SHORT COMMUNICATION

Biocatalysis in seawater: Investigating a halotolerant ω -transaminase capable of converting furfural in a seawater reaction medium

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The increasing demand for freshwater and the continued depletion of available resources has led to a deepening global water crisis. Significant water consumption required by many biotechnological processes contributes to both the environmental and economic cost of this problem. Relatively few biocatalytic processes have been developed to utilize the more abundant supply of seawater, with seawater composition and salinity limiting its use with many mesophilic enzymes. We recently reported a salt tolerant ω -transaminase enzyme, Ad2-TAm, isolated from the genome of a halophilic bacterium, *Halomonas* sp. CSM-2, from a Triassic period salt mine. In this study we aimed to demonstrate its applicability to biocatalytic reactions carried out in a seawater-based medium. Ad2-TAm was examined for its ability to aminate the industrially relevant substrate, furfural, in both seawater and freshwater-based reaction systems. Furfural was aminated with 53.6% conversion in a buffered seawater system, displaying improved function versus freshwater. Ad2-TAm outperformed the commonly employed commercial ω -TAMs from *Chromobacterium violaceum* and *Vibrio fluvialis*, both of which showed decreased conversion in seawater. Given the increasingly precarious availability of global freshwater, such applications of enzymes from halophiles have the ability to reduce demand for freshwater in large-scale industrial processes, delivering considerable environmental and economic benefits.

KEYWORDS

biocatalysis, furfural, halophile, seawater, transaminase

1 | INTRODUCTION

The demand for freshwater is rapidly increasing and presents considerable environmental and economic issues. This demand is starkly juxtaposed with the progressively more precarious shortage of freshwater on a global scale, which has already presented problems for food production [1,2]. Indeed, of all the water on Earth, only 3% is fresh water, with

2% trapped in glaciers and ice caps. Seawater accounts for the remaining 97% and yet it remains a relatively untapped resource for many processes. The problem has been exacerbated by the use of freshwater in biotechnology, such as the processing of industrial compounds in biorefineries and the increase of biocatalysis on larger scales, associated with sizeable freshwater consumption. For these reasons, enzymes capable of functioning in a seawater reaction medium could prove extremely valuable in the coming years.

There are relatively few examples of seawater-tolerant enzymes currently in use as industrial biocatalysts. Seawater has already been applied in biorefineries, with particular

Abbreviations: CFE, cell-free extract; FAM, furfurylamine; NPEA, 2-(4-nitrophenyl)ethan-1-amine; PLP, pyridoxal 5'-phosphate; TAm, transaminase.

relevance in coastal and arid areas [3,4]. Marine-derived cellulases and a D-glucose/xylose isomerase have also been shown to function in a seawater medium for use in chemical and biofuel production [5,6]. Salt-tolerant enzymes from halophilic microorganisms could also possess the required adaptations to function in a seawater medium. To date, examples of such applications are extremely limited in the literature. In a recent example, whole cell biocatalysis using the halotolerant yeast *Debaryomycesn etchellsii* was applied to the production of (R)-(-)-phenylacetylcarbinol, a precursor of the drug ephedrine [7].

An important group of enzymes currently experiencing widespread use in industrial biocatalysis is the transaminases (TAMs). TAMs are pyridoxal 5'-phosphate dependent enzymes used in the synthesis of chiral amines, important building blocks for the pharmaceutical and fine chemical industries [8,9]. Furfurylamines (FAMs) are a group of chiral amines used in the manufacture of pesticides, fibers, and perfumes [10], and have proven to be useful synthons in API production, as intermediates in the production of the diuretic furosemide, and in the synthesis of antiseptics and antihypertensives [11,12]. TAMs have emerged as a biocatalytic alternative for the synthesis of FAMs, with a number of groups recently producing FAMs using this approach [12,13].

To our knowledge, there have been no reports of TAMs operating in seawater for use in biocatalysis. Previously we reported a novel ω -TAM, Ad2-TAM, from the moderately halophilic bacterium, *Halomonas* sp. CSM-2, isolated from a salt mine formed during the Triassic period [14]. Ad2-TAM possessed a broad substrate range and showed no loss of activity up to 1.5 M NaCl (~8.8%). Herein, we expand the kinetic characterisation of Ad2-TAM, assess its ability to convert the industrially important substrate, furfural, in a seawater-based reaction medium, and compare this performance with commercially-available TAMs currently in widespread use.

2 | MATERIALS AND METHODS

2.1 | Preparation of cell-free extract for reactions

Lyophilized cell-free extract (CFE) for enzyme Ad2-TAM was prepared using a pET28a(+) plasmid and expressed in *Escherichia coli* BL21 DE3 cells, as described previously [14]. CFE for use as a negative control was produced in the same way using *E. coli* BL21 DE3 cells transformed with empty pET28a(+) plasmid. CFE for the commercially available TAMs from *Chromobacterium violaceum* (Cv-TAM) (NCBI Reference Sequence: WP_011135573.1) and *Vibrio*

PRACTICAL APPLICATION

Biocatalysis is the use of enzymes to provide a cost efficient and green alternative to conventional chemistry for a number of key industrial applications, including the production of APIs for the pharmaceutical industry. However, large scale biocatalytic processes and the use of enzymes in biorefineries are associated with significant freshwater consumption. Given the increasingly precarious availability of freshwater globally, enzymes capable of performing in the much more abundant seawater, would deliver considerable environmental and economic benefits. In this study, we have presented a transaminase enzyme (Ad2-TAM) isolated from a salt-loving bacterium (halophile), which not only maintains function in a seawater-based reaction medium, but exhibits improved performance under such conditions. Ad2-TAM has the potential to be applied in industrial biocatalysis using a seawater-based reaction medium in place of freshwater, in the production of chiral amines for the pharmaceutical industry, allowing significant environmental and economic advantages to be realised.

fluvialis (Vf-TAM) (PDB: 3NUI_A) were both prepared in the same way.

2.2 | Colorimetric assay using 2-(4-nitrophenyl)ethan-1-amine as amino donor

Reactions were set up in either a system containing freshwater or seawater. Seawater stocks were made by reconstituting API Aquarium Salt (Mars Fishcare, PA, USA), made from evaporated seawater, to 3.6%, mimicking the salinity of seawater. Freshwater and seawater systems were buffered at pH 9 using a universal buffer described previously [15]. Colorimetric assays using 2-(4-nitrophenyl)ethan-1-amine (NPEA) as amino donor were adapted from those described previously [16]. Screening assays were set up with the following conditions: buffered freshwater or buffered seawater (100 μ L) was added to a 96-well deepwell plate. NPEA stock solutions were prepared in water and 60 μ L added to give a final concentration of 25 mM. Pyridoxal 5'-phosphate (0.5 mM) and furfural in dimethyl sulfoxide (5 mM and 10% v/v respectively) were added to give a total volume of 180 μ L. The reaction was initiated by the addition of 20 μ L rehydrated CFE to a final concentration of 20 mg/mL. Assays were imaged following incubation for 16 h at 30°C and 1200 rpm, using an 8-megapixel camera and a Halco Copilite light box (Halco Sunbury, UK).

FIGURE 1 Colorimetric assay using 2-(4-nitrophenyl)ethan-1-amine (NPEA) as amino donor and furfural as substrate, comparing the performance of Ad2-TAM versus negative control in both freshwater and seawater-based reaction media. Wells A1-3: Ad2-TAM in a freshwater-based reaction medium; wells B1-3: negative control in a freshwater-based reaction medium; wells C1-3: Ad2-TAM in a seawater-based reaction medium; wells D1-3: negative control in a seawater-based reaction medium. Assay results are shown alongside the reaction schematic for the transaminase-catalyzed amination of furfural using NPEA as amino donor, adapted from a previously described assay [16]

2.3 | HPLC-based screening assay and kinetic characterization

Reactions were set up as described above, with (*S*)-methylbenzylamine (25 mM) in place of NPEA as amino donor. Reactions involving Ad2-TAM were buffered at pH 9 and 8 for the two commercial enzymes in accordance with the optimum pH for each. After incubation for 16 h at 30°C and 1200 rpm, the reaction was quenched with 800 μ L 62.5% acetonitrile and denatured protein removed by centrifugation. Two hundred microliter of supernatant was removed for analysis by HPLC using an XSelect[®] CSH[™] C18 5 μ m column (4.6 $\times$ 250 mm) at 25°C and an Agilent 1260 Infinity model with detection of acetophenone coproduct at 240 nm. The concentration of acetophenone coproduct formation was determined over 11 min using a gradient of 5–100% organic solvent (where A = dH₂O with 0.1% H₃PO₄ and B = acetonitrile with 0.1% H₃PO₄). Acetophenone eluted at a retention time of 8.6 min. Assays using Ad2-TAM, Vf-TAM, Cv-TAM, and vector only controls were all carried out in triplicate with an average conversion taken for each and values for negative control subtracted from TAM values.

Kinetic parameters were determined using the reaction described above in the buffered seawater system using a substrate range of 0.5–20 mM. Acetophenone formation was determined as described above and Ad2-TAM concentration was determined using a combination of a Qubit[®] 2.0 Fluorometer (Invitrogen, UK) and SDS-PAGE densitometry.

3 | RESULTS AND DISCUSSION

Colorimetric assays employing NPEA as amino donor showed Ad2-TAM had the ability to aminate furfural in both buffered freshwater and seawater reaction media, producing the orange-red color characteristic of the positive reaction

TABLE 1 Kinetic parameters and 16 h conversion for Ad2-TAM, determined using furfural as substrate and (*S*)-methylbenzylamine as amino donor, in seawater reaction medium buffered at pH 9

$V_{\max}$ (U/mg) ^a	K_m (mM)	K_{cat} (min ⁻¹)	Conversion at 16 h (%)
187.88	1.09	8.77	53.6

^aOne unit (U) is defined as the amount of enzyme required to produce 1 nmol of acetophenone in 1 min.

(Figure 1). Negative controls employing empty pET28a(+) plasmid remained unchanged.

Kinetic characterization of Ad2-TAM in seawater reaction medium was performed using (*S*)-methylbenzylamine as amino donor and furfural as substrate. This yielded a $V_{\max}$ of 187.88 U/mg, where one unit (U) is defined as the amount of enzyme required to produce 1 nmol of acetophenone in 1 min, a K_m value of 1.07 mM and K_{cat} of 8.77 min⁻¹ (Table 1). Following incubation for 16 h at 30°C, the overall conversion of furfural from a starting concentration of 5 mM was 53.6%. Characterization of Ad2-TAM in freshwater, including specific activity determination, was described previously [14].

Critically, Ad2-TAM exhibits a statistically significant improvement in performance in buffered seawater compared to buffered freshwater, with $110.2 \pm 2.7\%$ conversion observed, relative to the reaction in buffered freshwater (Figure 2). This is perhaps unsurprising, given the thalassohaline origin from which the CSM-2 organism is derived. It is reasonable to suggest that halophilic microorganisms found in environments formed by the evaporation of seawater would possess characteristics allowing them to function in a seawater medium. Overall conversion with both commercial enzymes decreased in buffered seawater compared to freshwater, with relative conversion of furfural decreasing to $95.0 \pm 1.5\%$ and $69.5 \pm 5.6\%$ for Vf-TAM and Cv-TAM, respectively (Figure 2). This highlights the potential of halotolerant enzymes in biocatalysis, whose function is not

FIGURE 2 Comparison of relative conversion over 16 h of Ad2-TAm and the commercially available TAmS, Cv-TAm, and Vf-TAm in both freshwater and seawater-based reaction media. For each pair, conversion in buffered freshwater was taken to be 100% for each enzyme, with performance in buffered seawater shown relative to this value

only unaffected by the use of seawater, but actually improves in this medium. Replacement of freshwater with seawater for large-scale industrial applications could have significant environmental benefits. The continued depletion of freshwater resources, coupled with rising demand, suggests the economic advantages of using seawater as a suitable substitute become increasingly profound. Biocatalytic processes employing halophiles and their enzymes represent an innovative solution to some of these issues.

Enzymes from halophiles are largely understudied in comparison to their mesophilic counterparts. As such, considerable engineering may be required in order to optimize their specificity and activity towards desirable substrates. However, their robust characteristics mean they could be used as scaffolds for protein engineering, informing the design of similarly durable enzymes. An enzyme such as Ad2-TAm, which displays halotolerance and the ability to convert industrially relevant substrates like furfural, may be an excellent place to start.

4 | CONCLUDING REMARKS

Herein we have investigated the ability of a ω -TAm from a halophilic bacterium, Ad2-TAm, to convert an industrially relevant substrate in a buffered seawater reaction medium, and compared this to commonly employed commercial ω -TAmS. Ad2-TAm-catalyzed conversion of furfural increased in seawater, whereas conversion decreased for both commercial TAmS. This highlights the potential for TAmS and other enzymes, from halophiles to be applied in biocatalysis using seawater-based reaction media. Given the increasingly precarious availability of freshwater globally, such applications have the ability to reduce demand for freshwater in large-scale industrial processes and deliver considerable environmental and economic benefits. Development and engineering

of enzymes such as Ad2-TAm represents a viable approach in achieving these goals using biotechnology.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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