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Catalytic Biomaterials for Atrazine Degradation

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Abstract: In this paper, triazine hydrolase from *Arthrobacter aurescens* TC1 (TrzN) was successfully immobilized in alginate beads (TrzN:alginate), alginate beads coated in chitosan (TrzN:chitosan), and tetramethylorthosilicate (TMOS) gels using the sol–gel method (TrzN:sol–gel) for the first time. TrzN:alginate and TrzN:chitosan hydrolyzed 50 μM of atrazine in 6 h with negligible protein loss with an ~80% conversion rate. However, the TrzN:sol–gel biomaterial converted >95% of a 50 μM atrazine solution in an hour with negligible protein loss. The treatment of each of these biomaterials with trypsin confirmed that the catalytic activity was due to the encapsulated enzyme and not surface-bound TrzN. All three of the biomaterials showed potential for long-term storage and reuse, with the only limitation arising from the loss of protein in the storage buffer for the TrzN:alginate and TrzN:chitosan biomaterials, not the denaturation of the encapsulated TrzN. TrzN:sol–gel stood out, with ~100% activity being retained after 10 consecutive reactions. Additionally, the materials stayed active in methanol concentrations <10%, suggesting the ability to increase the solubility of atrazine with organic solvents. The structural integrity of the TrzN:alginate and TrzN:chitosan materials became limiting in extreme pH conditions, while TrzN:sol–gel outperformed WT TrzN. Overall, the TrzN:sol–gel biomaterial proved to be the best atrazine dichlorination biocatalyst. As sol–gels can be cast into any desired shape, including pellets, which can be used in columns, the TrzN:sol–gel biomaterial provides a new avenue for the design of bioremediation methodologies for the removal of atrazine from the environment.

Keywords: zinc; dehalogenase; biomaterials; kinetics

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1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a water-soluble herbicide that inhibits photosynthesis in targeted plants [1,2]. Originally patented in 1958, commercial use in the United States began in 1959, and today, it is the second most used herbicide, with roughly 30,000 tons applied annually to sorghum, sugarcane, and corn crops [2–4]. Atrazine is persistent and mobile in aquatic environments, primarily through surface runoff, where it enters groundwater via leaching [2,5]. Once in the watershed, atrazine has the potential to travel miles, as it has a half-life of six months to several years, which is troublesome considering the potential downstream effects [2,6,7]. Atrazine toxicity impacts eukaryotes, including, but not limited to, crustaceans, insects, mollusk, fish, amphibians, and reptiles [2,8–10]. The effects observed in aquatic eukaryotes after atrazine exposure raises concerns for the possible effects that atrazine can have on humans, such as lung and kidney diseases, cardiovascular damage, retinal degeneration, and cancer, which is why the European union banned atrazine in 2003 [1]. In the US, the EPA Endocrine Disruptor Testing Advisory Committee recognized atrazine as an endocrine toxin in humans in 2000, and shortly thereafter, the agency for Toxic substances and Disease Registry (ATSDR) warned that people in rural communities near agricultural lands are at an increased risk of atrazine exposure primarily through their drinking water [11]. Given the widespread use of atrazine and its toxicity to both aquatic environments and humans, its biodegradation and environmental clean-up have become topics of significant importance [12].

Triazine hydrolase from *Arthrobacter aurescens* TC1 (TrzN, EC 3.8.1.8) has great potential as a biocatalyst for the bioremediation of atrazine because it irreversibly catalyzes the dichlorination of atrazine to its less toxic derivative hydroxyatrazine under physiological conditions (Figure 1) [13,14]. However, when utilizing enzymes for remediation purposes, a major concern arises regarding one's ability to separate the enzyme from the reaction mixture [15]. A related issue involves aprotic solvent contaminants typically used for extraction, as they degrade bacterial cells and decrease most free enzyme activity significantly. One way to overcome these challenges is through the immobilization of enzymes in optically transparent and sufficiently porous materials that permit small substrates access to the entrapped enzyme. Recent studies have demonstrated that encapsulated proteins retain their solution structure and native function while residing in a material and that nanoscopic confinement within materials stabilizes proteins against thermal and proteolytic degradation [16,17].

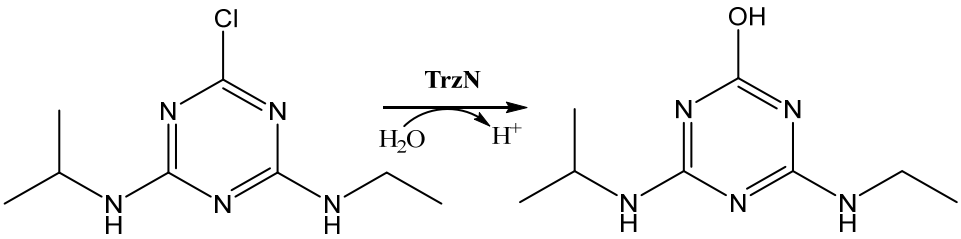


Figure 1. Hydrolysis of atrazine to hydroxyatrazine and chloride by TrzN.

Bioremediation methods utilizing TrzN have been limited to whole cells that naturally express TrzN or, in one case, simply adding pure TrzN to a contaminated drainage ditch [12,18]. While these methods have been somewhat successful in degrading atrazine, purified TrzN encapsulated in a material is necessary, as a major issue is the difficulty in recovering whole cells or free enzymes from the environment, as well as thermal and proteolytic degradation. Side reactions within a cell also pose problems. Herein, we report the immobilization of purified TrzN within an alginate matrix in the absence and presence of a chitosan outer layer and within silica glasses derived through the sol–gel process [16]. These novel biocatalytic materials are capable of the hydrolytic dichlorination of atrazine into its less toxic derivative hydroxyatrazine under mild conditions.

2. Results and Discussion

2.1. Encapsulation of TrzN

Wild-type (WT) TrzN was obtained by the overexpression of the gene from *Arthrobacter aurescens* TC1 with an engineered N-terminal hexa-histidine (His_6) affinity tag in *E. coli* that was synthesized with optimized *E. coli* codon usage. Per liter of culture, ~9 mg of pure TrzN was obtained after a single purification step utilizing immobilized metal affinity chromatography (IMAC). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single polypeptide band at ~51 kDa, consistent with previous studies. Purified TrzN was found to catalyze the hydrolysis of atrazine with a k_{cat} value of 4.0 ± 0.1 s^{-1} and a K_m value of 43 ± 3 μM in 50 mM HEPES buffer at pH 7.0 and 25 °C using a continuous spectrophotometric assay that monitors atrazine degradation at 264 nm (ε_{264} = 3.5 mM^{-1} cm^{-1}). These values are consistent with those previously reported [19].

Having pure, catalytically active TrzN in hand allowed for its encapsulation into alginate beads (TrzN:alginate), TrzN:alginate coated with chitosan (TrzN:chitosan), and tetramethylorthosilicate (TMOS) gels using the sol–gel method (TrzN:sol–gel). An analysis of both the buffer in which the biomaterials were stored and the buffer from all the washing steps using a Bradford assay indicated that, on average, >99% of the TrzN present was encapsulated within the materials, as < 0.5% of the TrzN used was present in either the wash or the storage buffer. Quantitating the reaction of atrazine with each of the TrzN biomaterials was difficult, as the exact quantity of encapsulated enzyme was not known; however,

assuming that ~99% of the enzyme used was encapsulated, ~99% of the solution activity was retained for TrzN:sol–gel at 25 °C, while TrzN:alginate and TrzN:chitosan retained approximately 28% and 30%, respectively. Interestingly, the TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel biomaterials readily reacted with atrazine (Figure 2) at 25 °C in the 50 mM HEPES buffer, pH 7, with ~80% and ~95% conversions of atrazine to the non-toxic hydroxyatrazine product over a 6 h reaction period for TrzN:alginate and TrzN:chitosan, respectively, while a 100% degradation of atrazine by TrzN:sol–gel was observed after only 1 h, a six-fold improvement compared to the alginate biomaterials. These data indicate that each of these biomolecular nanocomposites display the expected enzymatic properties, including substrate recognition, of TrzN in solution.

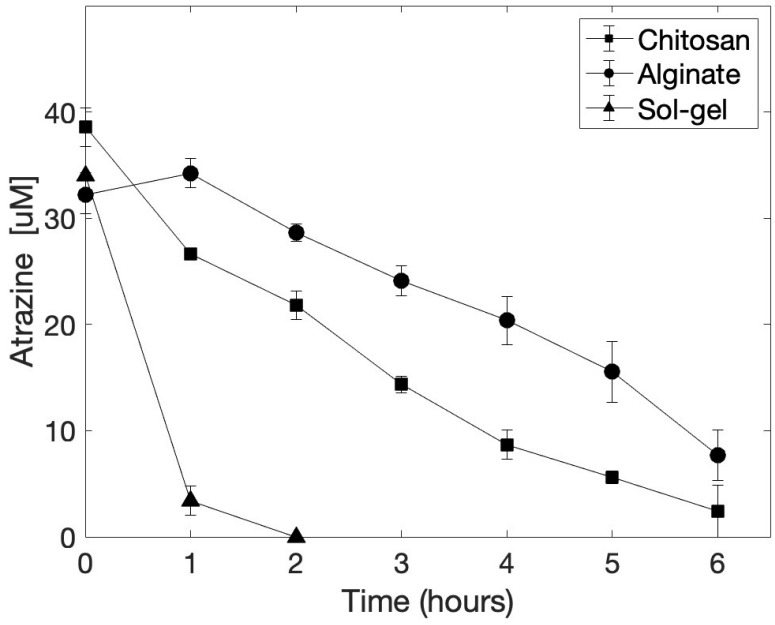
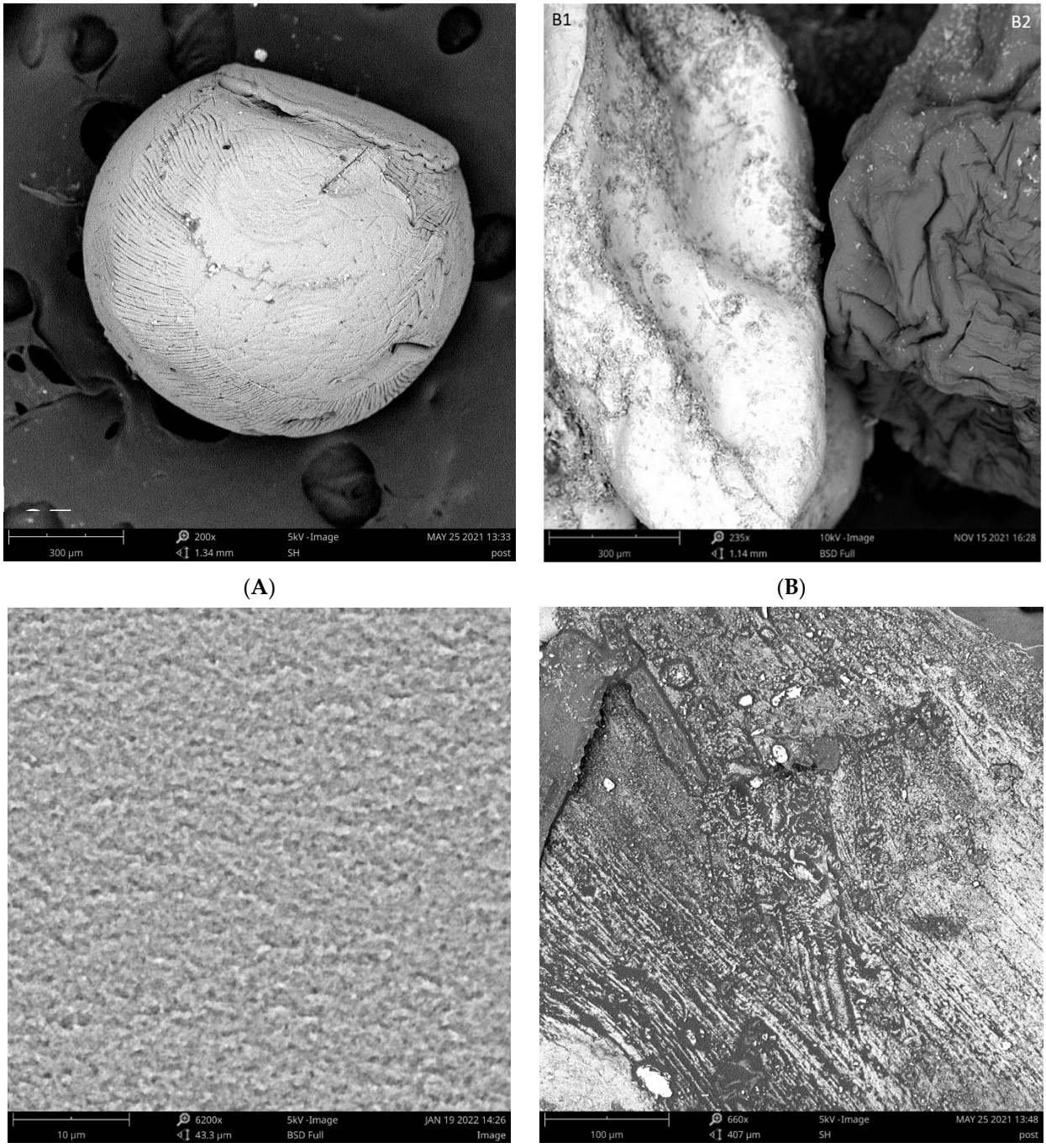


Figure 2. Time course for the hydrolysis of atrazine using TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel.

The scanning electron microscope (SEM) images of each of these biomaterials reveal the size of the TrzN:alginate and TrzN:chitosan beads, as well as the porous nature of the surfaces, confirming that the solution and substrate can access the encapsulated enzyme (Figure 3). The SEM data of TrzN:alginate (Figure 3A) and TrzN:chitosan (Figure 3B) reveal that the average bead size ranges from 1 to 2 mm in diameter. The chitosan coating is clearly visible in the SEM image (Figure 3B), as TrzN:chitosan appears dark in color when the data are collected with an electron detector, which detects back-scattered electrons. Conversely, TrzN:alginate, which has high-atomic-number atoms, such as Ca^{2+}, on its surface, appears lighter in color in the SEM image. Zooming in by 500× on the TrzN:chitosan and TrzN:sol–gel biomaterials reveals the porous nature of these materials to be consistent in the observed kinetics of substrate turnover for the TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel biomaterials, which appear to be governed by the mass transport of the substrate through the porous materials to the enzyme active site and product release.



(**A**) (**B**)

(**C**) (**D**)

Figure 3. (**A**) SEM image of TrzN:alginate biomaterial at 200× magnification. The scale bar is 300 µm. (**B**) SEM images of TrzN:alginate (**left**, **B1**) and TrzN:chitosan (**right**, **B2**) biomaterials at 235× magnification. The scale bar is 300 µm. TrzN:alginate and TrzN:chitosan averaged between 1 and 2 mm in diameter. (**C**) SEM image of the TrzN:sol–gel biomaterial at 510× magnification. The scale bar is 100 µm. (**D**) SEM image of a cross-sectional view of the dried TrzN:alginate biomaterial at 660× magnification. The scale bar is 100 µm.

2.2. Proteolytic Digestion of WT TrzN and the TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel Biomaterials

To ensure that the substrate has access to the fully immobilized enzyme and not the enzyme that is simply adhered to the surface, each of the biomaterials were treated with trypsin to proteolytically digest all surface-accessible proteins (Figure 4). TrzN:alginate and

TrzN:chitosan were digested with trypsin for 30 min at 35 °C with constant shaking. Preliminary studies (not shown) indicated that a shorter trypsin reaction time of 30 min, rather than the recommend 18 h, was necessary to avoid material degradation. The TrzN:alginate and TrzN:chitosan biomaterials retained 75 and 80 ± 5% of their original activities, respectively, compared to a TrzN enzyme solution sample, which retained 55 ± 5% of its activity. However, the TrzN:sol–gel biomaterial retained 85 ± 5% of its activity, even after 18 h in the presence of trypsin at 35 °C, compared to a TrzN enzyme control sample, which only retained 45 ± 5% of its activity over the same time period, while the TrzN:alginate and TrzN:chitosan biomaterials exhibited no detectable activity at the recommended 18 h for complete enzyme digestion. These data indicate that the substrate has access to the TrzN enzyme entrapped in the interior of each biomaterial, which is an active catalyst but protected from proteolytic digestion. Even though TrzN:sol–gel and TrzN:chitosan provided comparable protection against proteolytic cleavage over 30 min, the TrzN:sol–gel biomaterial was much more robust, as it retained its activity after 18 h, while TrzN:chitosan did not. These data also indicate that the surface-bound enzyme is hydrolyzed and that the internal TrzN is protected, resulting in a catalyst that is more robust than the free enzyme.

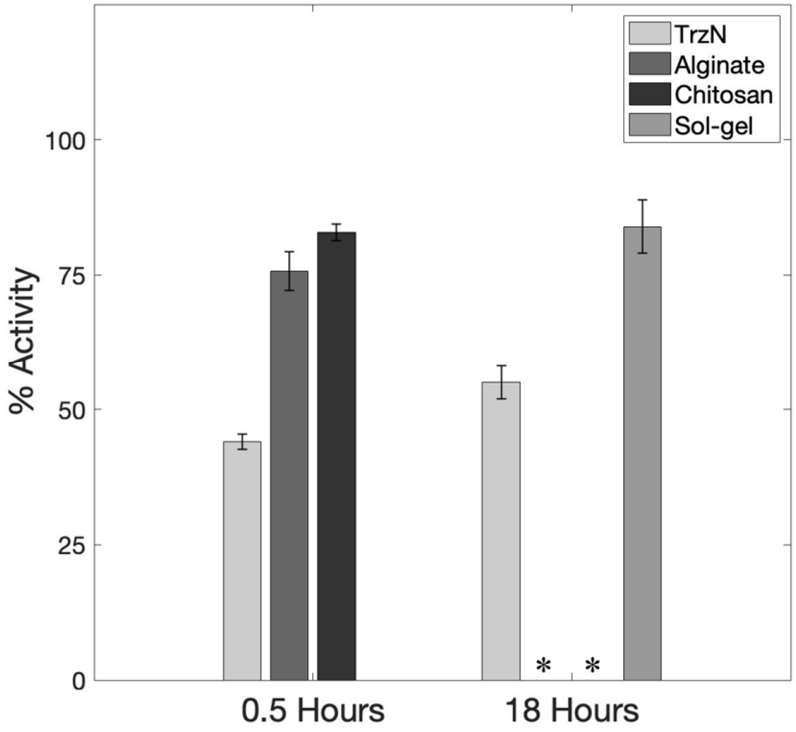


Figure 4. Residual activity of soluble TrzN, TrzN:sol–gel, TrzN:alginate, and TrzN:chitosan biomaterials proteolytically digested with trypsin. The soluble TrzN and the biomaterials were incubated at a 2:1 ratio of trypsin:TrzN at 35 °C. TrzN:alginate and TrzN:chitosan were digested for 30 min, while TrzN:sol–gel was digested for 18 h. A soluble TrzN control was digested for both 0.5 and 18 h, as 18 h is the recommended digestion time for complete protein degradation by trypsin digestion. * indicates that there was no detectable activity.

2.3. Thermostability of WT TrzN and the TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel Biomaterials

The thermostabilities of WT TrzN and the TrzN:sol–gel, TrzN:alginate, and TrzN:chitosan biomaterials were evaluated over a temperature range of 50–80 °C (Figure 5). WT TrzN and each of the TrzN biomaterials were thermally stable at 50 °C over a 30 min incubation time, with WT TrzN exhibiting a k_{cat} of 3.8 ± 0.1 s^{-1}, which is identical to that observed at 25 °C. As the temperature was increased to 80 °C, the k_{cat} value for WT TrzN increased to 5.0 ± 0.2 s^{-1}, while the TrzN:alginate and TrzN:sol–gel biomaterials were inactive. How-

ever, the TrzN:chitosan biomaterial retained ~30% of its original activity at 80 °C. An ICP-AES analysis of the reaction buffers showed a significantly higher concentration of Ca^{2+} ions in the TrzN:alginate reaction buffer compared to those of TrzN:chitosan. These data suggest that the polymer structure of the alginate tightened [20], resulting in reduced substrate accessibility to the encapsulated enzyme. Little or no Ca^{2+} ions in the chitosan reaction buffer indicates that the chitosan continued to act as a barrier in order to protect the alginate matrix, resulting in increased activity in the chitosan-coated biomaterial vs. the alginate [21]. Temperature has a direct relationship with the internal pore structure of alginate beads, and previous work has reported a significant loss of moisture during the heat shock of alginate beads, creating dense porous structures [20]. Therefore, the addition of a chitosan coating around the alginate matrix retains the integrity of the alginate material, resulting in increased thermostability [21].

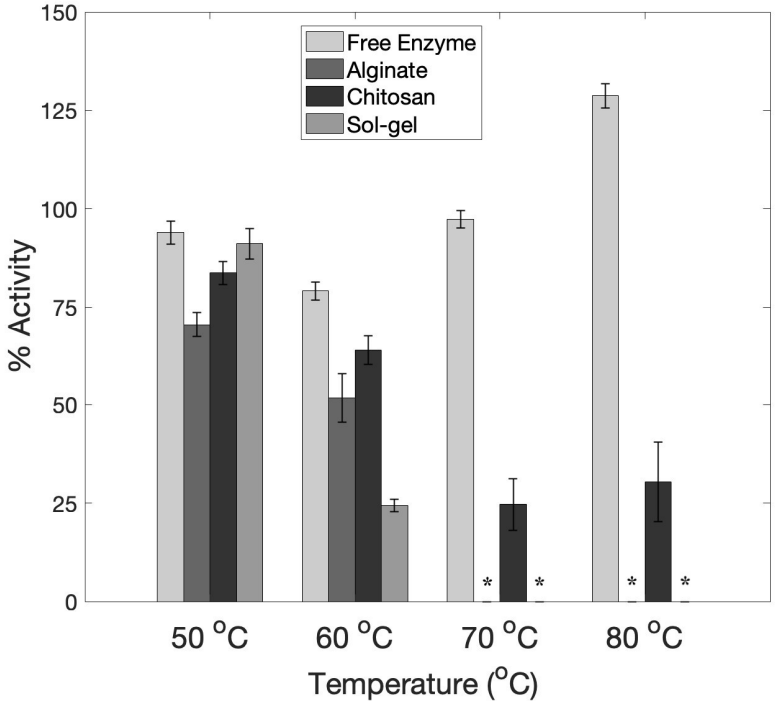


Figure 5. Thermostabilities of soluble TrzN, TrzN:alginate, and TrzN:chitosan biomaterials. The residual activities of the soluble enzyme and each biomaterial were recorded after a 30 min heat shock in a 50, 60, 70, and 80 °C water bath followed by measuring the dichlorination of atrazine at 25 °C for 6 h. * indicates that there was no detectable activity.

2.4. Reusability and Long-Term Stability of the TrzN:biomaterials

For commercial applications, a biocatalyst must be reusable and have long-term stability. With this is mind, we sought to investigate whether each of the biomaterials could be recycled in subsequent reactions (Figure 6). After every use, each of the TrzN biomaterials were thoroughly washed with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.0, to remove residual atrazine and hydroxyatrazine, and then they were stored in the buffer until they were subjected to the same reaction conditions. The conversion of atrazine to hydroxyatrazine decreased over a six-week period for each of the TrzN biomaterials, with ~65% of the initial activity remaining for TrzN:sol–gel and <20% for TrzN:chitosan and no observable activity for the TrzN:alginate biomaterial. Remarkably, soluble TrzN could also be stored at 4 °C in the buffer over a six-week period without a significant loss of activity (Figure 6). The observed loss of activity for the TrzN:alginate biomaterial compared to WT TrzN, which lost ~70% of its original activity, was due to the enzyme loss during the extended storage in the aqueous solution. Similarly, the TrzN:chitosan biomaterial lost ~55%. As previously reported, alginate beads tend

to be "leaky", and while a chitosan coating helps to decrease enzyme loss, it does not eliminate it [22]. The TrzN:sol–gel biomaterial also lost enzymes over the course of the six-week experiment but to a significantly lesser extent than either the TrzN:alginate or TrzN:chitosan biomaterials. These data indicate that the enzyme that remained in the biomaterials stayed active and that the loss of activity was due to the loss of immobilized enzyme, not enzymatic denaturation within the matrix or product build-up within the matrix hindering the diffusivity of the substrate to the enzyme [23]. The cyclical reusability of TrzN:sol–gel over ten reactions revealed no loss of activity, adding to the conclusion that enzyme loss during extended storage was the cause of activity loss. Overall, the TrzN:sol–gel biomaterial performed the best, and the encapsulation of TrzN in a sol–gel provides a matrix for the storage of TrzN, which can be recycled, stored, and reused for extended periods of time.

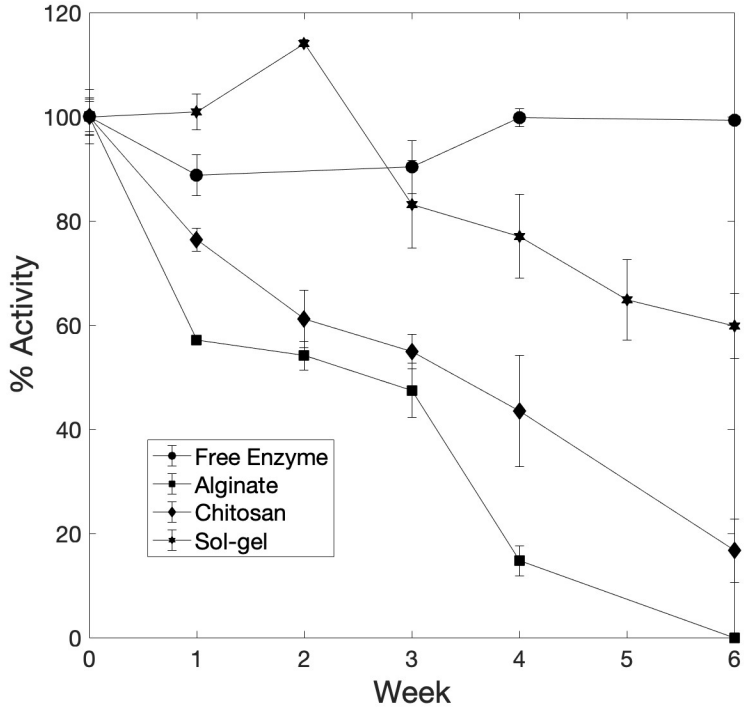


Figure 6. Long-term stabilities of soluble TrzN, TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel calculated over the 6-week experiment. The k_{cat} value of soluble TrzN was evaluated with 150 μM atrazine at 25 °C stored in 0.1 M sodium phosphate buffer pH 7.0 at 4 °C until the following week. The TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel biomaterials were evaluated weekly for the degradation of 50 mM atrazine in 50 mM HEPES, pH 7.0. The biomaterials were washed and stored at 4 °C in 50 mM HEPES pH 7.0 for 1 week before repeating the experiment.

2.5. Stability of the WT TrzN and TrzN:biomaterials in an Organic Co-Solvent

Atrazine presents itself traditionally in watersheds containing negligible amounts of organic solvents [24]; however, at higher concentrations, atrazine solubility becomes problematic and requires organic solvents. Thus, understanding the functions of biomaterials in solutions with organic solvents is important for exploring their potential as bioremediation catalysts, as enzymes typically denature when exposed to organic solvents [16]. The activities of the TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel biomaterials were examined in 5:95, 10:90, and 20:80 MeOH:water solvent mixtures (Figure 7). In the 5:95 and 10:90 MeOH:water solutions, each of the biomaterials retained at least 70% of the WT TrzN enzyme, but all exhibited a significant decrease in activity in the MeOH:water mixture of 20:80, with the TrzN:alginate biomaterial performing the worst with only 10% residual activity. At higher methanol concentrations, both the TrzN:alginate and TrzN:chitosan biomaterials lost all activity; however, the TrzN:sol–gel biomaterial retained ~5% of its

residual activity in the 70:30 MeOH:water solution. For comparison purposes, WT TrzN exhibited ~75% of its native enzyme's activity in the 20:80 MeOH:water mixtures. The poorer performance in organic solvents for the TrzN:alginate and TrzN:chitosan biomaterials was due to the increased Ca^{2+} ion concentration in the reaction solution, determined via ICP-AES. Previously, it was reported that ethanol at concentrations >15% reduces the hydrodynamic volume of alginate beads, resulting in a tightening of the polymer chains, which restricts substrate access to an encapsulated enzyme [25]. Therefore, of the three biomaterials tested, sol–gel encapsulation clearly performs the best in stabilizing against increasing protic solvents.

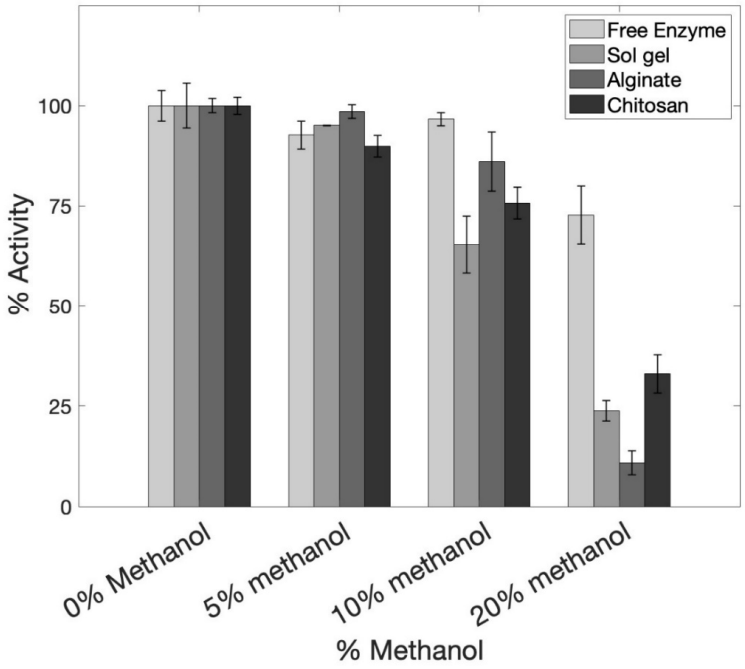


Figure 7. Activities of soluble TrzN, TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel for the conversion of 50 mM atrazine to hydroxyaztraine in 5:95, 10:90, and 20:80 MeOH:water (*v*/*v*) co-solvent solutions.

2.6. Stability of the WT TrzN and TrzN:biomaterials at Non-physiological pH Values

Typically, when using biomaterials in aqueous bioremediation strategies, the engineered material is expected to perform in native watershed conditions. Although watersheds typically present a pH of ~7, outside influences, such as polluted runoff, can cause the pH to fluctuate outside of the neutral range [26]. With this in mind, it is important to investigate the activities of TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel biomaterials in pH conditions at both higher (pH 9.0) and lower (pH 4.0) values than physiological pH (Figure 8). At pH 4.0 (50 mM citric acid buffer), both the TrzN:alginate and TrzN:chitosan biomaterials exhibited no detectable activity, while the TrzN:sol–gel biomaterial exhibited ~45% of its residual activity. At pH 9.0 (50 mM glycine buffer), TrzN:alginate again exhibited no detectable activity, while the TrzN:chitosan and TrzN:sol–gel biomaterials retained ~20 and ~95% of their residual activities, respectively. For comparison purposes, WT TrzN exhibited ~25 and ~45% of its residual activities at pH 4.0 and 9.0, respectively. Therefore, sol–gel encapsulation clearly affords significant stabilization against both increased and decreased proton ion concentrations, which is likely due to the strong electrostatic interaction between the silica sol and TrzN [27,28].

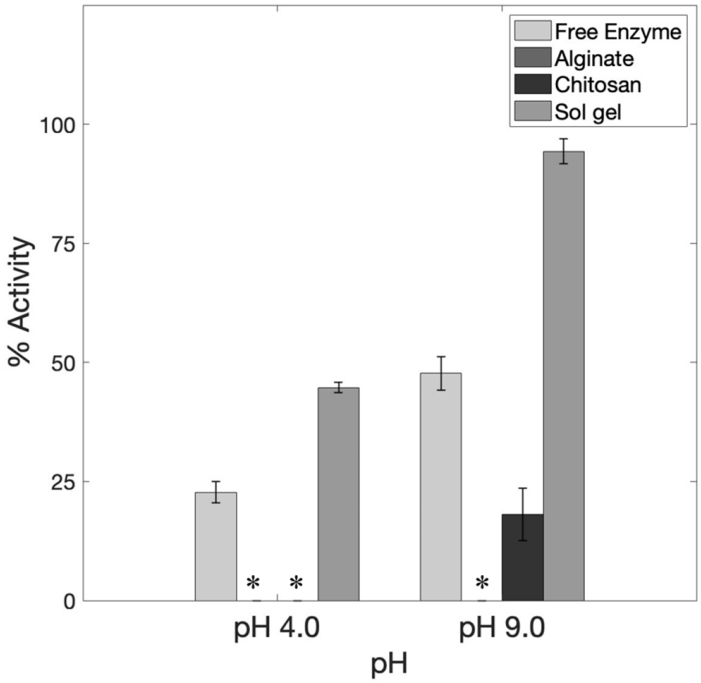


Figure 8. Activities of soluble TrzN, TrzN:alginate, and TrzN:chitosan for the conversion of 50 mM atrazine to hydroxyatrazine in 50 mM citric acid pH 4.0 buffer and 50 mM glycine buffer pH 9.0. * indicates that there was no detectable activity.

3. Materials and Methods

3.1. Materials

Sodium alginate was purchased from Spectrum Chemical MFG corp (New Brunswick, NJ, USA). Chitosan, atrazine, tetramethyl orthosilicate (TMOS, ≥99%), and type I trypsin from bovine pancreas were purchased from Sigma-Aldrich. All reagents were of the highest purity available, and they were received without further purification.

3.2. Expression and Purification of TrzN

The gene from *Arthrobacter aurescens* TC1 that encodes for TrzN with the D38N, L131P, and A159V mutations was synthesized with optimized *E. coli* codon usage that includes a polyhistidine (His_6) affinity tag engineered onto the N-terminus between the NdeI and XhoI restriction sites of kanamycin-resistant pET28a(+) in order to create a TrzN/pET28a(+) plasmid. The TrzN/pET28a(+) plasmid was transformed into *E. coli* BL21(DE3) competent cells (Stratagene) for expression. A 100 mL LB-Miller starter culture was inoculated from a single colony with 50 µg/mL of kanamycin. A 9 L culture was inoculated from this starter culture using 10 mL/liter supplemented with 5 µM isopropyl ß-d-1-thiogalactopyranoside (IPTG) and grown at 37 °C for 48 h [19]. Cells were harvested via centrifugation at 7000 rpm and 4 °C for 15 min. The cells were resuspended at 2 mL per gram of buffer A (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, pH 7.5) and then lysed via sonication on ice in three 10 min (30 s on and 45 s off) intervals using a Cole-Parmer 21 W Misonex sonicator 3000 (Vernon Hills, IL, USA). Cell debris was removed via centrifugation at 17,500 rpm and 4 °C for 40 min. The protein was purified via immobilized metal affinity chromatography (IMAC) using nickel–nitrilotriacetic acid (Ni-NTA) Superflow Cartridges (Qiagen). The column was equilibrated with buffer A, and the crude protein extract was loaded onto the IMAC column. Unbound protein was eluted with 15 column volumes (CV) of buffer A at a flow rate of 2 mL/min. The elution of TrzN was initiated with 15 CV of 3% buffer B (buffer A with 500 mM imidazole). The elution finished with a linear gradient (3–100%) of buffer B over 20 CVs at a flow rate of 2 mL/min. The peak fractions were pooled, resuspended in 50 mM HEPES pH 7.5, and concentrated with an Amicon Ultra-15

centrifugal filter device with a molecular weight cutoff (MWCO) of 30,000 (Millipore). SDS gel page revealed a single polypeptide band at ~51 kDa, consistent with previous studies. The protein concentration was determined via UV-Vis absorbance at 280 nm (61,670 Molar Absorptivity) and with a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). The expression of TrzN and purification using immobilized metal affinity chromatography (IMAC) resulted in ~10 mg/L of soluble TrzN enzyme [13,19].

3.3. Kinetic Activity Assay

The hydrolysis of atrazine by TrzN was quantified spectrophotometrically by continuously monitoring the decrease in absorbance at 264 nm (ε_{264} = 3.5 mM^{-1} cm^{-1}) that accompanies atrazine dichlorination [29]. This region contains no detectable product absorption. The activity of purified TrzN was determined by measuring the hydrolysis of a 150 µM atrazine solution in 0.1 M sodium phosphate buffer, pH 7.0 [19]. Assays were performed in a 1 mL quartz cuvette in triplicate on an Agilent 8453 UV-visible spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 µmol of atrazine per minute at 35 °C. Plots of the initial rates of hydrolysis of various concentrations of atrazine were fit to the Michaelis–Menten equation, which provided a k_{cat} of 3.97 ± 0.14 s^{-1} and a k_m value of 43.1 ± 2.9 µM, similar to those previously reported [19].

3.4. Immobilization of TrzN in a Sol–Gel and Alginate Beads in the Absence and Presence of a Chitosan Coating

Sodium alginate powder was added to the 50 mM sodium phosphate buffer to 1% (*w*/*v*) and heated to 50 °C with vigorous shaking. TrzN (5 mg) was added to 4.5 mL of the cooled alginate solution with gentle stirring and transferred to a 5 mL syringe with a 16 G needle. The TrzN:alginate mixture was dripped into 50 mL of a 1 M $CaCl_2$ solution with stirring (200 rpm) at 4 °C. After ~2 h, a 1 mL sample was removed, and a Coomassie (Bradford) Protein Assay Kit from Thermo Scientific (Waltham, MA, USA) was used to determine the amount of TrzN encapsulated into alginate beads. Next, 150 mL of nanopure water was added, and the beads were stirred for another 30 min to stop the gelation process. The resulting beads were then filtered and washed three times with 20 mL of nanopure water. The beads were air dried for ~30 min and then stored overnight at 4 °C in 5 mL of 50 mM HEPES buffer, pH 7.0.

TrzN:alginate beads were also coated in chitosan using a previously published procedure [30]. Briefly, a chitosan solution was prepared by dissolving 0.8 g of chitosan (Sigma-Aldrich St. louis, MO, USA) in 90 mL of nanopure water, after which 200 µL of glacial acetic acid (Fisher Hampton, NH, USA) was added to facilitate the dissolution. The solution was filtered, and the volume was increased to 100 mL with nanopure water providing a final concentration of 0.8% (*w*/*v*). The pH was adjusted to 5.6, and the TrzN:alginate beads were placed in 50 mL of this chitosan solution and stirred at 200 rpm for ~45 min. After the coating process, the TrzN:chitosan beads were washed three times with 20 mL of nanopure water and dried in air for 30 min. The beads were stored overnight at 4 °C in 5 mL of 50 mM HEPES buffer, pH 7.

The sol consisted of 1.57 mL of TMOS, 0.350 mL nanopure (18 Ω) H_2O, and 0.011 mL of 0.040 M HCl as previously described [16]. The mixture was sonicated on ice for 30 min and then left on ice for ~1 h prior to the addition of TrzN. An equal volume of TMOS sol (0.100 mL) was mixed with an equal volume of TrzN (2.5 mg) in 50 mM Tris-HCl, pH 7.5. The mixture was left on ice until gelation occurred, creating TrzN:sol–gel monoliths. The monoliths were washed three times with 0.400 mL of 50 mM Tris-HCl, pH 7.5 (sol–gel buffer), and stored at 4 °C in 0.400 mL of the same buffer. The next day, the aged monoliths were crushed with a metal spatula to produce a heterogenous material, and then they were washed three times with 0.400 mL of the sol–gel buffer. The wash and storage buffers were collected and tested for protein loss using a Coomassie (Bradford) Protein Assay kit.

3.5. Kinetic characterization of the TrzN:Alginate, TrzN:Chitosan, and TrzN:sol–gel biomaterials

The activities of the TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel biomaterials were determined by measuring the hydrolysis of atrazine by continuously monitoring the decrease in absorbance at 264 nm (ε_{264} = 3.5 mM^{-1} cm^{-1}) that accompanies atrazine dichlorination [29]. A solution of 50 µM atrazine in 50 mM HEPES pH 7.0 at 25 or 35 °C was reacted with TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel, with constant stirring at 200 rpm. Aliquots of the reaction mixture (0.3 mL) were taken at fixed time intervals, and the hydrolysis of atrazine was analyzed. The specific activity (U/mg) of each biomaterial was calculated from the reaction rate (µmol/L/min), the amount of TrzN immobilized, and the volume of the reaction. The concentration of the produced atrazine was determined using standard curves of absorbance versus known atrazine concentrations.

3.6. Proteolytic Digestion of Soluble TrzN, TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel

The trypsin digestion of soluble TrzN (0.007 mg), TrzN:alginate beads (5 mg of TrzN), TrzN:chitosan beads (5 mg TrzN), and TrzN:sol–gel (2.5 mg TrzN) was performed at a ratio of 2:1 trypsin to TrzN in 50 mM Tris-HCl and 1 mM $CaCl_2$ pH 7.6 (trypsin reaction buffer). TrzN:alginate and TrzN:chitosan were reacted for 30 min at 25 °C with constant stirring at 200 rpm, while TrzN:sol–gel was reacted for 18 h at 35 °C with constant stirring at 200 rpm. Along with the digested biomaterial samples, a control utilizing TrzN in a trypsin reaction buffer was also incubated in an identical fashion. Following digestion, the TrzN:alginate and TrzN:chitosan samples were filtered and washed three times with 20 mL of nanopure water, while the TrzN:sol–gel samples were filtered and washed three times with 0.4 mL sol–gel buffer to remove trypsin and digestion products. The biomaterials and digested TrzN were assayed for catalytic activities using standard assay conditions.

3.7. Recycling Experiments for TrzN:alginate, TrzN:chitosan and TrzN:sol–gel

A 20 mL solution of 50 µM atrazine in 50 mM HEPES, pH 7.0, at 25 °C was reacted with TrzN:alginate or TrzN:chitosan for 6 h, after which an aliquot (0.3 mL) was removed, and the amount of atrazine dichlorination was determined. The product mixture was then decanted, and the resulting biomaterial was washed with 20 mL nanopure water. A 1 mL aliquot was taken of the reaction buffer to test for protein loss from the biomaterial using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). The TrzN:alginate and TrzN:chitosan beads were stored at 4 °C in 5 mL of 50 mM HEPES pH 7.0 and reused on a weekly basis for six weeks.

A 5 mL solution of 50 µM atrazine in 50 mM HEPES pH 7.0 at 35 °C was reacted with TrzN:sol–gel for 1 h, after which the solution was centrifuged at 4000 rpm for 5 min. An aliquot (0.3 mL) of the supernatant was removed, and the amount of atrazine dichlorination was determined. A 1 mL aliquot of the supernatant was also taken to test for protein loss from the biomaterial using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). The TrzN:sol–gel biomaterial was washed three times with 0.4 mL of the sol–gel buffer and stored at 4 °C in 0.4 mL of the sol–gel buffer and reused on a weekly basis for six weeks. The reaction was also repeated on a different TrzN:sol–gel biomaterial 10 times over a 1 Hr time period as described above, resulting in 10 separate cycles with the same sample of TrzN:sol–gel biomaterial.

3.8. Activity of Soluble and Immobilized TrzN in Organic Co-Solvents

The activity of soluble TrzN with 5, 10, and 20% (v/v) methanol as the organic co-solvent toward atrazine was measured spectrophotometrically at 25 °C as described above. Each measurement was taken in triplicate. The degradation of atrazine using TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel with organic co-solvents at 5, 10, and 20% (v/v) was carried out in their respective reaction conditions described previously. Aliquots of 1 mL were taken at the start and end of the reactions, and they were analyzed spectrophotometrically as described above.

3.9. Activity of Soluble and Immobilized TrzN at Varying pH Values

The activity of soluble and encapsulated TrzN in 50 mM citric acid, pH 4.0, and 50 mM glycine buffer, pH 9.0, towards atrazine was measured spectrophotometrically as described above. Each measurement was taken in triplicate. Samples of 1.5 mL were taken at the end of the reactions, tested for protein loss using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific), and sent for ICP-MS analysis.

3.10. Thermostability of Soluble and Immobilized TrzN

The thermostabilities of TrzN, TrzN:alginate, and TrzN:chitosan were determined by incubating each for 30 min at 50, 60, 70, and 80 °C. The TrzN:alginate and TrzN:chitosan materials were suspended in 5 mL of 50 mM HEPES pH 7.0, and after the incubation period, a 1.5 mL sample was collected for ICP-MS and protein analyses using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific). The residual activities of the free enzyme and encapsulated TrzN were determined spectrophotometrically. The thermostability of the TrzN:sol–gel biomaterial was evaluated over a temperature range of 50–80 °C, with incubation times of 0, 30, 60, 180, 300, 420, and 600 min. After the incubation period was complete, the residual activity was determined spectrophotometrically.

4. Conclusions

In summary, this work presents the first immobilization of TrzN in any biomaterial. Alginate beads and sol–gels was chosen as the first biomaterials to test due to their well-documented properties. Table 1 summarizes the results of the three biomaterials tested.

Table 1. Summary of the activities of TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel.

Material	Activity Trypsin Digestion	Activity after Six Week Storage	Thermostability at 60 °C	MeOH as a Co-Solvent (20:80)	Activity at pH 4.0	Activity at pH 9.0
Alginate	75%	0%	52%	10%	0%	0%
Chitosan	80%	20%	30%	33%	0%	18%
Sol-Gel	85%	65%	24%	24%	45%	94%

Chitosan-coated alginate was also tested as a potential solution to the material limitations that can accompany alginate matrices. The resulting biomaterials (TrzN:alginate, TrzN:chitosan, and TrzN:sol–gel) all exhibited catalytic activities toward atrazine and protected the WT enzyme from proteolytic digestion. When evaluating reusability, the TrzN:sol–gel material remained active for a full 6 weeks and was clearly superior to the other biomaterials tested. Moreover, TrzN:sol–gel could be reused multiple times, making it a good candidate for bioremediation strategies. Similarly, TrzN:sol–gel was determined to be the superior biomaterial for use in organic solvents and non-physiological pH values, as it retained ~25% activity in a 20:80 MeOH:water mixture and was the only biomaterial to retain significant activity at both high and low pH values. Therefore, the TrzN:sol–gel biomaterial exhibited the best overall profile, making it the most effective biocatalyst for the dichlorination of atrazine into its less toxic derivative hydroxyartrazine. The beauty of the TrzN:sol–gel biocatalytic material as a bioremediation tool resides in the fact that TrzN:sol–gel materials can be cast into any desired shape, including pellets, which can be used in columns, providing a new avenue for the design of bioremediation methodologies for the removal of atrazine from the environment.

Author Contributions: K.D. prepared expression plasmids; carried out protein expression, purification, and enzymatic assays; prepared TrzN biomaterial samples; and characterized each of the reported biomaterials. R.C.H. conceived the idea and wrote the paper with K.D. All authors have read and agreed to the published version of the manuscript.

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