  $\mu\text{M}$  each) with the following program: 3 min of initial denaturation at 95 °C and 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 68 °C and 50 s extension 72 °C. The 35 cycles were followed by 5 min of final elongation at 72 °C. The gel-purified PCR product was digested with *EcoRI* and *BamHI* for 16 h following the manufacturer's guidelines and cloned into digested *pRSET B* and used to transform *E. coli* XL10-Gold® Ultracompetent cells. The construct was verified by sequencing and the obtained plasmid was named *pHESPUC*.

### 2.4. Expression of the *spuC* gene in *E. coli*

*E. coli* BL21(DE3) competent cells were transformed with *pHESPUC* plasmid. Expression was tested both in LB broth and ZYM-5052 auto-induction medium.

With the first method, cells were grown in 300 mL LB broth in the presence of ampicillin 100  $\mu\text{g}/\text{mL}$  at 37 °C shaking at 200 rpm starting with a 3 mL inoculum of seed culture previously grown overnight. The culture was grown until an OD<sub>600</sub> of around 0.6 was reached and then the expression was induced with isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) 1 mM and the culture was allowed

to grow at 37 °C, shaking at 200 rpm. After 20 h the cells were harvested, weighed and then frozen at -20 °C.

With the second method, cells were grown in 300 mL of ZYM-5052 medium for auto-induction [28] (*N*-Z-Amine 1%, yeast extract 0.5%, KH<sub>2</sub>PO<sub>4</sub> 50 mM, Na<sub>2</sub>HPO<sub>4</sub> 50 mM, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 25 mM, NaSO<sub>4</sub> 5 mM, MgSO<sub>4</sub> 2 mM, Glycerol 0.5%, Glucose 0.05%, α-Lactose 0.2%, 1000x trace elements 0.2%) in presence of ampicillin 100 µg/mL at 37 °C shaking at 200 rpm. The cells were harvested when the OD<sub>600</sub> of the culture stopped increasing, weighed and then frozen at -20 °C.

The pellet was thawed and resuspended in the lysis buffer containing potassium phosphate buffer pH 8.0 (50 mM) and pyridoxal 5'-phosphate (PLP) (0.1 mM) employing 7.5 mL per gram of pellet. Cells were disrupted by sonication at 4 °C using Sonics VCX 130 with 10 cycles of 30 s of sonication and 30 s of cooling. After centrifugation (38,000 × *g*, 4 °C, 45 min), the supernatant was clarified by filtration (0.45 µm filter) before chromatography and the activity of the raw extract was tested with the spectrophotometric enzymatic assay.

### 2.5. Purification

Chromatography was performed using an ÄKTA Purifier (GE Healthcare, Little Chalfont, UK). The filtered cellular extract was taken up in 0.5 M NaCl and loaded into a 5 mL column of Ni-NTA Superflow pre-charged with NiSO<sub>4</sub> (0.1 M). The column was washed at a flow rate of 5 mL/min with at least 10 column volumes of phosphate buffer (pH 8, 50 mM, containing 100 mM NaCl, 0.1 mM PLP and 30 mM imidazole), and the enzyme was eluted with phosphate buffer (pH 8, 50 mM, containing 100 mM NaCl, 0.1 mM PLP and 300 mM imidazole) after an intermediate step with the 10% of elution buffer to remove non-specifically bound proteins. The fractions containing the enzyme were desalted *via* size exclusion chromatography with a 20 mM phosphate buffer, pH 8, containing 0.1 mM PLP. The purified enzymes were conserved at 4 °C for short term storage or -20 °C for long term storage. The enzymatic activity was tested following the spectrophotometric enzymatic assays (see below) and the obtained pure enzyme was named with the acronym HEWT (*Halomonas elongata* ω-transaminase).

### 2.6. Size exclusion chromatography

The molecular mass of the native HEWT was determined by size exclusion chromatography on a Superdex 200 high resolution 10/30 column with a total bed volume of 24 mL (GE Healthcare) equilibrated with 20 mM sodium phosphate buffer, pH 8.0, containing 150 mM NaCl. Purified enzyme solution (100 µL) was injected and the experiment was run at a flow rate of 0.2 mL/min. Fractions were collected and those corresponding to the eluted peak were assayed for activity to confirm the protein was still correctly folded. Protein molecular weight markers (Sigma-Aldrich) used to prepare the calibration curve were β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa), with Blue Dextran (2000 kDa) being used to indicate the void volume. The samples for the calibration curve were run in 50 mM sodium phosphate buffer, pH 8.0.

### 2.7. Protein determination and SDS-PAGE analysis

The concentration of the purified protein was determined spectrophotometrically by UV absorption at 280 nm using urea 6 M as denaturing agent. The extinction coefficient 60,850 M<sup>-1</sup> cm<sup>-1</sup>, at 280 nm, measured in water, was estimated by ExpASy ProtParam tool, accessible from the ExpASy website (www.expasy.ch) [29]. SDS-PAGE was carried out employing a 12% polyacrylamide gels, stained with Coomassie Brilliant Blue R250 [30] and a broad range

protein marker (2–212 kDa) was used for determination of relative molecular weight.

### 2.8. Spectrophotometric enzymatic assays

A kinetic assay derived from Schatzle et al. [31] was used as standard enzymatic assay using pyruvate as the amino acceptor except where stated otherwise. The reactions were carried out at 25 °C employing a reaction mixture containing 1 mL phosphate buffer (50 mM, pH 8), 2.5 mM (*S*)-(-)-1-phenylethylamine ((*S*)-(-)-α-methylbenzylamine), 2.5 mM pyruvate, 0.25% DMSO, 0.1 mM PLP and an appropriate amount of enzyme. These parameters were modified in order to investigate the behaviour of the enzyme in different reaction conditions. Modifications to the standard assay are indicated when applied. The activity was estimated following the production of acetophenone during the first three minutes of reaction at 245 nm using a Varian Cary 50 Scan UV-Visible spectrophotometer equipped with a Cary single cell Peltier temperature controller.

In the study of the effect of the pH on enzyme activity and stability of the enzyme, instead of the phosphate buffer, a universal buffer was employed. This system contains 25 mM citric acid, 25 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM Tris, 12.5 mM Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, and 25 mM KCl [32,33].

The effect of additives on the transaminase was checked by incubating the enzyme at 25 °C in presence of the indicated concentration of each chemical. For the test on enzyme stability at different additive concentrations the solutions of additives were prepared in phosphate buffer pH 8, 0.5 M and the pH was adjusted to pH 8 before use (Fig. 4A). For the effect of interaction between (*S*)-(-)-1-phenylethylamine (5 mM) and pyridoxal 5'-phosphate (5 mM) on the enzyme stability phosphate buffer pH 8, 0.05 M was employed, (Fig. 4B). The residual activity was measured in both cases with the standard spectrophotometric assay.

The effect of cosolvents on HEWT activity and stability was studied storing the enzyme in the presence of either 10 or 20% (v/v) of cosolvent at 4 °C for 22 h. Before and after incubation the residual activity was determined with the standard spectrophotometric enzymatic assay in presence of the corresponding amount of cosolvents.

### 2.9. Enzymatic reaction

The enzymatic reactions were carried out at 37 °C in pH 8.0 potassium phosphate buffer 100 mM and 15% v/v purified enzyme solution corresponding to 0.89 U/mL for the screening of the amino donors, and 0.71 U/mL for the screening of the amino acceptor reactions. The reaction mixture contained 10 mM enantiopure amino donor (20 mM if racemic), 10 mM amino acceptor and 0.05 mg/mL of HEWT in a reaction volume of 200 µL. For the determination of enantioselectivity, reactions were carried out at 37 °C in pH 8.0 potassium phosphate buffer 100 mM in 1 mL, containing 20 mM *rac*-1-phenylethylamine, 10 mM pyruvate, 0.1 mM PLP with 0.1 mg/mL of HEWT. As a control, the reactions were set up as previously described but without the addition of HEWT, in which case only starting material was detected. The activity of the enzyme in the various reaction conditions was established employing (*S*)-(-)-1-phenylethylamine as amino donor and pyruvate as acceptor. One enzymatic unit was defined as the amount of enzyme that converts 1 µmol of (*S*)-(-)-1-phenylethylamine in 1 min. The initial rate was determined by monitoring the decrease of the concentration of (*S*)-(-)-1-phenylethylamine during the first three minutes. Frozen aliquots of the enzyme at -20 °C were used. *H. elongata* ω-transaminase was stable for several months without evident loss

of activity. Data were obtained by averaging the measurements on three independent samples.

## 2.10. Analytical methods

### 2.10.1. Spectrophotometric analysis

For the amino donor, final conversion and the relative initial rate were estimated spectrophotometrically employing a Varian Cary 50 Scan UV-Visible spectrophotometer equipped with a Cary single cell Peltier temperature controller. The concentration of pyruvate was determined after derivatization with *o*-phenylenediamine at a molar ratio of 20:1 respect to the initial concentration of the ketoacid in 80% acetic acid at 50 °C for 10 mins, followed by 1:10 dilution, and by measuring the adsorption at 337 nm of the corresponding quinoxaline derivative. The calibration curve of the quinoxaline derivative was determined at seven different concentrations ranging from 0 to 0.1 mM and for each reaction run, a sample was taken every 15–30 s to generate time points. Each run was performed in triplicate [34]. In this concentration range, a linear correlation between absorbance and concentration was observed. Errors are reported as standard deviations, where <10% was deemed acceptable. The kinetic catalytic constants  $V_{max}$  and  $K_m$  were measured at pH 8.0 and 25 °C using the two spectro-photometric coupled assays as described above in the presence of 0.25% DMSO, 0.1 mM PLP and an appropriate amount of enzyme (0.1 mg/mL). Reactions were carried out at 5 mM pyruvate and various concentrations (0.5–10 mM) of (*S*)-(-)-1-phenylethylamine, at 5 mM (*S*)-(-)-1-phenylethylamine and various concentrations (0.05–5 mM) of pyruvate. Concentrations higher than 5 mM of (*S*)-(-)-1-phenylethylamine gave significant interference with the UV detection signal and for this reason it was kept below saturation conditions. The initial-velocity data were fitted to the Michaelis-Menten equation using SigmaPlot software (Version 11.0) [35].

### 2.10.2. HPLC analysis

The relative initial reaction rate and the final conversion of the different amino acceptor were determined employing a Shi-madzu HPLC instrument equipped with a Supelcosil LC-18-T (250 mm × 4.6 mm, 5 μm particle size; Supelco, Sigma-Aldrich, Germany). The substrates were detected at 210 nm after an isocratic run with 25% acetonitrile/ 75% trifluoroacetic acid (TFA) 0.1%v/v at 25 °C with a flow rate of 1 mL/min. The depletion of aromatic amines, aldehydes and the formation of acetophenone were evaluated by calibration curve. The samples were injected after a dilution of 1:50 with TFA 0.2% in the quenching step (the reactions were monitored at 1, 2, 3, 5 and 10 mins by quenching an aliquot of the mix, in triplicates, generating 5 time-points per slope); at this concentration (≤0.2 mM) only the aromatic compounds are detectable at 210 nm between 5 and 25 mins [36]. The retention times in minutes are: acetophenone (16.10), benzaldehyde (14.42), benzylamine (7.23), cinamaldehyde (16.93), (*S*)-(-)-1-phenylethylamine (7.45), phenylacetaldehyde (10.94), 2-phenethylamine (13.80), 2-phenylpropionaldehyde (13.31), vanillin (5.73). All samples were quenched with TFA 0.2% and then centrifuged before the HPLC analysis. To facilitate the enantiomeric separation by chiral HPLC analysis, *rac*-1-phenylethylamine was derivatized to the corresponding amides by adapting the method reported by Andrade et al. and Codex® ATA Screening Kit [37]. Addition of 100 μL 5 M NaOH to the sample was followed by extraction in 900 μL methyl-*tert*-butyl ether (MTBE) and then by addition of 5 μL triethylamine and 5 μL acetic anhydride. For the determination of enantioselectivity, a normal-phase HPLC method was performed using an Agilent Technologies 1200 equipped with a Chiralpak® IC (250 mm × 4.6 mm, 5 μm particle size). Employing this method, product and starting materials were separated with

retention times in minutes: acetophenone (7.14), derivatized (*R*)-(+)-1-phenylethylamine (16.72) and (*S*)-(-)-1-phenylethylamine (19.14). Detection at 254 nm after an isocratic run 95:05 Heptane:Ethanol at 25 °C with a flow rate of 1 mL/min.

## 3. Results and discussion

The *spuC* gene was identified as a putative aminotransferase from *H. elongata*. The gene was cloned into the vector *pRSET B*, expressed in *E. coli* BL21(DE3) and purified by metal affinity chromatography. The His-tagged enzyme was characterized at different temperatures, pHs, salt concentrations and in the presence of different cosolvents and additives. The substrate specificity of the transaminase was studied against a library of amino donors and amino acceptors and compared with the  $\omega$ -TA from *V. fluvialis* and *C. violaceum*.

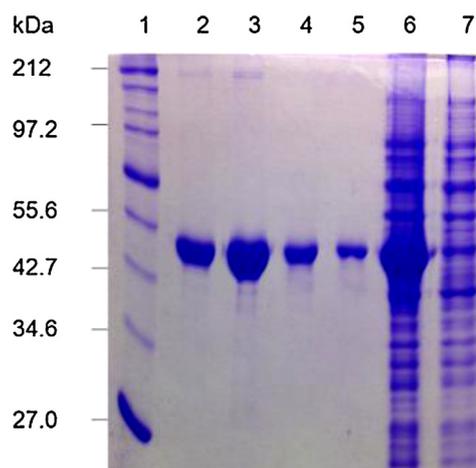
### 3.1. Purification and molecular mass of HEWT

The expression of the his-tagged transaminase was performed and compared in two different media: LB broth with IPTG and ZYM-5052 auto-induction medium and purified by immobilized metal affinity chromatography (IMAC). In both cases, the enzyme was obtained in the pure form (Fig. 1). The protein was expressed at higher concentration in auto induction medium (Purified protein specific activity 4.51 U/mg – Volumetric yield 40.6 mg/L) in comparison to the LB/IPTG broth (Purified protein specific activity 2.67 U/mg – Volumetric yield 10.9 mg/L). The enzyme was named with the acronym HEWT (*Halomonas elongata*  $\omega$ -transaminase). The molecular mass of native HEWT was estimated to be 109 kDa from the calibration curve, which indicated a dimeric quaternary structure (theoretical value 108 kDa).

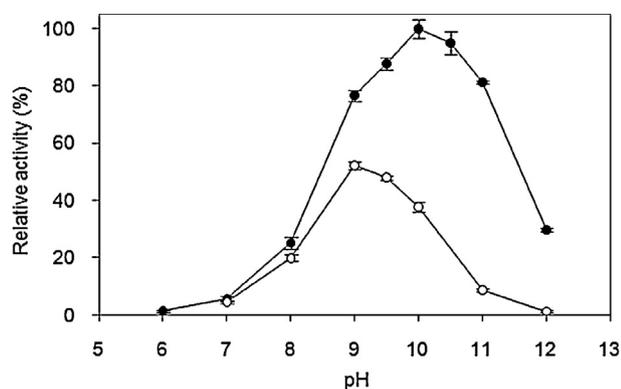
SDS-PAGE of *H. elongata* transaminase purification fractions. Lanes: 1. Protein marker (2–212 kDa), 2–5. Four different purification fractions containing different concentrations of the enzyme obtained with auto induction medium, 6. Cell-free extract, 7. Affinity column flow-through.

### 3.2. Optimal operation pH and temperature

The effect of pH on HEWT activity are reported in Fig. 2. HEWT activity reached the maximum at pH 10, but the catalyst was more



**Fig. 1.** SDS-PAGE of *H. elongata* transaminase purification fractions. Lanes: 1. Protein marker (2–212 kDa), 2–5. Four different purification fractions containing different concentrations of the enzyme obtained with auto induction medium, 6. Cell-free extract, 7. Affinity column flow-through.



**Fig. 2.** pH effect on enzyme activity (●) and stability (○). HEWT preparation was diluted 1:10 with a universal buffer solution at the desired pH and stored at 4 °C for 30 min. After incubation the residual activity was determined with the spectrophotometric enzyme assay carried out at each test conditions and 100% corresponds to 2.58 U/mL. Every reaction was performed in three replicates and the results are reported as the average of the data obtained.

stable at lower pHs. The optimal balance between activity and stability was found around pH 8–9.

pH effect on enzyme activity (●) and stability (○). HEWT preparation was diluted 1:10 with a universal buffer solution at the desired pH and stored at 4 °C for 30 mins. After incubation the residual activity was determined with the spectrophotometric enzyme assay carried out at each test conditions and 100% corresponds to 2.58 U/mL. Every reaction was performed in three replicates and the results are reported as the average of the data obtained.

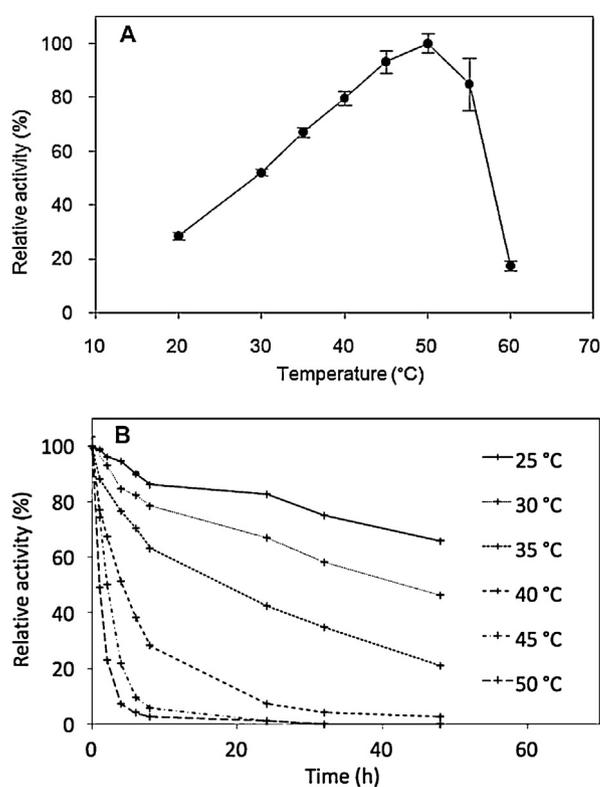
The effect of temperature on HEWT activity is displayed in Fig. 3A. Thermal stability was tested after incubation of the enzyme at variable temperatures as reported in Fig. 3B. The highest activity was reached at 50 °C and the stability is well preserved for temperatures up to 35 °C over 24 h.

Temperature effect on enzyme activity (A) and stability (B). The activity was determined with the spectrophotometric enzyme assay performed at the indicated temperature. For the stability test, the enzyme was stored in phosphate buffer 50 mM pH 8 in the presence of pyridoxal 5'-phosphate (PLP) 1 mM. The analysis of the residual activity was carried out at 25 °C and 100% corresponds to 3.05 U/mL. Every reaction was performed in three replicates and the results are reported as the average of the data obtained.

### 3.3. Effect of additives on enzyme stability

The stability of HEWT in the presence of organic molecules involved in the standard reaction was determined. The enzyme was stored with different concentrations of (S)-(-)-1-phenylethylamine, pyruvate and pyridoxal 5'-phosphate (PLP) at 25 °C and the activity was monitored over time under standard conditions (Fig. 4A). An increase in the enzyme stability was found at increasing pyruvate concentrations, while increasing the amine concentration resulted in a progressive inactivation of the catalyst. The destabilizing effect of (S)-(-)-1-phenylethylamine is, in any case, significantly lower with respect to what reported for *V. fluvialis* ω-TA: in that case the enzyme was completely inactivated when the concentration of the amine was higher than 5 mM [25]. The simultaneous presence of PLP with (S)-(-)-1-phenylethylamine alleviates the inactivating effect of the aromatic amine (Fig. 4B).

PLP is the prosthetic group of the enzyme and is necessary for enzymatic activity, but its concentration affects the enzyme stability. It is well known that PLP usually presents a destabilizing effect on ω-TAs at concentrations higher than 1 mM [25], therefore it is commonly used within 1 and 0.1 mM in enzymatic reactions



**Fig. 3.** Temperature effect on enzyme activity (A) and stability (B). The activity was determined with the spectrophotometric enzyme assay performed at the indicated temperature. For the stability test, the enzyme was stored in phosphate buffer 50 mM pH 8 in the presence of pyridoxal 5'-phosphate (PLP) 1 mM. The analysis of the residual activity was carried out at 25 °C and 100% corresponds to 3.05 U/mL. Every reaction was performed in three replicates and the results are reported as the average of the data obtained.

and storage buffer [38,39]. As shown, the enzyme at concentrations greater than 5 mM of cofactor loses the activity significantly faster than 0.1 mM, confirming the destabilization effect (Fig. 4A).

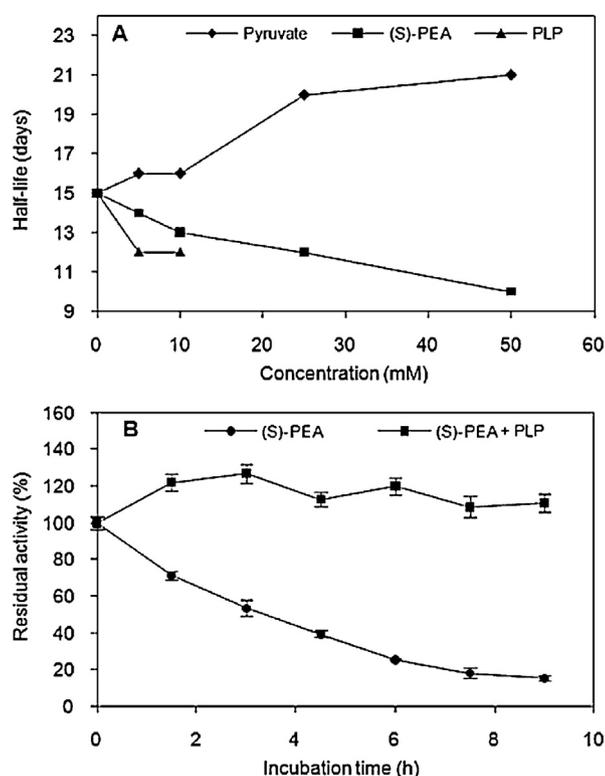
Enzyme stability at different additive concentrations (A). Stability interaction of pyridoxal 5'-phosphate and (S)-(-)-1-phenylethylamine (PEA), both 5 mM, on the enzyme (B). Every reaction was performed in three replicates and the results are reported as the average of the data obtained.

### 3.4. Effect of salt concentration and co-solvents on enzyme stability

The addition of a co-solvent to the reaction mixture was expected to affect both enzyme activity and stability. The effects of a selection of common water-miscible organic solvent were investigated (Table 1). In all cases, a decrease in enzyme activity was observed. However, the transaminase stability was unaffected by organic solvents. Hence, this enzyme is suitable for enantioselective amination of ketones and aldehydes where the presence of a co-solvent is necessary due to the poor solubility of the substrate in water.

The effect of different salt concentrations on enzyme stability and activity was also investigated. In all conditions, enzyme activity decreased with the increase of salt concentration, when compared to the enzyme at pH 8.0 in 50 mM phosphate buffer (Table 2). On the other hand, the salt presence shows from little to no effect on the stability of the enzyme.

Co-solvent effect on HEWT activity and stability. The activities are reported as relative values expressed respect to the activity of the reaction carried out with no co-solvent. Every reaction was



**Fig. 4.** Enzyme stability at different additive concentrations (A). Stability interaction of pyridoxal 5'-phosphate and (S)-(-)-1-phenylethylamine (PEA), both 5 mM, on the enzyme (B). Every reaction was performed in three replicates and the results are reported as the average of the data obtained.

**Table 1**

Co-solvent effect on HEWT activity and stability. The activities are reported as relative values expressed respect to the activity of the reaction carried out with no co-solvent. Every reaction was performed in three replicates and the results are reported as the average of the data obtained.

Co-solvent ratio	Relative activity (%)			
	10%		20%	
Incubation time	0 h	22 h	0 h	22 h
No co-solvent	100 ± 3	100 ± 6	100 ± 3	100 ± 6
MeOH	78 ± 4	68 ± 2	48 ± 4	47 ± 4
DMSO	51 ± 1	42 ± 2	38 ± 2	27 ± 5
EtOH	40 ± 1	46 ± 4	23 ± 5	25 ± 2
tBuOH	34 ± 6	36 ± 3	19 ± 1	20 ± 3
iPrOH	28 ± 1	30 ± 1	16 ± 6	16 ± 1
ACN	26 ± 3	n.d.	8 ± 1	n.d.
nPrOH	17 ± 4	n.d.	0	n.d.
THF	4 ± 5	n.d.	0	n.d.

Not determined (n.d.).

**Table 2**

Effect of the salt concentration on enzyme activity and stability. Incubation was carried out at the indicated salt concentration for 1.5 h and 24 h at 4 °C. The residual activity of the enzymatic solution was determined by the standard spectrophotometric assay carried out in presence of the salt concentration indicated and expressed in function of the activity under standard conditions. Every reaction was performed in three replicates and the results are reported as the average of the data obtained.

Incubation time	Relative activity (%)		
	0 h	1.5 h	24 h
Phosphate buffer 50 mM	100 ± 0.8	100 ± 1.5	100 ± 4.7
KCl 1 M	57 ± 2.5	55 ± 2.5	50 ± 0.9
KCl 2 M	49 ± 2.0	46 ± 6.5	36 ± 1.6
NaCl 1 M	42 ± 0.8	38 ± 0.4	40 ± 1.4
NaCl 2 M	27 ± 3.2	27 ± 2.0	29 ± 0.8
NaCl 3 M	21 ± 0.5	19 ± 2.2	19 ± 0.4

performed in three replicates and the results are reported as the average of the data obtained.

Effect of the salt concentration on enzyme activity and stability. Incubation was carried out at the indicated salt concentration for 1.5 h and 24 h at 4 °C. The residual activity of the enzymatic solution was determined by the standard spectrophotometric assay carried out in presence of the salt concentration indicated and expressed in function of the activity under standard conditions. Every reaction was performed in three replicates and the results are reported as the average of the data obtained.

### 3.5. Amino donor spectra

We investigated also a range of amino donors (Table 3). Interestingly, this enzyme accepts isopropylamine as amino donor with a relative initial rate 3-4 times higher respect to *C. violaceum* and *V. fluvialis*  $\omega$ -TAs with 41% of final conversion. This is a desirable substrate for  $\omega$ -transaminases and the ability to accept efficiently this compound as amino donor in an aqueous media is not common for  $\omega$ -TAs [40]. The  $\omega$ -TA from *V. fluvialis*, for example, had to be engineered in order to efficiently accept aliphatic amines like isopropylamine [5]. This donor has been increasingly used due the fact that it is cost-effective, easily obtainable and the generated acetone which can be easily removed thus shifting the equilibrium to the products side [41]. Alternative methods used to overcome the low equilibrium constant of the transaminase reaction require the presence of NADH/NAD<sup>+</sup> and other two enzymes in addition of  $\omega$ -TA, and this affects the overall cost of the process. On the other hand, employing isopropylamine the equilibrium can be shifted simply acting on reaction conditions such as substrate concentration, temperature and pressure [10,42-44].

HEWT shares a similar characteristic with *C. violaceum* and *V. fluvialis*, as it does not accept  $\beta$ -alanine and L-serine as substrates. HEWT lacks the particular preference showed by the *C. violaceum*  $\omega$ -TA for the racemic form of 1-phenylethylamine and presents a more common behavior exhibiting higher activity on the (S) enantiomer. HEWT is highly (S)-selective, being able to fully convert (S)-1-phenylethylamine to acetophenone and showing no detectable activity with the opposite enantiomer; a resolution of the racemate stops at 50% of conversion permitting the recovery of (R)-1-phenylethylamine with e.e > 99% (E > 100). This high stereoselectivity prompted further investigations into the asymmetric synthesis of amines; moreover, its ability to process  $\alpha,\alpha'$ -dihydroxyketones could be extended to chiral amino alcohols [45,46].

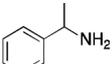
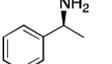
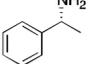
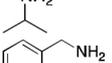
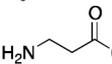
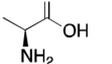
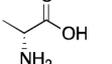
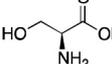
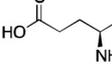
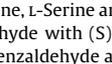
Fig. 3 HEWT specificity on different amino donor are reported in comparison with the data relative to *C. violaceum*  $\omega$ -TA [3] and *V. fluvialis*  $\omega$ -TA [47] obtained in similar conditions. The enzymatic reactions and the initial reaction rates and final conversions with HEWT are reported in the experimental section. Every reaction was performed in three replicates and the results are reported as the average of the data obtained. The detection limit of pyruvate after derivatization was 5%.

### 3.6. Amino acceptor spectrum

A library of compounds was selected in order to investigate the characteristics of the new amine transaminase in comparison to the two well-known structural homologues from *C. violaceum* and *V. fluvialis* [47] (Table 4). In spite of a higher sequence identity with the former, HEWT shows initial rates that are more comparable with the *V. fluvialis*  $\omega$ -TA activity. Higher initial reaction rates were measured for pyruvate, propanal and phenylacetaldehyde. Similarly to the other two transaminases, HEWT is inactive or shows a very low reaction rate with D-fructose, L-ribulose, 3-hydroxy-2-butanone and  $\alpha$ -ketoglutarate. The absence of activity

**Table 3**

HEWT specificity on different amino donor are reported in comparison with the data relative to *C. violaceum*-TA [3] and *V. fluvialis*-TA [47] obtained in similar conditions. The enzymatic reactions and the initial reaction rates and final conversions with HEWT are reported in the experimental section. Every reaction was performed in three replicates and the results are reported as the average of the data obtained. The detection limit of pyruvate after derivatization was 5%.

		<i>H. elongata</i> ω-TA		<i>C. violaceum</i> ω-TA [36]	<i>V. fluvialis</i> ω-TA [25]
		Final conversion (%)	Relative initial rate (%)	Relative initial rate (%)	Relative initial rate (%)
Amines					
rac-1-Phenylethylamine		88 ± 1	100 ± 6	100	100
(S)-(-)-1-Phenylethylamine		97 ± 1	106 ± 10	69.4	112.0
(R)-(+)-1-Phenylethylamine		<5	<5	<4	<1
Isopropylamine		41 ± 2	15 ± 3	<4	3.6
Benzylamine		64 ± 1	81 ± 5	133.9	115.6
β-Alanine		<5	<5	<4	<1
L-Alanine		41.0 ± 1.1 <sup>a</sup>	66.2 ± 1.0 <sup>a</sup>	165.0	10.0
D-Alanine		<5 <sup>a</sup>	<5 <sup>a</sup>	n.d.	<1
L-Serine		19.6 ± 0.2 <sup>a</sup>	8.2 ± 0.7 <sup>a</sup>	9.0	6.4
L-Glutamate		<5 <sup>a</sup>	<5 <sup>a</sup>	n.d.	<1

<sup>a</sup> The reactivity of L-Alanine, D-Alanine, L-Serine and L-Glutamate was measured employing benzaldehyde as amino acceptor. The relative initial rate is reported compared to the rate of conversion of benzaldehyde with (S)-(-)-1-phenylethylamine (1.41 ± 0.04 U/mg). Initial reaction rates and final conversion with HEWT were determined respectively by the concentration of benzaldehyde and benzylamine in the reaction measured by HPLC analysis. The detection limit of both the compound is 5%.

with α-ketoglutarate and glutamic acid, confirms the nature of the enzyme as a ω-TA. As expected, the reactivity with aldehydes and α-ketoacids is higher with respect to the ketones due to the relative low electrophilicity of their carbonyl group compared to aldehydes and ketoacids. Bulky aldehydes such as vanillin, cinnamaldehyde, and 2-phenylpropionaldehyde are well accepted by the enzyme Table 5.

Of particular interest are the activities with 1,3-dihydroxyacetone and L-erythrulose. Usually, in fact, α,α'-dihydroxyketones are not accepted by TAs; two notable exceptions are the ω-TAs from *C. violaceum* [22,23] and *P. aeruginosa* [48]. The lack of activity with D-fructose and L-ribulose could be caused by the excessive bulk of such substrates and impediment to be accommodated in the catalytic pocket. The final conversion was determined after 24 h of reaction time, even though it is accepted that the reactions reaches the equilibrium within 2–3 h. Complete conversions were observed for pyruvate, glyoxylate and benzaldehyde. For other compounds, like phenylacetaldehyde and propanal, despite a good initial rate, the reaction stopped before completion. This could be due to several reasons such as product inhibition and the chemical equilibrium.

Fig. 4 HEWT specificity with different amino acceptors are reported in comparison with the data reported for *C. violaceum* ω-TA [3] and for *V. fluvialis* ω-TA obtained in similar conditions. The enzymatic reactions with HEWT were carried out as reported in materials and methods. Every reaction was performed in three

replicates and results reported as the average value. The detection limit of (S)-(-)-1-phenylethylamine was 5%

### 3.7. Amine synthesis

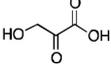
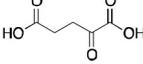
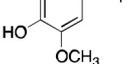
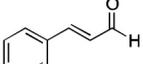
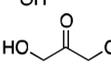
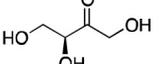
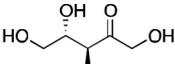
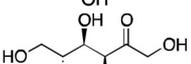
Five amino acceptors were tested with isopropylamine and *o*-xylylenediamine to assess the synthetic scope of HEWT. Although the equilibrium is unfavourable, increasing the amino donor equivalents or using *o*-xylylenediamine shifts it towards the products. HEWT had moderate conversions across the range of amino acceptors with isopropylamine (IPA) and *o*-xylylenediamine including acetophenone, cinnamaldehyde, vanillin and 2-phenylpropionaldehyde. Very efficient usage of isopropyl amine and *o*-xylylenediamine was found when benzaldehyde was employed as acceptor with 90–95% conversion.

The enantiospecific reaction of HEWT with acetophenone and IPA was investigated by chiral HPLC. The (S)-enantiopreference was highlighted with an excellent ee > 99% towards the production of (S)-(-)-1-phenylethylamine.

HEWT activity and conversion, exploiting isopropyl amine or *o*-xylylenediamine as amino donor. The reaction mixture contained 10 mM amino donor, 10 mM amino acceptor and 0.05 mg/mL of HEWT in a reaction volume of 200 μL. Specific activity was determined using isopropyl amine as amino donor. Activity and conversion were determined monitoring the aromatic signals as described in paragraph 2.10.2. Isopropylamine was also tested

**Table 4**

HEWT specificity with different amino acceptors are reported in comparison with the data reported for *C. violaceum*–TA [3] and for *V. fluvialis*–TA obtained in similar conditions. The enzymatic reactions with HEWT were carried out as reported in materials and methods. Every reaction was performed in three replicates and results reported as the average value. The detection limit of (S)-(-)-1-phenylethylamine was 5%

	<i>H. elongata</i> ω-TA		<i>C. violaceum</i> ω-TA[36]		<i>V. fluvialis</i> ω-TA [25]	
	Final conversion (%)	Relative initial rate (%)	Conversion at equilibrium (%)	Relative initial rate (%)	Relative initial rate (%)	
<b>Ketoacids</b>						
Pyruvic acid		97.5 ± 2.5	100 ± 2.2	95–100	100	100
β-Hydroxypyruvic acid		39.7 ± 0.5	14.8 ± 0.3	65	144.1	29.2
α-Ketoglutaric acid		<5	<5	<5	<5	0.3
<b>Aldehydes</b>						
Glyoxylic acid		97.5 ± 2.5	22.5 ± 2.9	95–100	176.2	60.2
Acetaldehyde		39.2 ± 2.8	33.0 ± 0.6	n.d.	n.d.	15.8
Propanal		55.1 ± 2.6	49.9 ± 4.6	60–70	132.3	36.4
Benzaldehyde		95.0 ± 5.0 <sup>a</sup>	20.4 ± 0.5 <sup>a</sup>	90	91.2	72.8
Phenylacetaldehyde		48.1 ± 2.4 <sup>b</sup>	48.1 ± 6.2 <sup>b</sup>	68	107.0	68.5
2-Phenylpropionaldehyde		67.1 ± 1.1	21.2 ± 0.6	n.d.	n.d.	n.d.
Vanillin		45.5 ± 6.0 <sup>a</sup>	4.3 ± 0.1 <sup>a</sup>	>60 <sup>d</sup>	61.7	n.d.
Cinnamaldehyde		56.1 ± 1.6	5.5 ± 0.3	>60 <sup>d</sup>	79.7	31.5
<b>Ketones</b>						
Cyclohexanone		30.5 ± 1.7	16.2 ± 1.7	12.4 <sup>c</sup>	29.2	6.1
3-Hydroxy-2-butanone		<5	<5	1.3 <sup>c</sup>	5.3	4.8
1,3-Dihydroxyacetone		35.3 ± 1.5	5.4 ± 0.0	2.1 <sup>c</sup>	3.5	n.d.
L-Erythrulose		19.1 ± .8	15.9 ± 4.4	3.2 <sup>c</sup>	13.0	n.d.
L-Ribulose		<5	<5	n.d.	n.d.	n.d.
D-Fructose		<5	<5	n.d.	<5	n.d.

Concentration of substrates was kept constant at 10 mM.

<sup>a</sup> Benzylamine and vanillin peak overlap the (S)-(-)-1-phenylethylamine in the HPLC analysis. The initial reaction rate was determined by the acetophenone formation and the final conversion was estimated with the complete depletion of the benzaldehyde peak in 3 h. The conversion standard deviation of 5% is assigned due to some degree of benzaldehyde evaporation during the reaction.

<sup>b</sup> Phenylacetaldehyde has a low water solubility. The substrate was added in a methanol solution to guarantee the correct concentration of the substrate inside the reaction mixture. For this reason these reactions were carried out in presence of 2.5% of methanol.

<sup>c</sup> Conversion after one hour.

<sup>d</sup> The reactions were not run to equilibrium.

**Table 5**

HEWT activity and conversion, exploiting isopropyl amine or *o*-xylylenediamine as amino donor. The reaction mixture contained 10 mM amino donor, 10 mM amino acceptor and 0.05 mg/mL of HEWT in a reaction volume of 200  $\mu$ L. Specific activity was determined using isopropyl amine as amino donor. Activity and conversion were determined monitoring the aromatic signals as described in paragraph 2.10.2. Isopropylamine was also tested for conversion only in excess with 20 eq. (200 mM). Activity was assayed at 37 °C, while the conversion at 24 h was kept at room temperature.

	Isopropylamine		<i>o</i> -Xylylenediamine	
	Specific activity (U/mg)	Conversion 1 eq. (%)	Conversion 20 eq. (%)	Conversion 1 eq. (%)
Acetophenone	n.d. <sup>a</sup>	9.7 $\pm$ 0.4	13.0 $\pm$ 0.1	29.2 $\pm$ 0.2
Benzaldehyde	0.58 $\pm$ 0.01	85.4 $\pm$ 2.2	95.3 $\pm$ 0.1	90.9 $\pm$ 2.0
Cinnamaldehyde	0.07 $\pm$ 0.01	20.1 $\pm$ 0.7	21.8 $\pm$ 0.1	28.5 $\pm$ 0.1
Vanillin	0.12 $\pm$ 0.01	10.2 $\pm$ 0.7	13.2 $\pm$ 0.4	15.6 $\pm$ 0.1
2-Phenylpropionaldehyde	0.24 $\pm$ 0.02	16.0 $\pm$ 0.9	19.0 $\pm$ 2.0	22.7 $\pm$ 1.8

<sup>a</sup> Activity with acetophenone was not detectable (n.d.) under conditions tested.

for conversion only in excess with 20 eq. (200 mM). Activity was assayed at 37 °C, while the conversion at 24 h was kept at room temperature.

### 3.8. Enzyme kinetics

The maximum velocity ( $V_{max}$ ) was 0.18  $\mu$ M/s and the Michaelis–Menten constants ( $K_m$ ) were 2.57 mM and 0.56 mM for (S)-(-)-1-phenylethylamine and pyruvate, respectively. The turnover number was also determined as  $k_{cat}$  0.094  $s^{-1}$  (Fig. 5). The cooperative profile found for (S)-(-)-1-phenylethylamine was outlined by the Hill coefficient of ca. 2 and confirmed by size exclusion

chromatography that highlighted that HEWT is a dimeric protein in its active state.

### 4. Conclusions

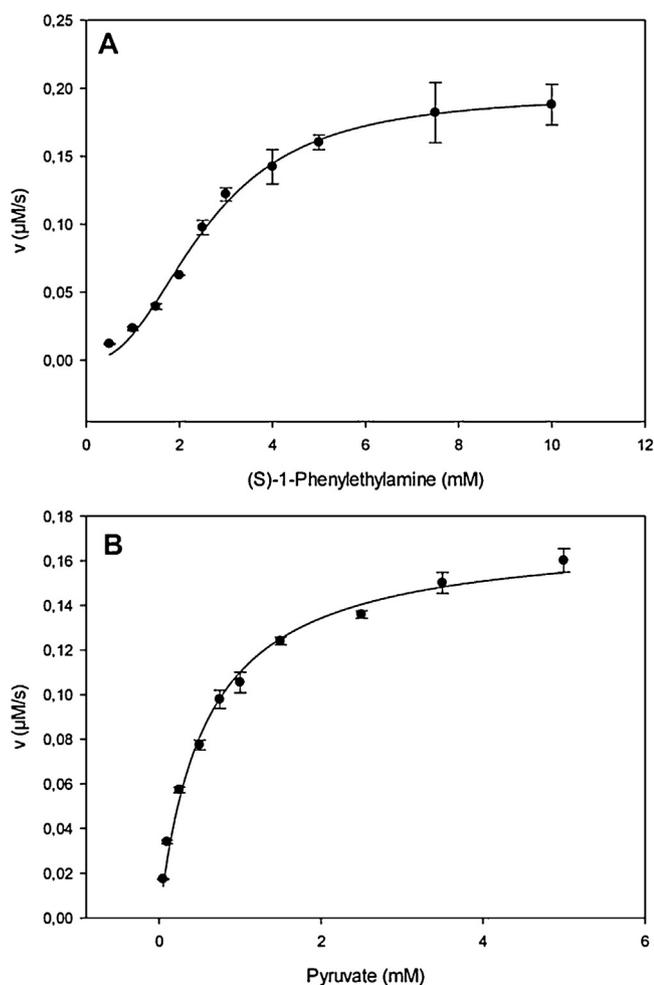
This is the first study reported on an amine transaminases from the moderate halophile bacterium *H. elongata*. HEWT tolerated 20% (v/v) co-solvents over 22 h, the best solvents were MeOH (47%) and DMSO (27%). Among the amino acceptors tested, benzaldehyde presented 60% conversion, shifting the equilibrium using IPA or *o*-xylylenediamine, conversion increased up to 95%. Acceptance of isopropylamine as amino donor is an advantage in asymmetric synthesis and a cost-effective benefit for industrial applications. The activity observed with L-erythrose and 1,3-dihydroxyacetone gives scope for further investigation into structural properties and as well as reinforcing the potential for this enzyme for the application in the synthesis of chiral amino alcohols. With its high enantioselectivity, large substrate spectra and stability in organic solvents, HEWT is a promising enzyme for biotechnological applications in the production of chiral amines.

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**Fig. 5.** Michaelis–Menten plot of the HEWT reaction for (S)-(-)-1-phenylethylamine (A) and pyruvate (B).

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